

**INVESTIGATION OF CIRCULATING FACTORS AS MEDIATORS OF MUSCLE
MITOCHONDRIAL BIOENERGETIC CHANGES ASSOCIATED WITH AGING, DIET,
AND EXERCISE**

BY

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DEDICATION

This dissertation is dedicated to those of us who dreamed of leaving a small town, dreamed of going to college, and dared to have our dreams come true. Never say never, don't back down, and don't forget that you deserve to be here.

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LIST OF ABBREVIATIONS

1RM	One repetition maximum
³¹ P-MRS	Phosphorus magnetic resonance spectroscopy
ANCOVA	Analysis of covariance
ATP	Adenosine triphosphate
AUC	Area under the curve
BCA	Bicinchoninic acid
BCAA	Branched-chain amino acid
BMI	Body mass index
Ca ²⁺	Calcium
CETF	Electron transferring flavoprotein complex
CO ₂	Carbon dioxide
CPT	Carnitine palmitoyl transferase
CR	Caloric restriction
CRP	C-reactive protein
CS	Citrate synthase
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ETS	Electron transport system
FADH ₂	Flavin adenine dinucleotide
FAO	Fatty acid oxidation

FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GH	Growth hormone
GPDH	Glycerol phosphate dehydrogenase
HOMA-IR	Homeostatic model of insulin resistance
IGF-1	Insulin-like growth factor 1
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
I'M FIT	Improving Muscle for Functional Independence Trial
iv-GTT	Intravenous glucose tolerance test
MAM	Mitochondria-associated endoplasmic reticulum membrane
Mat-sf	Mobility assessment tool - short form
mtDNA	Mitochondrial deoxyribonucleic acid
mTOR	Mechanistic target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NIRS	Near-infrared spectroscopy
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rot	Rotenone
RT	Resistance training
SAA	Serum amyloid A
SASP	Senescence associated secretory phenotype
SDH	Succinate dehydrogenase

SEM	Standard error of the mean
SOD	Superoxide dismutase
SPPB	Short physical performance battery
SUIT	Substrate-uncoupler-inhibitor titration
SRC	Spare respiratory capacity
T2D	Type II diabetes
TCA	Tricarboxylic acid
TNF- α	Tumor necrosis factor- α
UCP1	Uncoupling protein 1
UCP3	Uncoupling protein 3
VDAC	Voltage dependent anion channel

ABSTRACT

As older adults continue to live longer and, as a result, with more comorbidities, there is a need to understand the cellular and molecular processes, including mitochondrial dysfunction, that are implicated in healthy aging and age-related diseases. Our lab and others have pioneered the use of blood cells as reporters of systemic mitochondrial bioenergetic capacity based on the premise that these circulating cells are continuously exposed to a myriad of factors that mediate bioenergetic capacity across multiple tissues. In support of this theory, our lab has demonstrated that blood cell mitochondrial bioenergetics can recapitulate the bioenergetics of brain, skeletal muscle, and cardiac muscle and that increased circulating IL-6 is associated with decreased mitochondrial bioenergetic capacity in blood cells. However, direct evidence supporting the effect of circulating factors on bioenergetics is limited. Therefore, the overarching hypothesis of my work is that there are circulating factors in blood associated with aging (and consequently, age-related diseases and interventions to improve healthspan) that can mediate systemic bioenergetic capacity. In Chapter Two, we found that long-term consumption of a Western diet resulted in elevated skeletal muscle mitochondrial bioenergetics of female cynomolgus macaques when compared to Mediterranean diet and that increased respiration was associated with increased insulin resistance, particularly with Western diet. In Chapter Three, we used a heterochronic parabiosis mouse model to investigate the effects of circulating factors on mitochondrial structure and function. We found a greater negative effect of old blood on young heterochronic mice, demonstrating the profound deleterious effects of circulating factors from old mice on mitochondrial structure and function. In Chapter Four, we developed a novel *in vitro* method to determine the effects of human serum from older adults who completed

resistance training or resistance training plus caloric restriction interventions on muscle cells. We demonstrated that our method recapitulated the expected results of the intervention, i.e. improved bioenergetic capacity and differences between intervention groups, and identified potential circulating factors responsible for mediating mitochondrial bioenergetics associated with diet and exercise intervention. Taken together, this body of work provides evidence that circulating factors mediate bioenergetic capacity in multiple contexts: aging, diet, and exercise.

CHAPTER ONE:

INTRODUCTION

Jenny L. Gonzalez-Armenta

I. AGING POPULATIONS

A. The aging world population and shifting demographics

Since the 1950s, the global average lifespan has dramatically increased, allowing people across the world to live an average of 10 to 20 years longer (1). In 2020, the World Health Organization estimated that over 1 billion people, or 13.5% of the global population, are adults over the age of 60 which is 2.5 times larger than the population of older adults in 1980 (1). The population of older adults is expected to grow and reach 2.1 billion people by 2050 (1). The increase in lifespan is largely due to improvements in quality of life, the prevention and treatment of diseases, and decreases in mortality at birth (2–5). In low- to middle-income countries, the increase is driven by reduced mortality in young people, particularly in childhood, while in high-income countries lifespan continues to increase due to reduced mortality of older adults (6).

In the United States, the average lifespan has increased from 69.7 years in 1960 to 79.4 years in 2015 and is projected to increase to 85.6 years in 2060 as shown in **Figure 1** (adapted from (7)). Moreover, the proportion of older adults in the United States is increasing. By 2030, the entire Baby Boomer generation, individuals born between 1946-1964, will be over the age of 65 and older adults are projected to outnumber children (<18 years old) for the first time in history (8). This has resulted in a demographic shift from a pyramid shape showing a large number of children and young adults and small numbers of older adults in 1960 to a pillar or column where projected population age groups are more evenly distributed with a large number of older adults living into their 70s and 80s as shown in **Figure 2**. These trends are also mirrored in the global population (9). This demographic shift associated with increased lifespan raises concerns about society's ability to plan for and accommodate longer lives, as aging will affect nearly every aspect of society, including: economic growth, retirement, and the

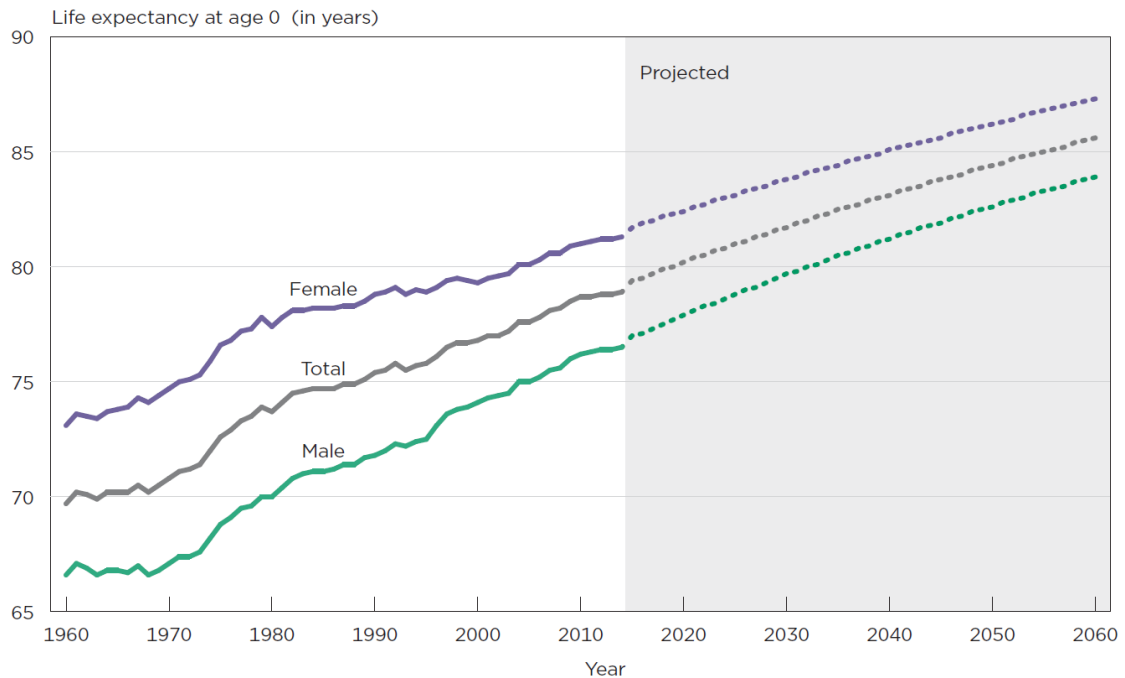


Figure 1. Historical and projected life expectancy of U.S. population at birth from 1960 to 2060. Adapted from L. D. Medina, et al., *Living Longer: Historical and Projected Life Expectancy in the United States, 1960 to 2060. Curr. Popul. Rep.*, 1–25 (2020).

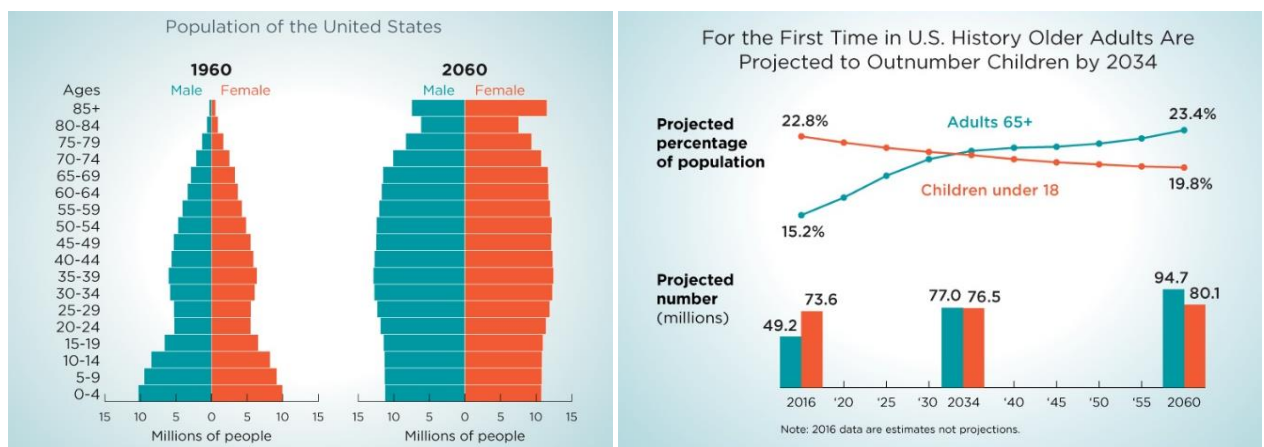


Figure 2. Historical and projected demographic shifts in the U.S. population. Adapted from U.S. Census Bureau, *National Population Projections. (2017)* and U.S. Census Bureau, *Older People Projected to Outnumber Children. (2018)*

ability of governments and communities to provide resources for older adults (10). On the other hand, it should be noted that increased lifespan and shifting demographics have benefits for society as well. Economically, longer lifespans, and consequently, an increased proportion of the population is of working age, results in increased per capita income, tax revenues, and the potential for increased personal savings and investments (11). Additionally, older adults are more likely to be law-abiding, politically active, volunteer and help the wider community, and regularly provide family support and care for children and grandchildren (12, 13).

B. Increased lifespan is associated with increased morbidity in older adults

The global average lifespan has increased, while healthspan, the period in which individuals are healthy and disease-free, has not increased at the same rate (14). An average of 16-20% of life is spent in late-life morbidity, defined as the incidence of one or more chronic diseases or disabilities (14, 15). This period of morbidity is more pronounced among women, individuals who are obese, and people from lower socioeconomic statuses (14). Aging itself is the greatest risk factor for the leading causes of morbidity and mortality in older adults: heart disease, stroke, cancer, diabetes, kidney disease, Alzheimer's disease, and Parkinson's disease (14, 16). In addition, these diseases often occur together and are sometimes referred to as "comorbidities of aging" (16). In 2014, 80% of Medicare beneficiaries had at least two chronic conditions, and 60% reported at least 3 chronic conditions (17). We can expect these rates of comorbidities to increase as the aging population continues to grow.

In 1980, James Fries introduced the compression of morbidity paradigm (18). He noted that most illnesses were chronic and occurred later in life and hypothesized that the lifetime burden of illness could be reduced if the onset of chronic illness was delayed (19). This theory has influenced gerontologists in the biology of aging to shift their focus

from lifespan extension to identifying biological mechanisms and strategies to increase healthspan (16). Indeed, baby boomers report a strong interest in remaining healthy, active, productive, and independent until the end of life (16). Therefore, optimal longevity for both gerontologists and older adults is to live a long life but remain healthy and well until the end as shown in **Figure 3** (adapted from (16)).

C. Chronological versus biological age

In gerontology, we often hear a common anecdote about older adults. On one hand, some 80-year-olds are completely independent, physically active, and mentally alert and appear younger than their age (think Dr. Anthony Fauci). On the other, there are some 55-year-olds with multiple comorbidities who seem to have aged more rapidly than others in their age group. While chronological age, the number of years someone has been alive, is useful in aging research, it would be more beneficial if we could use a metric to account for these differences in overall health. Biological age, or a composite age that takes into account a person's functional status and biomarkers of aging, has been introduced as an alternative to chronological age. To date, biological age has been measured using many methods, including frailty index, measures of DNA methylation, composite scores of blood-based biomarkers, and other age-related phenotypes (autophagy, mitochondrial function, cellular senescence, etc.) (20–23). Current and future research is focused on determining the biomarkers and methods that are most closely related to function, can predict health-related outcomes, and can be used to test and develop interventions to prevent or delay the progression of multimorbidity and disability with aging (22).

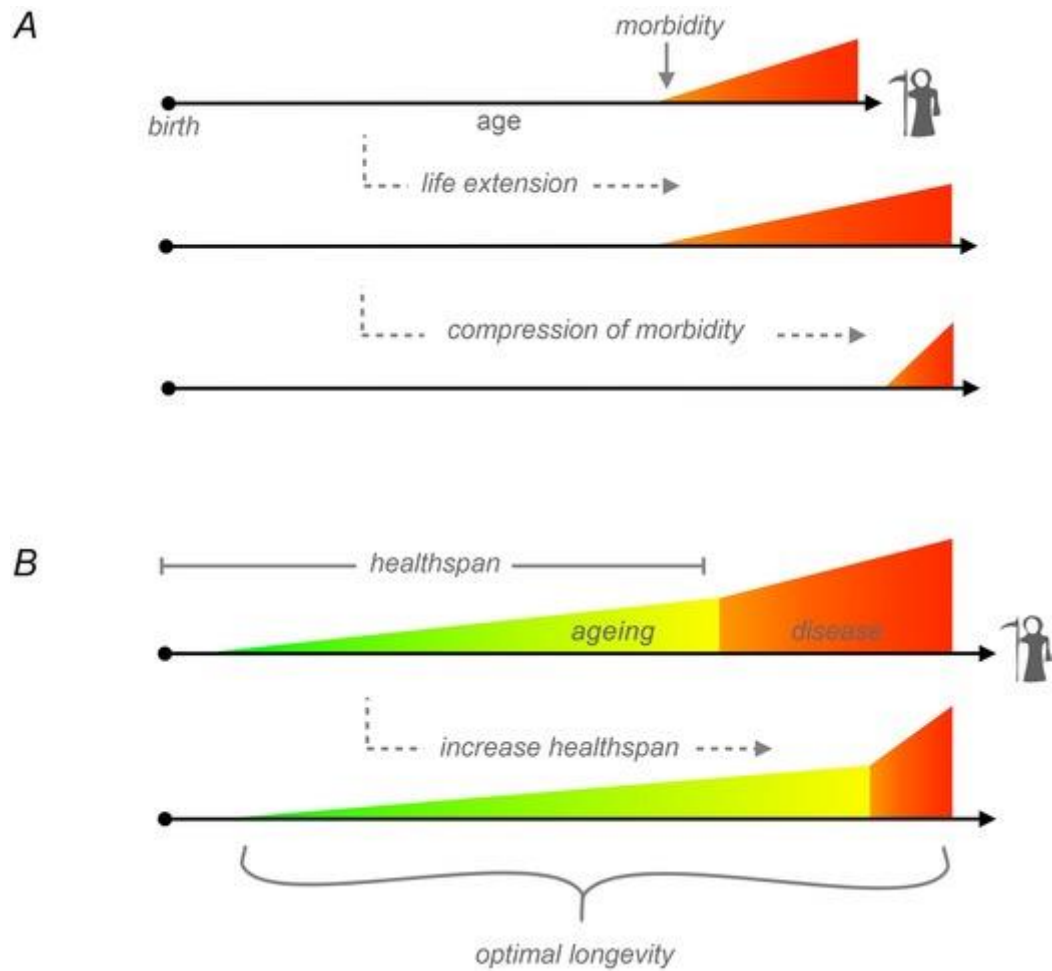


Figure 3. Compression of morbidity, healthspan, and optimal longevity. Adapted from D.R. Seals, et al., *Physiological geroscience: targeting function to increase healthspan and achieve optimal longevity. J Physiol.*, 2001-2024 (2016).

II. THE BIOLOGY OF AGING

A. Theories of aging

Over 70 years of biological, epidemiological, and demographic research has generated several theories that have attempted to identify causes or processes that explain aging (24). However, aging is an extremely complex and multifactorial process. Therefore, the theories of aging are not mutually exclusive and may describe some or all aspects of the aging process and may need to be combined with other theories (24). For the purpose of this dissertation, I have described several key theories of aging that can be broadly classified into four categories: evolutionary, molecular, cellular, and system theories of aging.

i. Evolutionary theories

Broadly, the evolutionary theories of aging argue that evolution primarily occurs to maximize reproductive fitness and that longevity is a trait that would be selected only if it was beneficial during the reproductive period (24). These theories began to form in the 1940s based on observations of Huntington's Disease (25). Huntington's Disease is the result of a lethal autosomal dominant mutation that remains in the population even though it should, in theory, be selected against. However due to the late onset of the disease in patients well into their 30s and even 40s, allows for the carrier to reproduce before dying, therefore avoiding natural selection and allowing the mutation to be passed on. This observation inspired the mutation accumulation theory of aging in 1952 which states that detrimental, late-acting mutations may accumulate in the population and ultimately lead to pathology and senescence in individuals as they age (26). Initial experiments in *Drosophila* appeared to support mutation accumulation theory: increased mutations late in life seemed to contribute to aging and senescence, though the authors

could not rule out other theories of aging, such as antagonistic pleiotropy (27–29). However, later experiments and further analyses have cast doubt on these conclusions (30, 31).

Other evolutionary theories of aging focus on evolutionary trade-offs as the basis of aging. For instance, antagonistic pleiotropy theory, proposed in 1957, posits that genes selected for their beneficial effects early in life may have unselected deleterious effects later in life that lead directly to senescence (32). Additionally, the disposable soma theory (1977), argues that a somatic organism is only maintained for reproductive success and, therefore, the soma becomes disposable after reproduction (33). There must be trade-offs in balancing resources between growth, reproduction, and somatic maintenance, leading to progressive cellular damage and senescence with age (34). There is experimental evidence for these trade-offs in *Drosophila* and *C. elegans*. Increased body size, longer development times, and reduced early fecundity, or reproductive ability, are all associated with longer lifespans in *Drosophila* and reduced fecundity has been causally linked to increased lifespan (35, 36). Similar trade-offs between longevity and early life fitness have been reported in *C. elegans*, though there are contradicting reports, and there is evidence that the effects of mutations can be strongly influenced by the environment (37–39).

It should be noted that these evolutionary theories were developed before the discovery of the mitochondrial genome and were based on the available knowledge of the nuclear genome (40). Consequently, the role of the mitochondrial genome, the coordination between the mitochondrial and nuclear genomes, and the influence of maternal inheritance of mitochondrial DNA are not taken into account in these theories (40).

ii. Molecular theories

Gene regulation theory was first introduced in 1975 which argues that senescence is a result of changes in gene expression and that longevity is influenced by the selection of genes that promote longevity (41). Indeed, many genes show changes in expression over the lifespan (42–45). Studies of centenarians and their relatives have also found a significant influence of genetics on longevity (46–50). However, it is more likely that aging is driven primarily by a balance of damage and repair processes influenced by environmental factors and genetic variation (51). Further evidence for gene regulation theory is the discoveries of signaling pathways that regulate lifespan in model organisms, such as insulin-like growth factor 1 (IGF-1) (52–54) and the mechanistic target of rapamycin (mTOR) pathways (54–56).

iii. Cellular theories

The cellular senescence theory of aging was first presented in 1965 and described senescence as the process that limits the number of cell divisions that normal human cells can undergo in culture (57). This number of cell divisions, also referred to as the Hayflick limit, results in terminally arrested cells that exhibit altered physiology (24). Replicative senescence is ultimately driven by the shortening and eventual loss of telomeres with each cell division and has been extensively studied in cell culture (24, 58). Additionally, early cell culture experiments demonstrated that replicative potential was negatively associated with donor age and cells from short-lived organisms appear to senesce earlier than longer-lived organisms (24). Telomerase-deficient mice do not exhibit accelerated aging and the phenotypic effects of telomerase deficiency are not observed for several generations, suggesting that telomere shortening alone cannot explain normal aging in mice (59, 60). However, in an aging animal model using the African turquoise killifish, it was found that telomeres shorten with age, have similar

telomere length as humans, and telomerase mutants exhibit some signs of accelerated aging (61–64). It is difficult to test replicative senescence in humans, but evidence from genetic disorders such as Dyskeratosis congenita, a disease resulting in altered telomerase RNA subunit metabolism, suggests that telomere shortening plays a role in human aging, although severe pathology develops over multiple generations similar to rodent models of telomere deficiency (65, 66). Senescence can also occur in response to stressors such as DNA damage, oxidative stress, modifications in heterochromatin structure, or signals in response to oncogene expression (24, 58). This stress-induced senescence is a cellular response to age-related molecular changes that can aggravate or accelerate aging (24). Senescence likely contributes to the aging process through the senescence-associated secretory phenotype or SASP (67). The SASP is composed of various cytokines, chemokines, growth factors, and extracellular matrix proteases that reinforce senescence, affect the local tissue environment, and the entire organism (67).

The free radical theory of aging (1957) argues that free radical reactivity is inherent in biology and results in cumulative damage and senescence (68). Reactive oxygen species (ROS) are found in all cellular environments (69). ROS are produced during mitochondrial respiration (complex I, complex III, glycerol-3-phosphate dehydrogenase, and α -ketoglutarate dehydrogenase all produce free radicals) and superoxide dismutase (SOD), the cellular superoxide scavenger, is found in all aerobic organisms (69, 70).

There is evidence of oxidative damage to DNA, proteins, and lipids (24). It is clear that oxidative damage increases with age, but it is not clear if this damage contributes to aging in all organisms. Alterations in ROS production or ROS scavengers alter lifespan in *Drosophila* and *C. elegans* (24, 71–73). However, in rodent models, dietary antioxidants and ubiquitous overexpression of SOD do not result in lifespan extension (74, 75). Mutations in SOD produce mice that are sick and die quickly, but there is

limited evidence for accelerated aging (24). Additionally, chronic exposure to ionizing radiation, which generates free radicals, actually results in lifespan extension in mice (76).

iv. System theories

In these systems theories of aging, the decline of the nervous, endocrine, and immune systems is considered essential to the aging process. The neuroendocrine theory of aging was first introduced in 1954 and describes aging as the progressive loss of hypothalamic receptor sensitivity to negative feedback inhibition (40). A potential neuroendocrine regulator of aging, the growth hormone (GH) and insulin-like growth factor 1 (IGF-1) axis, has been studied for many years. GH is a key regulator of metabolism and somatic growth, either directly or via other pathways, such as IGF-1 signaling (77). GH signaling blocks insulin action, stimulates lipolysis, and inhibits lipogenesis, while IGF-1 signaling has the opposite effects (77). Many genes that regulate lifespan in model organisms are direct or indirect homologs of these pathways (52, 78, 79). Also, mutations in these genes have been linked to increased stress resistance and lifespan in yeast, worms, and flies (80–82). In centenarians, an overrepresented heterozygous mutation in the IGF-1 receptor results in reduced activity, suggesting that IGF-1 signaling and longevity are linked (83).

The immune system is essential for clearing infections, foreign matter, tissue debris, and wound healing (40). This type of immune response is adaptive and acute inflammation that returns to baseline levels after responding to these events (84). However, resting levels of inflammation increase with age (85). This progressive, age-related, chronic, and sterile low-grade inflammation forms the basis of another theory of aging called inflammaging (85). Levels of inflammatory cytokines are consistently elevated in older adults, particularly interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) (86–89), and

these increased levels of pro-inflammatory cytokines are associated with several age-related diseases, including atherosclerosis, Alzheimer's disease and other dementias, insulin resistance and diabetes, and cancer (90–96). Further, macrophages and senescent cells secrete many pro-inflammatory mediators and have been implicated as major sources of chronic inflammation (97–100), thus these associations suggest that inflammaging plays an important role in aging and the development of age-related diseases.

v. Summary of theories of aging and implications

Aging is an extremely complex and multifactorial process that involves multiple biological mechanisms. Through several decades of research, scientists have produced many theories of aging. The evolutionary theories seek to explain the detrimental effects of aging by arguing that natural selection drives improved development and reproduction, rather than longevity. According to molecular theories, aging is the result of changes in gene expression and DNA damage. The cellular theories argue that aging is due to cellular senescence and is driven by shortened telomeres, cellular stress, and the effects of ROS. In the system theories of aging, the neuroendocrine and immune systems decline over the lifespan and drive aging processes.

While each of these theories presents valid arguments and evidence to support their claims, they cannot explain the entire aging process on their own. On their own, they cannot show the interconnectedness between various theories of aging, how one aspect of aging could impact another, what aspects of aging should be targeted, and how aging should be studied. Instead, it is far more useful to use them together in an attempt to explain the aging process, rather than as individual theories.

B. Hallmarks of aging

To study the progression of aging and identify targets to combat aging and age-related diseases, nine cellular and molecular hallmarks have been proposed and are shown in **Figure 4** (101). These hallmarks include: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication (101). These hallmarks are widely considered to contribute to the aging process and form the aging phenotype when taken together (101). Moreover, these hallmarks integrate all of the various theories of aging and provide a framework to guide future research: each hallmark is observed during normal aging and should accelerate and slow aging when experimentally manipulated (101). It should be noted that the hallmarks of aging are not independent factors driving aging, but are often intertwined and interrelated processes.

Mitochondria have been linked to many of the other hallmarks of aging, including the decline of stem cell function by regulation of metabolites such as NADH (nicotinamide adenine dinucleotide) and the regulation of cellular senescence by modulating metabolism and ROS generation (102, 103). Additionally, modulating the mitochondrial unfolded protein response results in increased longevity and improved proteostasis, improved mitochondrial quality control increases lifespan, and mitochondrial dysfunction has been linked to increased inflammation by activation of the innate immune system in response to damaged unmethylated mtDNA entering the cytosol (102, 103). An overview of the links between mitochondrial dysfunction and the other hallmarks of aging is shown in **Figure 5**.

Mitochondria seem to be playing a central role in the aging process. Our lab is interested in mitochondrial function and its role in healthy aging, the progression of age-related

diseases, and interventions to improve healthspan. This dissertation will focus on two of those aspects: the roles of mitochondrial function in healthy aging and interventions that improve healthspan.



Figure 4. The hallmarks of aging. Adapted from C Lopez-Otin, et al., *The Hallmarks of Aging*. *Cell*, 1194-1217 (2013).

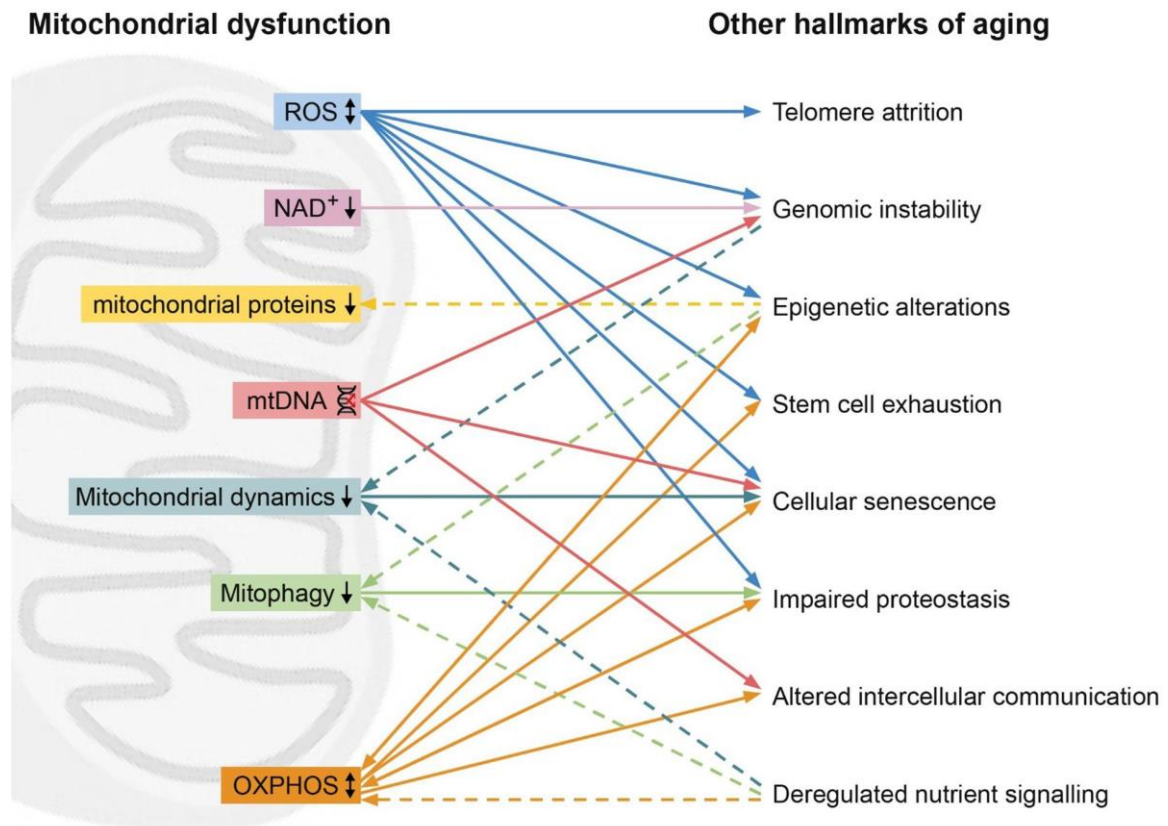


Figure 5. Links between mitochondrial dysfunction and the other hallmarks of aging. Adapted from S van der Rijt, et al., *Integrating the Hallmarks of Aging Throughout the Tree of Life: A Focus on Mitochondrial Dysfunction*. *Front Cell Dev Biol*, 1-12 (2020).

III. MITOCHONDRIA

Mitochondria are organelles found in most eukaryotic organisms (104). The prevailing hypothesis is that mitochondria were originally prokaryotic cells that were engulfed by a proto-eukaryotic cell forming an endosymbiotic relationship that enabled eukaryotic cells to evolve (105). Modern mitochondria are organelles that are responsible for generating most of the cellular energy in the form of ATP (adenosine triphosphate) (106).

Mitochondria are also involved in many other cellular processes, including: signaling, differentiation, cell death, and cell growth (106, 107).

A. Mitochondrial structure

Mitochondria are dynamic organelles that exist in various sizes and networks within each cell. Each mitochondrion consists of two membranes: an inner membrane and an outer membrane. The two membranes create three other distinct parts of the mitochondrion: the intermembrane space, cristae (folds of the inner membrane), and matrix (space within the inner membrane). A view of mitochondrial networks and the structures of an individual mitochondrion are shown in **Figure 6** (adapted from (108, 109)).

The mitochondrial outer membrane contains large numbers of channel-forming proteins called porins (also called voltage-dependent anion channels or VDAC) (104). These porins allow for the free flow of small molecules less than 5 kDa (104). Other proteins embedded in this membrane include enzymes involved in lipid synthesis and conversion of lipid substrates to be transported and metabolized in the matrix, import receptors for mitochondrial proteins, and the enzymatic machinery for fission and fusion of the mitochondrial outer membrane (104). This membrane can also associate with the endoplasmic reticulum (ER) via a mitochondria-associated ER-membrane (MAM) structure (110).

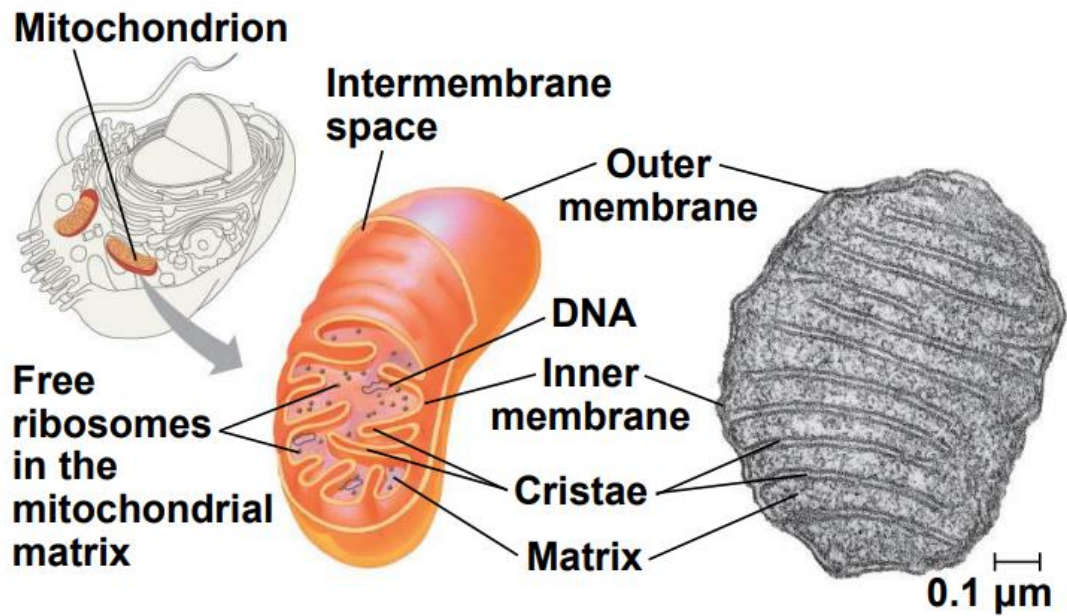
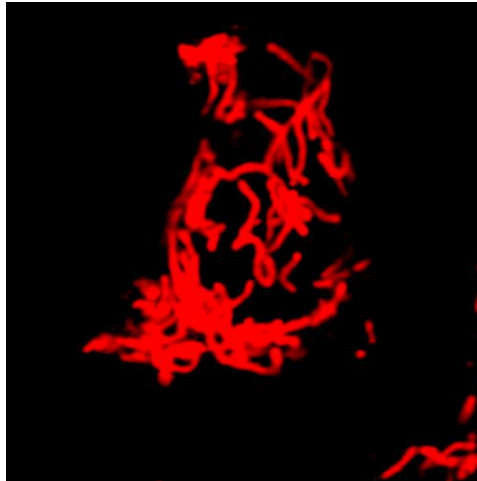


Figure 6. Mitochondrial networks in a pancreatic β -cell and the structure of an individual mitochondrion. Adapted from AJA Molina et al, *Mitochondrial Networking Protects β -Cells from Nutrient-Induced Apoptosis, Diabetes*, 2303-2315 (2009) and JB Reece et al, *Campbell Biology*, 10th ed. (2014).

The intermembrane space has a similar concentration of small molecules as the cytosol (104). This space also contains several enzymes that use ATP transported from the matrix to phosphorylate other nucleotides (104).

The inner membrane contains no porins, is rich in cardiolipin, and has a very high protein to phospholipid ratio (3:1) (104, 110). Thus, the inner membrane is highly impermeable to most molecules and requires transport proteins that regulate the passage of metabolites in and out of the mitochondrial matrix (104). This membrane also contains the proteins of the electron transport system (ETS) and ATP synthase (104). An electrochemical gradient of H^+ forms a membrane potential across the inner membrane and is used to drive ATP synthase (104).

The inner membrane is folded many times and forms structures called cristae. These cristae increase the surface area of the inner membrane and allow for an enhanced ability to produce ATP (104). Cells that have higher energy demands such as cardiac or skeletal muscle have greater amounts of cristae (104).

The mitochondrial matrix contains hundreds of enzymes, including the enzymes involved in the oxidation of pyruvate and fatty acids and the enzymes of the tricarboxylic acid (TCA) cycle (104). The matrix also contains mitochondrial ribosomes, transfer RNA, and several copies of mtDNA (104).

B. Mitochondrial function

i. Energy conversion

The major role of mitochondria in the cell is to produce ATP by oxidative phosphorylation using pyruvate and fatty acids, from the breakdown of sugars and fats, as fuel (104). An overview of this process is shown in **Figure 7** (adapted from (111)). These fuels are

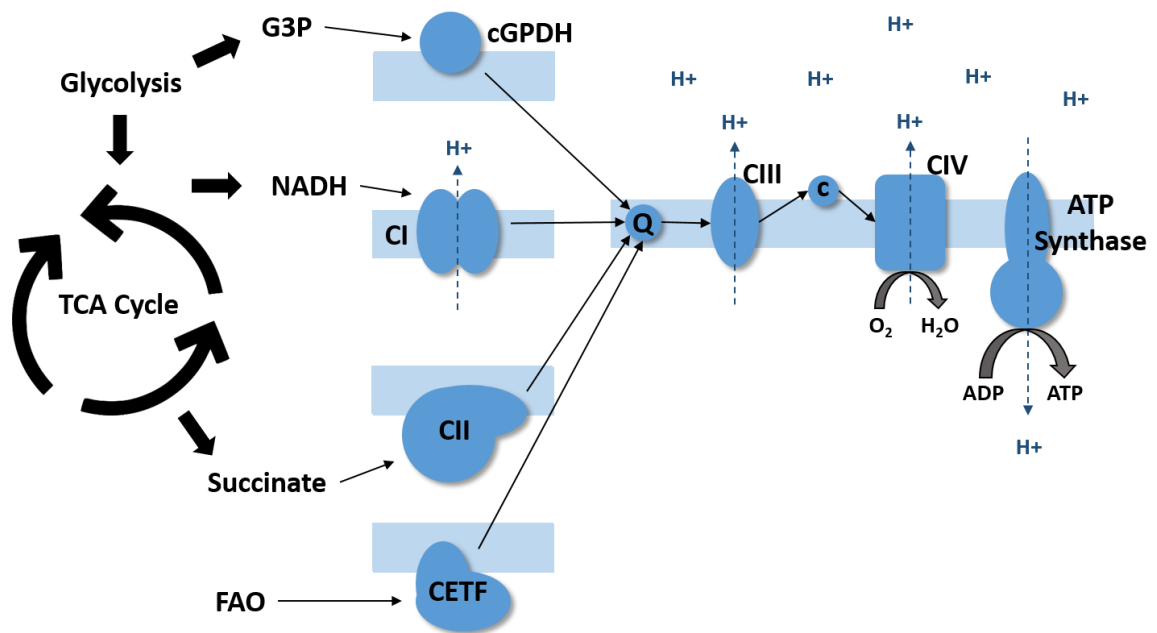


Figure 7. Overview of the electron transport system. Adapted from E Gnaiger, *Mitochondrial Pathways and Respiratory Control: An Introduction to OXPHOS Analysis* (2014).

transported across the mitochondrial inner membrane where they are converted to acetyl CoA in the matrix and oxidized in the TCA cycle to produce NADH, FADH₂ (flavin adenine dinucleotide), and CO₂ (carbon dioxide) (104). NADH and FADH₂ are high-energy carrier molecules that allow for the transfer of electrons through complexes I and II of the ETS in the mitochondrial inner membrane (104). In the ETS, a series of redox reactions occur as electrons are passed from complexes I and II (or from alternate sources such as GPDH from G3P or CETF from fatty acids), to coenzyme Q, complex III, cytochrome c, and complex IV (104, 111). Oxygen is reduced to water as the final electron acceptor at complex IV (104). The energy produced by the redox reactions is used to pump protons across the mitochondrial inner membrane at complexes I, III, and IV (104). The movement of protons produces an electrochemical gradient that exerts a proton-motive force that drives protons back to the mitochondrial matrix through complex V or ATP synthase (104). ATP synthase is made up of two parts: a transmembrane proton carrier (F₀) and a rotating portion (F₁ ATPase) (104). The proton-motive force pushes a proton through the F₀ portion which physically rotates the F₁ ATPase to drive the reaction of ADP and P_i to create ATP (104). This allows energy to be stored in this chemical bond for future reactions throughout the cell (104).

Oxidative phosphorylation allows eukaryotic cells to produce more energy than prokaryotes and the majority of energy is produced in animal cells via this mechanism. For example, oxidative phosphorylation of one molecule of glucose has a net yield of ~30 ATP molecules compared to a net yield of ~2 molecules of ATP from glycolysis alone (104). A large amount of ATP is also produced from the oxidation of fatty acids which has a net yield of ~129 ATP molecules from the complete oxidation of palmitate, a 16-carbon fatty acid (104).

ii. Other functions

In some cells, mainly brown adipose tissue, mitochondrial respiration is uncoupled from ATP synthesis (104). Uncoupling protein 1 (UCP1) allows protons to freely move across the mitochondrial inner membrane and bypass ATP synthase, allowing the energy to dissipate by the production of heat (104). In brown adipose tissue, this allows the cells to metabolize fatty acid stores and generate heat through non-shivering thermogenesis (112). There is also increasing evidence that white adipose tissue can become “beige” adipose tissue by expressing UCP1 which contributes to systemic energy homeostasis (112).

Mitochondria also play a role in calcium (Ca^{2+}) homeostasis by transiently storing Ca^{2+} . Some functions of Ca^{2+} and mitochondria include: (1) to regulate mitochondrial metabolism by accumulating Ca^{2+} in the mitochondrial matrix, resulting in transient membrane potential depolarization, (2) to act as a Ca^{2+} buffer by regulating the Ca^{2+} concentration in cellular microdomains and therefore regulating Ca^{2+} signaling in the cell, and (3) to trigger cell death by mitochondrial uptake of large amounts of cellular Ca^{2+} under pathological conditions (113).

C. Methods for measuring mitochondrial function

Mitochondrial isolation techniques were developed in 1948 and accelerated the progress of understanding mitochondrial function (104). Most of the early studies were performed using isolated mitochondria from the liver, where each cell contains about 1000-2000 mitochondria that make up about 20% of total cell volume (104). Since molecular oxygen is the final electron acceptor of the ETS, the change in oxygen concentration (commonly referred to as oxygen flux or oxygen consumption rate) can be measured to calculate mitochondrial respiratory rates (111). This technique is referred to as mitochondrial

respirometry. Isolated mitochondria, whole cells, or tissues can be probed with fuels, inhibitors, and uncouplers to examine specific complexes of the ETS or the ETS as a whole. Today, mitochondrial respirometry is still the gold standard for measuring mitochondrial function because it allows for the direct investigation of the ETS in real-time, though the context of the isolated mitochondria, cells, or tissues must be considered when interpreting the results. There are three main technologies available for performing mitochondrial respirometry: Clark-type electrodes, the Oroboros O2k, and the Seahorse Extracellular Flux Analyzer. The bulk of experiments in this dissertation will include respirometry, either high-resolution respirometry performed with the Oroboros O2k or high-throughput respirometry performed with the Seahorse Extracellular Flux Analyzer.

Other methods of measuring mitochondrial function have various advantages and disadvantages. ATP content can be measured in vitro using various colorimetric, fluorescent, or luminescent probes that can often be purchased as a kit. While relatively easy to measure, the amount of cellular ATP does not consistently report on actual mitochondrial function and further experiments are needed to determine if changes in the amount of ATP are connected to changes in mitochondrial function (114).

The rate of ATP synthesis can be measured kinetically using a luciferase-based bioluminescent approach developed by Lanza and Nair (115). This approach also allows for the introduction of various substrates and inhibitors and uses small amounts of isolated mitochondria, but will need multiple conditions to probe individual pathways through the ETS (i.e. succinate and rotenone to measure complex II-mediated respiration) (115). This method is a cost-effective alternative to mitochondrial respirometry, but the interpretation must consider that isolated mitochondria are taken

out of cellular context and that various aspects of mitochondrial function do not always end in ATP production (i.e. leak respiration, ROS production, uncoupling, etc.).

Microscopy allows for the examination of changes in mitochondrial shape, distribution, and appearance and various fluorescent probes can be used to measure other parameters such as mitochondrial membrane potential (114). This allows for the observation of these changes in real-time and within cellular context. While changes in shape, distribution, appearance, and membrane potential are associated with changes in mitochondrial function, these changes do not directly report on mitochondrial respiration and should be carefully interpreted or used in combination with mitochondrial respirometry (114).

The gene expression, protein expression, and activities of representative complexes of the ETS, enzymes of the TCA cycle, transcription factors involved in mitochondrial biogenesis, or markers of fusion and fission are often measured as surrogates of mitochondrial function (114). Again, though the expression of these genes or proteins and enzyme activities are associated with mitochondrial function, these changes do not directly report on mitochondrial respiration and should be carefully interpreted or used in combination with mitochondrial respirometry.

More recently, imaging techniques have been used to measure mitochondrial function *in vivo*, including phosphocreatine recovery following depletion after exercise by phosphorus magnetic resonance spectroscopy (^{31}P -MRS) and monitoring tissue oxygenation during exercise with near-infrared spectroscopy (NIRS) (116). ^{31}P -MRS is used as a measure of ATP production and NIRS is used as a measure of oxygen consumption (116). These techniques are non-invasive, safe, and physiologically relevant, but require equipment and expertise, can be expensive, and require

assumptions and modeling to interpret them (116). These *in vivo* techniques also lack specificity and the ability to explore mechanisms when compare to *in vitro* methods.

D. Mitochondria and aging

i. General features of aging mitochondria

In general, aging is accompanied by a decline in mitochondrial function as shown in **Figure 8**. Studies have found reduced respiratory capacity in older adults, both by reduced phosphocreatine recovery time and reduced mitochondrial respiration (117–119). Many mitochondrial enzyme activities decrease with increasing age, such as succinate dehydrogenase, citrate synthase, cytochrome c oxidase, and β -hydroxyacyl-CoA dehydrogenase (120–122). Reduced mitochondrial DNA (mtDNA) copy number and increased number of mtDNA mutations have been positively associated with age in both animal and human subjects (122–125). Additionally, decreased mitochondrial content and decreased mitochondrial dynamics are associated with aging (124, 126).

ii. Links between aging, body composition, physical function, and mitochondria

The prevalence of obesity increases with age and obesity is associated with increased incidences of cardiovascular diseases, diabetes, physical function decline, sarcopenia, frailty, and mortality (127–131). Increasing age is also associated with decreased lower limb strength (132, 133). This decline in strength leads to slower gait speed, short physical performance battery (SPPB) scores, and reduced 400-meter walk time; all of these measures are potent predictors of disability and mortality in older adults (134–140). Active older adults have higher mitochondrial content and respiratory capacity than sedentary older adults and higher mitochondrial function is associated with improved walking performance in 400-meter walk time (141, 142). Increased subcutaneous,

- ↓ respiratory capacity
- ↓ mito enzyme activities
- ↓ mtDNA copy number
- ↑ mtDNA mutations
- ↑ ROS production
- ↓ mito content
- ↓ mito dynamics



Figure 8. General features of aging mitochondria.

intermuscular, and intramyocellular lipid impairs muscle function and can lead to physical function decline (143, 144). Waist circumference can predict the development of insulin resistance and abdominal obesity is associated with reduced mitochondrial respiration (130, 145). Reductions in the number of subsarcolemmal mitochondria and reduced expression and activities of mitochondrial electron transport chain proteins have been observed in insulin-resistant, obese subjects (146, 147). In addition, our lab has shown that reduced mitochondrial function is associated with increased BMI and adiposity and that improved physical function is associated with improved mitochondrial function in older adults in both blood cells and skeletal muscle tissue (148–150). While the connections between aging, adiposity, physical function, and mitochondrial function are fairly established, few studies have examined the molecular mechanisms involved.

IV. INTERVENTIONS TO IMPROVE HEALTHSPAN

Exercise and lifestyle interventions have shown promise in attenuating the hallmarks of aging and improving healthspan. For example, exercise has been shown to decrease DNA damage, prevent telomere shortening, and change DNA methylation; increase autophagy for improved proteostasis; activate nutrient signaling pathways; improve mitochondrial function and content; reduce senescence; and reduce inflammation (151). The effects of exercise on hallmarks of aging are summarized in **Figure 9** (adapted from (151)). Diet and exercise have been shown to improve mitochondrial function in a variety of models and age groups (152–157). Similar changes have also occurred in severely obese adults that had lost weight, including: reduced energy expenditure, increased work efficiency, and increased protein expression of mitochondrial electron transport chain proteins (158). When sedentary adults are compared to active adults, mitochondrial respiration and markers of fusion and fission are not associated with chronological age but rather strongly associated with biological age, through measures of adiposity and cardiorespiratory fitness (159). Even though caloric restriction and exercise can independently improve insulin sensitivity in older adults, only aerobic exercise increased mitochondrial content and the activities of the electron transport chain and fatty acid oxidation enzymes in one study (160). In combination, caloric restriction and aerobic exercise consistently result in improvements in oxidative capacity, mitochondrial content, and the activities of respiratory chain enzymes (161–164). Resistance training alone has been associated with increases in mitochondrial content, mitochondrial enzyme activities, and mitochondrial respiration, even without weight loss (165–167). Mitochondrial changes induced by diet and exercise are not limited to skeletal muscle and have been observed in the brain, pancreatic beta cells, and skin

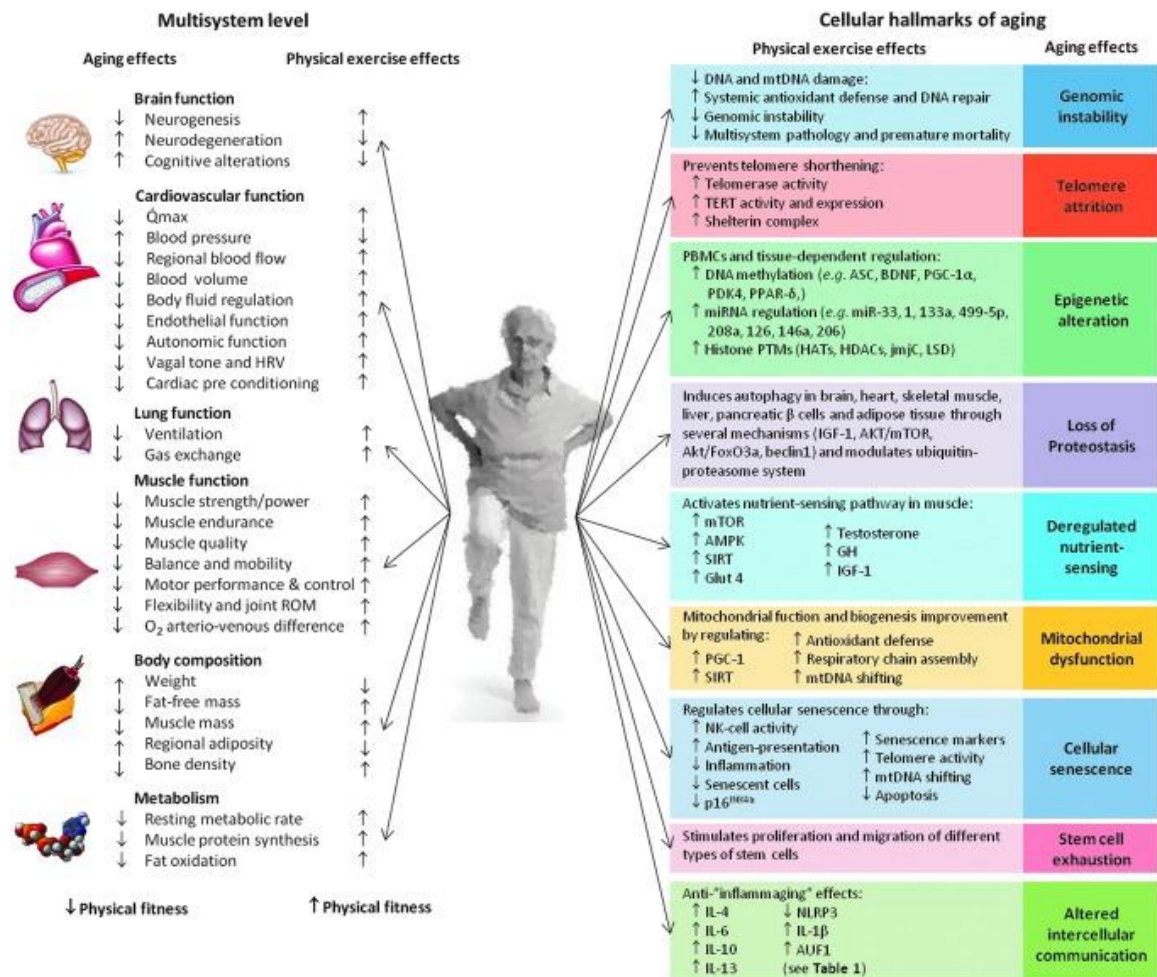


Figure 9. Exercise attenuates many hallmarks of aging. Adapted from N Garatachea et al, Exercise attenuates the major hallmarks of aging, *Rejuvenation Res*, 57-89 (2015).

(168–170). The mechanisms of these mitochondrial changes are not known and further investigation should be completed to begin to understand this process.

Additionally, early lifestyle interventions for individuals with prediabetes and type II diabetes (T2D) have been shown to prevent or delay the progression of the disease (171). The American Diabetes Association recommends weight loss for individuals who are obese or overweight that have or are at risk for developing T2D as the first line of treatment (171). A meta-analysis of lifestyle weight-loss interventions in obese and overweight adults with T2D showed overall trends for improved lipid profiles, decreased blood pressure, and hemoglobin A1c (171). While the overall trends were not statistically significant, the studies that reported greater than 5% weight loss showed statistically significant improvements (171). Another study indicated that a combination of diet and exercise, through caloric restriction and resistance training, can result in 5% weight loss and significantly reduce the presence of metabolic syndrome in obese and overweight older adults when compared to resistance training alone (172). However, losing 5% or more of total body weight is considered an intensive intervention, and the feasibility of such weight loss outside of a clinical trial is questionable. While the majority of participants experience improvements in physical function during lifestyle interventions, other studies have shown that responses are heterogeneous with wide ranges of improvement and a subset of participants that show no changes or a decline after an intervention (173). There is a need to determine the mechanism of how lifestyle interventions can improve metabolic parameters and determine which individuals will respond best to what intervention.

V. BLOOD AND CIRCULATING FACTORS

Circulating factors seem to play a central role in aging, disease processes, and the beneficial effects of lifestyle interventions. Using parabiosis, researchers have been able to show that circulating factors play a role in many processes, such as aging, in a variety of tissues: skeletal muscle, liver, brain, skin, and bone (174–180). Our lab has recently completed experiments that reveal heterochronic parabiosis leads to decreased mitochondrial bioenergetic capacity in young mice connected to old mice (see **Chapter Three** for more detail). Previous work in our lab has also shown that blood cell bioenergetics can recapitulate brain, skeletal muscle, and cardiac muscle respiration. Maximal respiration in mitochondria isolated from the frontal cortex was significantly and positively correlated with maximal respiration in monocytes (181). Respiration in permeabilized skeletal muscle fibers and cardiac muscle isolated mitochondria were also significantly and positively correlated with maximal respiration in monocytes (150). Our lab has also shown that increased levels of circulating interleukin-6 (IL-6) are associated with decreased mitochondrial bioenergetic capacity in peripheral blood mononuclear cells, further supporting a role for circulating factors in mediating mitochondrial metabolism (148). Furthermore, many of the mitochondrial changes associated with aging are systemic and have been shown in multiple tissues, including the brain, heart, skeletal muscle, and liver (122, 182). Taken together, this information suggests that there is a potential role for circulating factors in mediating the age-related decline in mitochondrial function and that this decline can have multiple mechanisms of action, including changes in mitochondrial enzyme expression, mitochondrial content, and reduced mitochondrial dynamics.

Many studies have also included investigations of individual circulating factors in various contexts, such as: aging, obesity, diabetes, diet, and exercise. Circulating levels of IL-6,

IL-1 β , tumor necrosis factor- α (TNF- α), and c-reactive protein (CRP) increase with age (85, 183–187). In centenarians, an increased level of IL-10 has been associated with lower amounts of inflammation and longevity (183). Circulating adipokines, such as adiponectin, increase with age and have been linked to the risk of mortality and developing cardiovascular disease (188). Circulating levels of various hormones, including triiodothyronine, growth hormone, insulin-like growth factor-1 (IGF-1), cortisol, luteinizing hormone, and follicle-stimulating hormone have all been associated with age (189). Sirtuins have been associated with longevity and have been protective against age-related diseases in rodent models (189).

Several circulating factors have been identified as being associated with age-related diseases, such as obesity and T2D. In the general population, inflammatory markers such as CRP, fibrinogen, and IL-6 are associated with cardiovascular disease (CVD) and death and, within the context of T2D, IL-6 is a strong and independent predictor of CVD (190–194). Obesity alone is associated with increased levels of growth factors and binding proteins involved in the growth hormone and insulin-like growth factor system (195). However, obese individuals with T2D had a different combination of upregulation and downregulation of these factors and proteins (195). Other studies have associated metabolites with adiposity and insulin resistance, including: carnitine, triacylglycerols, branched-chain amino acids, and other lipids and hormones (196–199).

In resistance training interventions with older adults, studies have found reductions in CRP, IL-6, leptin, and TNF- α and increases in IL-10 and adiponectin when compared to controls (200–203). Other studies have found that caloric restriction and weight loss are associated with decreases in IL-6, leptin, CRP, and serum amyloid A (SAA) (204, 205). Another study identified specific circulating microRNAs that predicted physical function response to exercise in older adults (206). A summary of potential circulating factors

implicated in aging, diet, and exercise is presented in **Figure 10**. When combined, these data suggest that circulating factors are not only involved in aging and the progression of age-related diseases such as obesity and T2D, but also the response to exercise and weight loss. Additionally, many studies involving exercise and weight-loss interventions in older adults have focused mainly on circulating factors associated with inflammation, highlighting the need to identify other circulating factors for future investigation.

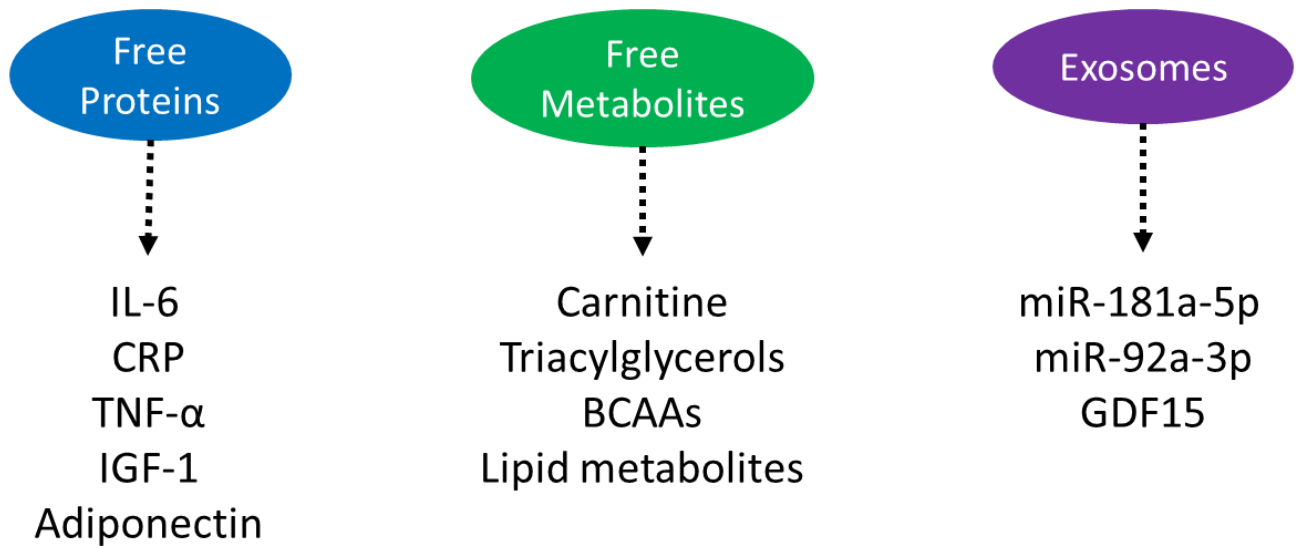


Figure 10. Potential circulating factors present in blood that may impact mitochondrial function in the context of aging and diet and exercise interventions.

VI. SUMMARY OF RESEARCH

As older adults continue to live longer and, as a result, with more comorbidities, there is a continuing need to understand the cellular and molecular processes underlying both healthy aging and age-related diseases. In 2013, cellular and molecular hallmarks were proposed to guide the study of aging, including mitochondrial dysfunction. Mitochondrial dysfunction is multifaceted and includes: reduced respiratory capacity, decreased mitochondrial enzyme activities, decreased mtDNA copy number, increased mtDNA mutations, increased ROS production, decreased mitochondrial content, and decreased mitochondrial dynamics in older adults and organisms. These changes are systemic and have been observed in many tissues, including the brain, heart, skeletal muscle, and liver. Diet and exercise have been shown to improve healthspan and many of the hallmarks of aging, including mitochondrial function, in a variety of models and age groups. Again, these changes are systemic and have been shown in many tissues. Furthermore, our lab and others have shown that blood cell mitochondrial bioenergetics can recapitulate the bioenergetics of the brain, skeletal muscle, and cardiac muscle and that increased circulating IL-6 is associated with decreased mitochondrial bioenergetic capacity in blood cells. Moreover, this information suggests that there is a potential role for circulating factors in mediating the age-related decline in mitochondrial function and the corresponding improvements from interventions such as diet and exercise.

The overarching hypothesis of my work is that there are circulating factors in blood associated with aging, and consequently, age-related diseases and interventions to improve healthspan, that can mediate systemic bioenergetic capacity. This hypothesis guided the body of work presented in this dissertation. In Chapter Two, we examined the effects of long-term consumption of whole dietary patterns, either a Mediterranean or Western diet, on skeletal muscle mitochondrial bioenergetics of female cynomolgus

macaques. In Chapter Three, we investigated the effects of circulating factors on skeletal muscle mitochondrial structure and function in a heterochronic parabiosis mouse model. In Chapter Four, we developed a novel method to determine the effects of human serum on muscle cells in vitro and used metabolomics to identify potential circulating factors responsible for mediating changes in mitochondrial bioenergetics in response to resistance training and caloric restriction. Finally, in Chapter Five, I discuss the implications of the work described in this dissertation.

REFERENCES

1. World Health Organization, Decade of Healthy Aging Baseline Report (2020) (December 17, 2020).
2. J. Oeppen, J. W. Vaupel, Broken Limits to Life Expectancy. *Science* **296**, 1029–1031 (2002).
3. L. J. Waite, Aging, health, and public policy: demographic and economic perspectives. *Popul. Counc.* (2004) <https://doi.org/10.1111/j.1741-6612.2006.00192.x> (December 20, 2020).
4. R. W. Fogel, D. L. Costa, A theory of technophysio evolution, with some implications for forecasting population, health care costs, and pension costs. *Demography* **34**, 49–66 (1997).
5. J. W. Vaupel, *et al.*, Biodemographic Trajectories of Longevity. *Science* **280**, 855–860 (1998).
6. World Health Organization, World Report on Ageing and Health (2015) (December 17, 2020).
7. L. D. Medina, S. Sabo, J. Vespa, Living Longer: Historical and Projected Life Expectancy in the United States, 1960 to 2060. *Curr. Popul. Rep.*, 1–25 (2020).
8. US Census Bureau, Older People Projected to Outnumber Children. *U. S. Census Bur.* (2018) (December 18, 2020).
9. United Nations, World Population Ageing 2017 (2017) (December 17, 2020).
10. , NIA Strategic Directions 2020-2025. *Natl. Inst. Aging* (December 18, 2020).

11. National Institute on Aging, Why Population Aging Matters. 1–32 (2007).
12. E. Palmore, Advantages of Aging. *The Gerontologist* **19**, 220–224 (1979).
13. S. Harper, The positive impacts of an ageing population. *Age Int.* (2020) (March 9, 2021).
14. L. Partridge, J. Deelen, P. E. Slagboom, Facing up to the global challenges of ageing. *Nature* **561**, 45–56 (2018).
15. L. P. Fried, T. L. Bush, MORBIDITY AS A FOCUS OF PREVENTIVE HEALTH CARE IN THE ELDERLY. *Epidemiol. Rev.* **10**, 48–64 (1988).
16. D. R. Seals, J. N. Justice, T. J. LaRocca, Physiological geroscience: targeting function to increase healthspan and achieve optimal longevity. *J. Physiol.* **594**, 2001–2024 (2016).
17. E. Fabbri, *et al.*, Aging and Multimorbidity: New Tasks, Priorities, and Frontiers for Integrated Gerontological and Clinical Research. *J Am Med Dir Assoc* **16**, 640–647 (2015).
18. J. F. Fries, Aging, Natural Death, and the Compression of Morbidity. *N. Engl. J. Med.* **303**, 245–250 (1980).
19. J. F. Fries, Measuring and Monitoring Success in Compressing Morbidity. *Ann. Intern. Med.* **139**, 455–459 (2003).
20. S. Kim, L. Myers, J. Wyckoff, K. E. Cherry, S. M. Jazwinski, The frailty index outperforms DNA methylation age and its derivatives as an indicator of biological age. *GeroScience* **39**, 83–92 (2017).

21. M. E. Levine, Modeling the Rate of Senescence: Can Estimated Biological Age Predict Mortality More Accurately Than Chronological Age? *J. Gerontol. A. Biol. Sci. Med. Sci.* **68**, 667–674 (2013).
22. L. Ferrucci, *et al.*, Measuring biological aging in humans: A quest. *Aging Cell* **19**, e13080 (2020).
23. D. C. Parker, *et al.*, Association of Blood Chemistry Quantifications of Biological Aging With Disability and Mortality in Older Adults. *J. Gerontol. A. Biol. Sci. Med. Sci.* **75**, 1671–1679 (2019).
24. B. T. Weinert, P. S. Timiras, Invited Review: Theories of aging. *J. Appl. Physiol.* **95**, 1706–1716 (2003).
25. J. Haldane, *New Paths in Genetics* (Allen & Unwin, 1941).
26. P. B. Medawar, *An unsolved problem of biology* (Published for the College by H.K. Lewis, 1952).
27. K. A. Hughes, B. Charlesworth, A genetic analysis of senescence in *Drosophila*. *Nature* **367**, 64–66 (1994).
28. B. Charlesworth, K. A. Hughes, Age-specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. *Proc. Natl. Acad. Sci.* **93**, 6140–6145 (1996).
29. K. A. Hughes, J. A. Alipaz, J. M. Drnevich, R. M. Reynolds, A test of evolutionary theories of aging. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 14286–14291 (2002).

30. D. E. Promislow, M. Tatar, A. A. Khazaeli, J. W. Curtsinger, Age-specific patterns of genetic variance in *Drosophila melanogaster*. I. Mortality. *Genetics* **143**, 839–848 (1996).
31. F. H. Shaw, D. E. Promislow, M. Tatar, K. A. Hughes, C. J. Geyer, Toward reconciling inferences concerning genetic variation in senescence in *Drosophila melanogaster*. *Genetics* **152**, 553–566 (1999).
32. G. C. Williams, Pleiotropy, Natural Selection, and the Evolution of Senescence. *Evolution* **11**, 398–411 (1957).
33. T. B. L. Kirkwood, Evolution of ageing. *Nature* **270**, 301–304 (1977).
34. F. Drenos, T. B. L. Kirkwood, Modelling the disposable soma theory of ageing. *Mech. Ageing Dev.* **126**, 99–103 (2005).
35. S. C. Stearns, M. Ackermann, M. Doebeli, M. Kaiser, Experimental evolution of aging, growth, and reproduction in fruitflies. *Proc. Natl. Acad. Sci.* **97**, 3309–3313 (2000).
36. C. M. Sgrò, L. Partridge, A delayed wave of death from reproduction in *Drosophila*. *Science* **286**, 2521–2524 (1999).
37. W. A. V. Voorhies, S. Ward, Genetic and environmental conditions that increase longevity in *Caenorhabditis elegans* decrease metabolic rate. *Proc. Natl. Acad. Sci.* **96**, 11399–11403 (1999).
38. T. E. Johnson, E. W. Hutchinson, Absence of strong heterosis for life span and other life history traits in *Caenorhabditis elegans*. *Genetics* **134**, 465–474 (1993).

39. D. W. Walker, G. McColl, N. L. Jenkins, J. Harris, G. J. Lithgow, Evolution of lifespan in *C. elegans*. *Nature* **405**, 296–297 (2000).
40. C. P. Bwiza, J. M. Son, C. Lee, “Integrated Theories of Biological Aging” in *Oxford Research Encyclopedia of Psychology*, (Oxford University Press, 2019)
<https://doi.org/10.1093/acrefore/9780190236557.013.334> (December 20, 2020).
41. M. S. Kanungo, A model for ageing. *J. Theor. Biol.* **53**, 253–261 (1975).
42. S. D. Pletcher, *et al.*, Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr. Biol. CB* **12**, 712–723 (2002).
43. V. Reinke, Functional exploration of the *C. elegans* genome using DNA microarrays. *Nat. Genet.* **32 Suppl**, 541–546 (2002).
44. R. Weindruch, T. Kayo, C. K. Lee, T. A. Prolla, Gene expression profiling of aging using DNA microarrays. *Mech. Ageing Dev.* **123**, 177–193 (2002).
45. S. Zou, S. Meadows, L. Sharp, L. Y. Jan, Y. N. Jan, Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13726–13731 (2000).
46. T. Perls, L. Kunkel, A. Puca, The genetics of aging. *Curr. Opin. Genet. Dev.* **12**, 362–369 (2002).
47. T. T. Perls, *et al.*, Life-long sustained mortality advantage of siblings of centenarians. *Proc. Natl. Acad. Sci.* **99**, 8442–8447 (2002).

48. A. A. Puca, *et al.*, A genome-wide scan for linkage to human exceptional longevity identifies a locus on chromosome 4. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10505–10508 (2001).
49. H. Bae, *et al.*, Effects of FOXO3 Polymorphisms on Survival to Extreme Longevity in Four Centenarian Studies. *J. Gerontol. Ser. A* **73**, 1439–1447 (2018).
50. N. van den Berg, *et al.*, Longevity defined as top 10% survivors and beyond is transmitted as a quantitative genetic trait. *Nat. Commun.* **10**, 35 (2019).
51. D. Melzer, L. C. Pilling, L. Ferrucci, The genetics of human ageing. *Nat. Rev. Genet.* **21**, 88–101 (2020).
52. M. Tatar, A. Bartke, A. Antebi, The endocrine regulation of aging by insulin-like signals. *Science* **299**, 1346–1351 (2003).
53. M. Barbieri, M. Bonafè, C. Franceschi, G. Paolisso, Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *Am. J. Physiol. Endocrinol. Metab.* **285**, E1064-1071 (2003).
54. C. J. Kenyon, The genetics of ageing. *Nature* **464**, 504–512 (2010).
55. T. Weichhart, mTOR as Regulator of Lifespan, Aging, and Cellular Senescence: A Mini-Review. *Gerontology* **64**, 127–134 (2018).
56. D. E. Harrison, *et al.*, Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392–395 (2009).
57. L. Hayflick, The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614–636 (1965).

58. T. Kuilman, C. Michaloglou, W. J. Mooi, D. S. Peeper, The essence of senescence. *Genes Dev.* **24**, 2463–2479 (2010).
59. M. A. Blasco, *et al.*, Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91**, 25–34 (1997).
60. H. W. Lee, *et al.*, Essential role of mouse telomerase in highly proliferative organs. *Nature* **392**, 569–574 (1998).
61. Y. Kim, H. G. Nam, D. R. Valenzano, The short-lived African turquoise killifish: an emerging experimental model for ageing. *Dis. Model. Mech.* **9**, 115–129 (2016).
62. S. Valdesalici, A. Cellerino, Extremely short lifespan in the annual fish *Nothobranchius furzeri*. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, S189–S191 (2003).
63. I. Harel, *et al.*, A Platform for Rapid Exploration of Aging and Diseases in a Naturally Short-Lived Vertebrate. *Cell* **160**, 1013–1026 (2015).
64. N. Hartmann, *et al.*, Telomeres shorten while Tert expression increases during ageing of the short-lived fish *Nothobranchius furzeri*. *Mech. Ageing Dev.* **130**, 290–296 (2009).
65. J. R. Mitchell, E. Wood, K. Collins, A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* **402**, 551–555 (1999).
66. T. Vulliamy, *et al.*, The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature* **413**, 432–435 (2001).

67. R. Di Micco, V. Krizhanovsky, D. Baker, F. d'Adda di Fagagna, Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat. Rev. Mol. Cell Biol.* **22**, 75–95 (2021).
68. D. Harman, Aging: A Theory Based on Free Radical and Radiation Chemistry. *J. Gerontol.* **11**, 298–300 (1956).
69. T. Finkel, N. J. Holbrook, Oxidants, oxidative stress and the biology of ageing. *Nature*, 239–247 (2000).
70. A. Sanz, R. K. A. Stefanatos, The Mitochondrial Free Radical Theory of Aging. *Curr. Aging Sci.* **1**, 10–21 (2008).
71. J. Tower, Transgenic methods for increasing *Drosophila* life span. *Mech. Ageing Dev.*, 1–14 (2000).
72. R. Arking, *et al.*, Forward and reverse selection for longevity in *Drosophila* is characterized by alteration of antioxidant gene expression and oxidative damage patterns☆. *Exp. Gerontol.* **35**, 167–185 (2000).
73. P. Larsen, Aging and resistance to oxidative damage in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci.*, 8905–8909 (1993).
74. R. J. Mehlhorn, “Oxidants and antioxidants in aging” in *Physiological Basis of Aging and Geriatrics*, 3rd Ed., (CRC, 2003), pp. 61–83.
75. T. T. Huang, E. J. Carlson, A. M. Gillespie, Y. Shi, C. J. Epstein, Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. *J. Gerontol. A. Biol. Sci. Med. Sci.* **55**, B5-9 (2000).

76. A. Caratero, M. Courtade, L. Bonnet, H. Planel, C. Caratero, Effect of a continuous gamma irradiation at a very low dose on the life span of mice. *Gerontology* **44**, 272–276 (1998).
77. A. Vijayakumar, S. Yakar, D. Leroith, The Intricate Role of Growth Hormone in Metabolism. *Front. Endocrinol.* **2** (2011).
78. O. Altintas, S. Park, S.-J. V. Lee, The role of insulin/IGF-1 signaling in the longevity of model invertebrates, *C. elegans* and *D. melanogaster*. *BMB Rep.* **49**, 81–92 (2016).
79. L. Fontana, L. Partridge, Promoting Health and Longevity through Diet: from Model Organisms to Humans. *Cell* **161**, 106–118 (2015).
80. V. D. Longo, Evolutionary Medicine: From Dwarf Model Systems to Healthy Centenarians? *Science* **299**, 1342–1346 (2003).
81. G. A. Kleeman, C. T. Murphy, The endocrine regulation of aging in *Caenorhabditis elegans*. *Mol. Cell. Endocrinol.*, 51–57 (2009).
82. M. Giannakou, L. Partridge, Role of insulin-like signalling in *Drosophila* lifespan. *Trends Biochem. Sci.* **32**, 180–188 (2007).
83. Y. Suh, *et al.*, Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proc. Natl. Acad. Sci.* **105**, 3438–3442 (2008).
84. C. N. Serhan, N. Chiang, J. Dalli, The Resolution Code of Acute Inflammation: Novel Pro-Resolving Lipid Mediators in Resolution. *Semin. Immunol.* **27**, 200–215 (2015).

85. C. Franceschi, *et al.*, Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann. N. Y. Acad. Sci.* **908**, 244–54 (2000).
86. W. B. Ershler, Interleukin-6: A Cytokine for Gerontologists. *J. Am. Geriatr. Soc.* **41**, 176–181 (1993).
87. U. Fagiolo, *et al.*, Increased cytokine production in mononuclear cells of healthy elderly people. *Eur. J. Immunol.* **23**, 2375–2378 (1993).
88. T. Singh, A. B. Newman, Inflammatory markers in population studies of aging. *Ageing Res. Rev.* **10**, 319–329 (2011).
89. J. Wei, H. Xu, J. L. Davies, G. P. Hemmings, Increase of plasma IL-6 concentration with age in healthy subjects. *Life Sci.* **51**, 1953–1956 (1992).
90. P. Libby, Inflammation in atherosclerosis. *Nature* **420**, 868–874 (2002).
91. H. Bruunsgaard, *et al.*, A High Plasma Concentration of TNF- α Is Associated With Dementia in Centenarians. *J. Gerontol. Ser. A* **54**, M357–M364 (1999).
92. E. M. Sutinen, T. Pirttilä, G. Anderson, A. Salminen, J. O. Ojala, Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid- β production in human neuron-like cells. *J. Neuroinflammation* **9**, 199 (2012).
93. H. Xu, *et al.*, Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **112**, 1821–1830 (2003).
94. K. E. Wellen, G. S. Hotamisligil, Inflammation, stress, and diabetes. *J. Clin. Invest.* **115**, 1111–1119 (2005).

95. F. Balkwill, A. Mantovani, Inflammation and cancer: back to Virchow? *Lancet Lond. Engl.* **357**, 539–545 (2001).
96. L. M. Coussens, Z. Werb, Inflammation and cancer. *Nature*, 860–867 (2002).
97. D. Sarkar, P. B. Fisher, Molecular mechanisms of aging-associated inflammation. *Cancer Lett.* **236**, 13–23 (2006).
98. S. U. Amano, *et al.*, Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation. *Cell Metab.* **19**, 162–171 (2014).
99. H. Tilg, A. R. Moschen, Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat. Rev. Immunol.* **6**, 772–783 (2006).
100. J.-P. Coppé, *et al.*, Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLOS Biol.* **6**, e301 (2008).
101. C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, G. Kroemer, The Hallmarks of Aging. *Cell* **153**, 1194–1217 (2013).
102. S. van der Rijt, M. Molenaars, R. L. McIntyre, G. E. Janssens, R. H. Houtkooper, Integrating the Hallmarks of Aging Throughout the Tree of Life: A Focus on Mitochondrial Dysfunction. *Front. Cell Dev. Biol.* **8** (2020).
103. N. Sun, R. J. Youle, T. Finkel, The Mitochondrial Basis of Aging. *Mol. Cell* **61**, 654–666 (2016).
104. B. Alberts, *et al.*, *Molecular Biology of the Cell*, 5th Ed. (Garland Science, Taylor and Francis Group, LLC, 2008).

105. N. Lane, W. Martin, The energetics of genome complexity. *Nature* **467**, 929–934 (2010).
106. J. Nunnari, A. Suomalainen, Mitochondria: In Sickness and in Health. *Cell* **148**, 1145–1159 (2012).
107. H. M. McBride, M. Neuspiel, S. Wasiak, Mitochondria: More Than Just a Powerhouse. *Curr. Biol.* **16**, R551–R560 (2006).
108. A. J. A. Molina, *et al.*, Mitochondrial Networking Protects β -Cells From Nutrient-Induced Apoptosis. *Diabetes* **58**, 2303 (2009).
109. J. B. Reece, *et al.*, *Campbell Biology*, 10th Ed. (Pearson Education, 2014).
110. T. Hayashi, R. Rizzuto, G. Hajnoczky, T.-P. Su, MAM: more than just a housekeeper. *Trends Cell Biol.* **19**, 81–88 (2009).
111. E. Gnaiger, *Mitochondrial Pathways and Respiratory Control: An Introduction to OXPHOS Analysis*, 4th Ed. (Oroboros Instruments, 2014).
112. K. Ikeda, T. Yamada, UCP1 Dependent and Independent Thermogenesis in Brown and Beige Adipocytes. *Front. Endocrinol.* **11** (2020).
113. M. R. Duchen, Mitochondria and calcium: from cell signalling to cell death. *J. Physiol.* **529**, 57–68 (2000).
114. M. D. Brand, D. G. Nicholls, Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435**, 297–312 (2011).
115. I. R. Lanza, K. S. Nair, Functional Assessment of Isolated Mitochondria In Vitro. *Methods Enzymol.* **457**, 349–372 (2009).

116. I. R. Lanza, K. S. Nair, Mitochondrial Metabolic Function Assessed In Vivo and In Vitro. *Curr. Opin. Clin. Nutr. Metab. Care* **13**, 511–517 (2010).
117. M. Tonkonogi, *et al.*, Reduced oxidative power but unchanged antioxidative capacity in skeletal muscle from aged humans. *Pflugers Arch.* **446**, 261–269 (2003).
118. K. Falk Petersen, *et al.*, Mitochondrial Dysfunction in the Elderly: Possible Role in Insulin Resistance. *Science* **300**, 1140–1142 (2003).
119. D. L. Johannsen, *et al.*, Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *J. Clin. Endocrinol. Metab.* **97**, 242–250 (2012).
120. A. R. Coggan, *et al.*, Histochemical and Enzymatic Comparison of the Gastrocnemius-Muscle of Young and Elderly Men and Women. *J. Gerontol.* **47**, B71–B76 (1992).
121. O. E. Rooyackers, D. B. Adey, P. A. Ades, K. S. Nair, Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15364–9 (1996).
122. R. Barazzoni, K. R. Short, K. S. Nair, Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. *J Biol Chem* **275**, 3343–3347 (2000).
123. S. Melov, J. M. Shoffner, A. Kaufman, D. C. Wallace, Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. *Nucleic Acids Res.* **23**, 4938 (1995).

124. K. E. Conley, S. A. Jubrias, P. C. Esselman, Oxidative capacity and ageing in human muscle. *J. Physiol.* **526**, 203–210 (2000).
125. K. R. Short, *et al.*, Decline in skeletal muscle mitochondrial function with aging in humans. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 5618–23 (2005).
126. S. Srivastava, The Mitochondrial Basis of Aging and Age-Related Disorders. *Genes* **8** (2017).
127. M. D. Jensen, *et al.*, 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: A report of the American College of cardiology/American Heart Association task force on practice guidelines and the obesity society. *Circulation* **129** (2014).
128. M.-P. St-Onge, Relationship between body composition changes and changes in physical function and metabolic risk factors in aging. *Curr Opin Clin Nutr Metab Care* **8**, 523–528 (2005).
129. K. N. Manolopoulos, F. Karpe, K. N. Frayn, Gluteofemoral body fat as a determinant of metabolic health. *Int. J. Obes.* **34**, 949–959 (2010).
130. G. Nilsson, *et al.*, Waist circumference alone predicts insulin resistance as good as the metabolic syndrome in elderly women. *Eur. J. Intern. Med.* **19**, 520–526 (2008).
131. D. K. Houston, B. J. Nicklas, C. A. Zizza, Weighty Concerns: The Growing Prevalence of Obesity among Older Adults. *J. Am. Diet. Assoc.* **109**, 1886–1895 (2009).

132. G. Onder, *et al.*, Change in physical performance over time in older women: the Women's Health and Aging Study. *J. Gerontol. - Ser. Biol. Sci. Med. Sci.* **57**, M289-93 (2002).
133. F. Lauretani, *et al.*, Age-associated changes in skeletal muscles and their effect on mobility: an operational diagnosis of sarcopenia. *J. Appl. Physiol.* **95**, 1851–1860 (2003).
134. G. E. Hicks, *et al.*, Absolute strength and loss of strength as predictors of mobility decline in older adults: The InCHIANTI study. *J. Gerontol. - Ser. Biol. Sci. Med. Sci.* **67 A**, 66–73 (2012).
135. M. Rantakokko, M. Mänty, T. Rantanen, Mobility decline in old age. *Exerc. Sport Sci. Rev.* **41**, 19–25 (2013).
136. G. Onder, *et al.*, Measures of Physical Performance and Risk for Progressive and Catastrophic Disability: Results From the Women's Health and Aging Study. *J. Gerontol. - Ser. Biol. Sci. Med. Sci.* **60**, 74–79 (2005).
137. S. Studenski, *et al.*, Gait speed and survival in older adults. *JAMA - J. Am. Med. Assoc.* **305**, 50–58 (2011).
138. M. Chang, *et al.*, Incidence of loss of ability to walk 400 meters in a functionally limited older population. *J. Am. Geriatr. Soc.* **52**, 2094–2098 (2004).
139. J. M. Guralnik, *et al.*, Lower Extremity Function and Subsequent Disability: Consistency Across Studies, Predictive Models, and Value of Gait Speed Alone Compared With the Short Physical Performance Battery. *J. Gerontol. A. Biol. Sci. Med. Sci.* **55**, M221–M231 (2000).

140. S. Vasunilashorn, *et al.*, Use of the short physical performance battery score to predict loss of ability to walk 400 meters: Analysis from the InCHIANTI study. *J. Gerontol. - Ser. Biol. Sci. Med. Sci.* **64**, 223–229 (2009).
141. A. J. Santanasto, *et al.*, Skeletal Muscle Mitochondrial Function and Fatigability in Older Adults. *J. Gerontol. A. Biol. Sci. Med. Sci.* **70**, 1379–1385 (2015).
142. A. J. Santanasto, *et al.*, The relationship between mitochondrial function and walking performance in older adults with a wide range of physical function. *Exp. Gerontol.* **81**, 1–7 (2016).
143. B. H. Goodpaster, *et al.*, Attenuation of skeletal muscle and strength in the elderly: The Health ABC Study. *J. Appl. Physiol. Bethesda Md 1985* **90**, 2157–65 (2001).
144. S. J. Choi, *et al.*, Intramyocellular lipid and impaired myofiber contraction in normal weight and obese older adults. *J. Gerontol. - Ser. Biol. Sci. Med. Sci.* **71**, 557–564 (2016).
145. E. Chanseume, *et al.*, Muscle mitochondrial oxidative phosphorylation activity, but not content, is altered with abdominal obesity in sedentary men: Synergism with changes in insulin sensitivity. *J. Clin. Endocrinol. Metab.* **95**, 2948–2956 (2010).
146. V. B. Ritov, *et al.*, Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes* **54**, 8–14 (2005).
147. H. Hwang, *et al.*, Proteomics analysis of human skeletal muscle reveals novel abnormalities in obesity and type 2 diabetes. *Diabetes* **59**, 33–42 (2010).

148. D. J. Tyrrell, *et al.*, Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. *Exp. Gerontol.* **70**, 84–91 (2015).
149. M. S. Bharadwaj, *et al.*, Relationships between mitochondrial content and bioenergetics with obesity , body composition and fat distribution in healthy older adults. *BMC Obes.*, 1–11 (2015).
150. D. J. Tyrrell, M. S. Bharadwaj, M. J. Jorgensen, T. C. Register, A. J. A. Molina, Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: Implications for a minimally invasive biomarker of mitochondrial health. *Redox Biol.* **10**, 65–77 (2016).
151. N. Garatachea, *et al.*, Exercise Attenuates the Major Hallmarks of Aging. *Rejuvenation Res.* **18**, 57–89 (2015).
152. E. V Menshikova, *et al.*, Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J. Gerontol. A. Biol. Sci. Med. Sci.* **61**, 534–40 (2006).
153. I. R. Lanza, K. S. Nair, Muscle mitochondrial changes with aging and exercise. *Am. J. Clin. Nutr.* **89**, 467S–71S (2009).
154. F. G. S. Toledo, B. H. Goodpaster, The role of weight loss and exercise in correcting skeletal muscle mitochondrial abnormalities in obesity, diabetes and aging. *Mol. Cell. Endocrinol.* **379**, 30–34 (2013).
155. J. C. Drake, R. J. Wilson, Z. Yan, Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle. *FASEB J.* **30**, 13–22 (2016).

156. H. W. Hyatt, *et al.*, A Ketogenic Diet in Rodents Elicits Improved Mitochondrial Adaptations in Response to Resistance Exercise Training Compared to an Isocaloric Western Diet. *Front. Physiol.* **7**, 533 (2016).
157. K. Barbosa de Queiroz, *et al.*, Physical activity prevents alterations in mitochondrial ultrastructure and glucometabolic parameters in a high-sugar diet model. *PLOS ONE* **12**, e0172103 (2017).
158. K. C. Hames, *et al.*, Resting and exercise energy metabolism in weight-reduced adults with severe obesity. *Obesity* **24**, 1290–1298 (2016).
159. G. Distefano, *et al.*, Chronological Age Does not Influence Ex-vivo Mitochondrial Respiration and Quality Control in Skeletal Muscle. *J. Gerontol. Ser. A* **72**, 535–542 (2017).
160. E. V. Menshikova, *et al.*, Calorie Restriction-induced Weight Loss and Exercise Have Differential Effects on Skeletal Muscle Mitochondria Despite Similar Effects on Insulin Sensitivity. *J. Gerontol. Ser. A* **73**, 81–87 (2017).
161. E. V. Menshikova, Effects of weight loss and physical activity on skeletal muscle mitochondrial function in obesity. *AJP Endocrinol. Metab.* **288**, E818–E825 (2004).
162. F. G. S. Toledo, S. Watkins, D. E. Kelley, Changes Induced by Physical Activity and Weight Loss in the Morphology of Intermyo-fibrillar Mitochondria in Obese Men and Women. *J. Clin. Endocrinol. Metab.* **91**, 3224–3227 (2006).
163. F. G. S. Toledo, *et al.*, Effects of Physical Activity and Weight Loss on Skeletal Muscle Mitochondria and Relationship With Glucose Control in Type 2 Diabetes. *Diabetes* **56**, 2142–2147 (2007).

164. T. Fritz, *et al.*, Low-intensity exercise increases skeletal muscle protein expression of PPAR δ and UCP3 in type 2 diabetic patients. *Diabetes Metab. Res. Rev.* **22**, 492–498 (2006).
165. E. Phielix, R. Meex, E. Moonen-Kornips, M. K. C. Hesselink, P. Schrauwen, Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. *Diabetologia* **53**, 1714–1721 (2010).
166. R. C. R. Meex, *et al.*, Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes* **59**, 572–579 (2010).
167. C. Porter, P. T. Reidy, N. Bhattarai, L. S. Sidossis, B. B. Rasmussen, Resistance Exercise Training Alters Mitochondrial Function in Human Skeletal Muscle. *Med. Sci. Sports Exerc.* **47**, 1922–1931 (2015).
168. A. M. Gusdon, *et al.*, Exercise increases mitochondrial complex I activity and DRP1 expression in the brains of aged mice. *Exp. Gerontol.* **90**, 1–13 (2017).
169. F. M. Cerqueira, *et al.*, Diluted serum from calorie-restricted animals promotes mitochondrial b-cell adaptations and protect against glucolipotoxicity. *FEBS J.* **283**, 822–833 (2016).
170. M. F. Forni, *et al.*, Caloric Restriction Promotes Structural and Metabolic Changes in the Skin. *Cell Rep.* **20**, 2678–2692 (2017).

171. M. J. Franz, J. L. Boucher, S. Rutten-Ramos, J. J. VanWormer, Lifestyle Weight-Loss Intervention Outcomes in Overweight and Obese Adults with Type 2 Diabetes: A Systematic Review and Meta-Analysis of Randomized Clinical Trials. *J. Acad. Nutr. Diet.* **115**, 1447–1463 (2015).
172. E. Normandin, E. Chmelo, M. F. Lyles, A. P. Marsh, B. J. Nicklas, Effect of Resistance Training and Caloric Restriction on the Metabolic Syndrome. *Med. Sci. Sports Exerc.* **49**, 413–419 (2017).
173. E. A. Chmelo, *et al.*, Heterogeneity of Physical Function Responses to Exercise Training in Older Adults. *J. Am. Geriatr. Soc.* **63**, 462–469 (2015).
174. I. M. Conboy, T. A. Rando, Heterochronic parabiosis for the study of the effects of aging on stem cells and their niches. *Cell Cycle* **11**, 2260–2267 (2012).
175. H. Yousef, *et al.*, Systemic attenuation of the TGF- β pathway by a single drug simultaneously rejuvenates hippocampal neurogenesis and myogenesis in the same old mammal. *Oncotarget* **6**, 11959–78 (2015).
176. J. Rebo, *et al.*, ARTICLE A single heterochronic blood exchange reveals rapid inhibition of multiple tissues by old blood. *Nat. Commun.* **7** (2016).
177. S. Salpeter, *et al.*, Systemic regulation of the age-related decline of pancreatic β -cell replication. *Diabetes* 2013;62:2843–2848. *Diabetes* **62**, 3300 (2013).
178. S. A. Villeda, *et al.*, The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* **477**, 90–94 (2011).
179. F. S. Loffredo, *et al.*, Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell* **153**, 828–839 (2013).

180. X.-L. Bu, *et al.*, Blood-derived amyloid- β protein induces Alzheimer's disease pathologies. *Mol. Psychiatry* **23**, 1948–1956 (2018).
181. D. J. Tyrrell, *et al.*, Blood-based bioenergetic profiling reflects differences in brain bioenergetics and metabolism. *Oxid. Med. Cell. Longev.* **2017**, 1–9 (2017).
182. C. Desler, *et al.*, Is There a Link between Mitochondrial Reserve Respiratory Capacity and Aging? *J. Aging Res.* **2012**, 1–9 (2012).
183. K. H. Wagner, D. Cameron-Smith, B. Wessner, B. Franzke, Biomarkers of aging: From function to molecular biology. *Nutrients* **8** (2016).
184. S. G. Lakoski, *et al.*, Adiposity, inflammation, and risk for death in black and white men and women in the United States: the Reasons for Geographic and Racial Differences in Stroke (REGARDS) study. *J. Clin. Endocrinol. Metab.* **96**, 1805–14 (2011).
185. E. K. Kabagambe, *et al.*, Inflammation biomarkers and risk of all-cause mortality in the Reasons for Geographic And Racial Differences in Stroke cohort. *Am. J. Epidemiol.* **174**, 284–92 (2011).
186. Emerging Risk Factors Collaboration, *et al.*, C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *The Lancet* **375**, 132–140 (2010).
187. M. Visser, *et al.*, Relationship of Interleukin-6 and Tumor Necrosis Factor- With Muscle Mass and Muscle Strength in Elderly Men and Women: The Health ABC Study. *J. Gerontol. A. Biol. Sci. Med. Sci.* **57**, M326–M332 (2002).

188. J. Poehls, *et al.*, Association of adiponectin with mortality in older adults: the Health, Aging, and Body Composition Study. *Diabetologia* **52**, 591–5 (2009).
189. P. M. Engelfriet, E. H. J. M. Jansen, H. S. J. Picavet, M. E. T. Dolle, Biochemical Markers of Aging for Longitudinal Studies in Humans. *Epidemiol. Rev.* **35**, 132–151 (2013).
190. G. Lowe, *et al.*, Circulating Inflammatory Markers and the Risk of Vascular Complications and Mortality in People With Type 2 Diabetes and Cardiovascular Disease or Risk Factors: The ADVANCE Study. *Diabetes* **63**, 1115 LP – 1123 (2014).
191. P. A. Kern, S. Ranganathan, C. Li, L. Wood, G. Ranganathan, Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am. J. Physiol.-Endocrinol. Metab.* **280**, E745–E751 (2001).
192. J. N. Fain, A. K. Madan, M. L. Hiler, P. Cheema, S. W. Bahouth, Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* **145**, 2273–2282 (2004).
193. J. S. Yudkin, M. Kumari, S. E. Humphries, V. Mohamed-Ali, Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* **148**, 209–14 (2000).
194. S. W. Coppack, Pro-inflammatory cytokines and adipose tissue. *Proc. Nutr. Soc.* **60**, 349–356 (2001).

195. J. Frystyk, C. Skjærbæk, E. Vestbo, S. Fisker, H. Ørskov, Circulating levels of free insulin-like growth factors in obese subjects: the impact of Type 2 diabetes. *Diabetes Metab. Res. Rev.* **15**, 314–322 (1999).
196. R. A. Murphy, *et al.*, Metabolites Associated With Lean Mass and Adiposity in Older Black Men. *J. Gerontol. Ser. A* **72**, 1352–1359 (2017).
197. U. N. Das, Is there a role for bioactive lipids in the pathobiology of diabetes mellitus? *Front. Endocrinol.* **8** (2017).
198. V. T. Samuel, K. F. Petersen, G. I. Shulman, Lipid-induced insulin resistance: unravelling the mechanism. *The Lancet* **375**, 2267–2277 (2010).
199. D. E. Kelley, B. H. Goodpaster, Stewing in Not-So-Good Juices: Interactions of Skeletal Muscle With Adipose Secretions. *Diabetes* **64**, 3055–7 (2015).
200. A. S. Ribeiro, *et al.*, Effect of resistance training on C-reactive protein, blood glucose and lipid profile in older women with differing levels of RT experience. *Age* **37**, 109 (2015).
201. L. N. Forti, *et al.*, Strength training reduces circulating interleukin-6 but not brain-derived neurotrophic factor in community-dwelling elderly individuals. *Age* **36**, 9704 (2014).
202. M. D. Phillips, *et al.*, Resistance training reduces subclinical inflammation in obese, postmenopausal women. *Med. Sci. Sports Exerc.* **44**, 2099–2110 (2012).
203. B. F. De Salles, *et al.*, Effects of resistance training on cytokines. *Int. J. Sports Med.* **31**, 441–450 (2010).

204. I. Imayama, *et al.*, Effects of a caloric restriction weight loss diet and exercise on inflammatory biomarkers in overweight/obese postmenopausal women: a randomized controlled trial. *Cancer Res.* **72**, 2314–2326 (2012).
205. K. M. Beavers, W. T. Ambrosius, B. J. Nicklas, W. J. Rejeski, Independent and combined effects of physical activity and weight loss on inflammatory biomarkers in overweight and obese older adults. *J. Am. Geriatr. Soc.* **61**, 1089–1094 (2013).
206. T. Zhang, *et al.*, Circulating MiRNAs as biomarkers of gait speed responses to aerobic exercise training in obese older adults. *Aging* **9**, 900–913 (2017).

CHAPTER TWO:

SKELETAL MUSCLE MITOCHONDRIAL RESPIRATION IS ELEVATED IN FEMALE CYNOMOLGUS MACAQUES FED A WESTERN VERSUS MEDITERRANEAN DIET

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Skeletal muscle mitochondrial respiration is elevated in female cynomolgus macaques fed a Western versus Mediterranean diet^{1,2,3,4}

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²Abbreviations

AUC: area under the curve

BCA: bicinchoninic acid

CPT: carnitine palmitoyl transferase

CPT1: carnitine palmitoyl transferase 1

CS: citrate synthase

ER: endoplasmic reticulum

ETS: electron transport system

FAO: fatty acid oxidation

FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

HOMA-IR: homeostatic model of insulin resistance

iv-GTT: intravenous glucose tolerance test

Rot: rotenone

SDH: succinate dehydrogenase

SUIT: substrate-uncoupler-inhibitor titration

UCP3: uncoupling protein 3

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Anthony J.A. Molina, no conflict of interest

Abstract

Background. Western diets are associated with increased incidences of obesity, hypertension, diabetes, and hypercholesterolemia, while Mediterranean diets, richer in polyphenols, monounsaturated fats, fruits, vegetables, poultry, and fish, appear to have cardiometabolic health benefits. Previous work has included population-based studies with limited evidence for causation or animal studies focused on single macro or micronutrients; therefore, primate animal models provide an opportunity to determine potential mechanisms underlying the effects of dietary patterns on health and disease.

Objective. Our objective was to determine the effects of whole dietary patterns, either a Western or Mediterranean diet, on skeletal muscle mitochondrial bioenergetics in cynomolgus macaques.

Methods. In this study, 22 adult female cynomolgus macaques (approximately 11-14 years by dentition) were fed either a Western or Mediterranean diet for 30 months. The Western diet was designed to mimic the diet of a middle-aged American woman and the Mediterranean diet included key aspects of Mediterranean diets studied in humans, such as plant-based proteins and fat, complex carbohydrates, and fiber. Diets were matched on macronutrient composition (16% protein, 54% carbohydrate, and 31% fat) and cholesterol content. Skeletal muscle was collected for high-resolution respirometry, citrate synthase activity, and western blot measurements. Pearson correlation analysis between respirometry measures and measures of carbohydrate metabolism was also performed.

Results. We found that consumption of a Western diet resulted in significantly higher mitochondrial respiration with FAO (53%), FAO + complex I (52%), complex I + II (31%), max ETS (31%), and ETS Rot sensitive (31%) when compared to the Mediterranean diet.

Additionally, measures of respiration in response to fatty acids were significantly and positively correlated with both insulin resistance and plasma insulin concentrations.

Conclusions. This study highlights the importance of dietary composition in mitochondrial bioenergetics and that diet can influence skeletal muscle mitochondrial respiration, independent of other factors, such as macronutrient composition.

Keywords: Western, Mediterranean, diet, skeletal muscle, mitochondria, bioenergetics, non-human primates

Introduction

Typical Western diets contain high amounts of saturated fats, sucrose and fructose, proteins from red meats, sodium, and low amounts of monounsaturated and polyunsaturated fats. Consumption of a Western diet has been associated with increased incidences of obesity, all-cause mortality, cancer, kidney disease, osteoporosis, hypertension, type 2 diabetes, and hypercholesterolemia (1–3). In contrast, Mediterranean diets are composed of high amounts of monounsaturated fats from mainly plant sources such as olive oil, fruits, vegetables, and proteins from poultry and fish sources. Consumption of a Mediterranean diet has been associated with reduced risk of developing diabetes, cancer, cardiovascular disease, and Alzheimer's disease (4–7). These associations with dietary patterns have been elucidated primarily by population-based epidemiological studies; therefore, there is limited evidence for causation and a need for mechanistic insights.

Animal studies have reported that a high-fat diet increases fatty acid, but not pyruvate, malate, or glutamate, mediated respiration of mitochondria isolated from skeletal muscle (8–10). Increased expression of oxidative phosphorylation proteins and uncoupling protein 3 (UCP3) were also observed. When mice were fed a high fat and high sucrose diet, increased mitochondrial respiration in permeabilized skeletal muscle fibers, increased citrate synthase (CS) and carnitine palmitoyl transferase (CPT) activity, and increased expression of UCP3, CS, and complex I was observed (11). Rodents in these studies were obese and insulin resistant, but not overtly diabetic. In humans, previous studies demonstrate decreased mitochondrial enzyme activity in subjects with type 2 diabetes and obesity. Obese subjects had lower succinate dehydrogenase (SDH or complex II) activity (12), lower CPT activity (13), and lower cytochrome c oxidase activity (complex IV) (14). When mitochondrial function was examined by respirometry, no differences in respiration

between obese and lean subjects were observed (15). Diabetic subjects had lower NADH:ubiquinone oxidoreductase (complex I) activity and CS activity (16). Diabetics also exhibited lower skeletal muscle mitochondrial respiration than healthy controls (17). These subjects also had decreased levels of mitochondrial DNA and CS activity, suggesting that there was lower mitochondrial density in their skeletal muscle, a potential cause for their decreased mitochondrial respiration.

In this study, we have examined the effects of whole dietary patterns, either a Mediterranean or a Western diet, on skeletal muscle mitochondrial bioenergetics. It is unlikely that the negative effects of a Western diet or the positive effects of a Mediterranean diet are due to a single dietary component. To our knowledge, the study presented here is the first to report on the effects of whole dietary patterns on skeletal muscle mitochondrial bioenergetics. We utilized cynomolgus macaques to examine the effects of whole diets administered for 30 months, approximately equivalent to 8 human years. We performed bioenergetic profiling of skeletal muscle comprised of high-resolution respirometry of permeabilized muscle fibers, with and without fatty acid substrates, tissue citrate synthase activity, and western blot analyses.

Methods

Experimental model and study design

The ancillary study described here examined 22 randomly selected female cynomolgus macaques (*Macaca fascicularis*) ranging from approximately 11 to 14 years that were available from a larger parent study. The parent study included 42 female cynomolgus macaques and was designed to test the effects of Mediterranean versus Western diet on a broad range of health outcomes (18).

Animals were housed in small social groups of 3-4 in pens measuring 3.3 m x 3.3 m x 3.3 m, on a 12 h/12 h light/dark schedule. Animals were fed either a Western or Mediterranean diet designed and produced by the Primate Nutrition and Diet Laboratory at Wake Forest School of Medicine for 30 months. For the parent study, animals were assigned to either diet treatment using stratified randomization, balanced on pretreatment characteristics that reflect overall health, including body weight, body mass index, basal cortisol, and plasma lipid concentrations. Diet composition is shown in **Supplemental Table 1** and macronutrient composition is shown in **Supplemental Table 2**. These semi-purified diets were matched on protein, fat, carbohydrate, and cholesterol content. The Western diet was designed to be similar to consumption of middle-aged American women, with protein and fat derived from mainly animal sources and high in saturated fats and sodium (19). The Mediterranean diet was designed to mimic key aspects of the Mediterranean diet, including protein and fats from mainly plant sources, higher amounts of monounsaturated fats, high in complex carbohydrates and fiber, and lower amounts of sodium (20,21). In particular, the Mediterranean diet included English walnut powder and extra virgin olive oil, which were key ingredients included in the PREDIMED study (22). Water was available *ad libitum*. Skeletal muscle tissue (*Vastus lateralis*) for bioenergetics analyses was collected at the time of necropsy. Animals were first sedated with intramuscular ketamine hydrochloride (15 mg/kg), then intravenous sodium pentobarbital (approximately 13 mg/kg) was administered to achieve surgical anesthesia, and exsanguinated in accordance with guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association. All procedures were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services, and the Animal Care and Use Committee of Wake Forest School of Medicine. All procedures and protocols were reviewed and approved by the Wake Forest School of Medicine Animal Care and Use Committee.

Body mass measurements

Body length (suprasternal notch to pubic symphysis) was measured during month 27 of the treatment phase and body weight was measured on the day of necropsy. BMI (kg/m^2) was calculated as described previously (23).

Insulin, glucose, and insulin sensitivity measurements

Intravenous glucose tolerance tests (iv-GTT) with insulin responses were done during treatment phase month 26 as previously described (24). Briefly, animals were sedated (15 mg/kg) after an 18 hour fast and administered 500 mg/kg dextrose. Blood samples were taken at 0, 5, 10, 20, 30, 40, and 60 min. Glucose AUC and k were calculated as previously described (23). Area under the insulin curve was calculated using insulin responses between 10-40 min. Glucose concentrations were determined by colorimetric assay using reagents and instrumentation (ACE Alera autoanalyzer) from Alfa Wasserman Diagnostic Technologies (West Caldwell, NJ, (25)). Insulin was determined by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). HOMA-IR was calculated as described previously (26).

Energy intake measurements

Energy intake was measured in the parent study (18). Briefly, each monkey was offered 120 kcal of diet per kilogram of body weight per day for the duration of the study (30 months) and experimental diets were weighed before and after the meal to calculate calories consumed. Additionally, all monkeys were provided enrichment several times per week, including flavored noncaloric ice cubes two times per week, rice krispies one time per week, and low-calorie vegetables such as celery one time per week.

Preparation of permeabilized skeletal muscle fibers

Immediately after euthanasia, skeletal muscle tissue (*Vastus lateralis*) was removed. Approximately 10-15 mg of tissue was selected for mechanical separation as described previously (27). The tissue was placed in ice-cold BIOPS (10 mM Ca-EGTA buffer, 0.1 μ M free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl_2 , 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) The tissue was cut into smaller bundles of approximately 5 mg and separated mechanically with sharp angular forceps under magnification, permeabilized with saponin (30 mg/mL) for 30 min on ice, and washed with MiR05 (110 mM sucrose, 60 mM K^+ -lactobionate, 0.5 mM EGTA, 3 mM MgCl_2 , 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 1 mg/mL BSA, pH 7.1) or buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4 , 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mg/mL BSA, pH 7.4) for 15 min on ice before analysis.

High-resolution respirometry of permeabilized skeletal muscle fibers

Two substrate-uncoupler-inhibitor titration (SUIT) protocols were used to examine mitochondrial bioenergetics with and without fatty acid oxidation. Approximately 2.5 mg of tissue was added to each chamber and steady-state rate of respiration measurements were obtained after every substrate addition and expressed as $\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$.

For the SUIT protocol with fatty acids, high-resolution O_2 flux measurements were conducted in 2 mL of MiR06Cr (MiR05 containing 20 mM creatine and 280 U/mL catalase) using the Oroboros Oxygraph-2k (O2k; Oroboros Instruments, Innsbruck, Austria). This protocol was adapted from Pesta and Gnaiger (27) and completed as follows: 7.5 mM ADP, 0.5 mM octanoylcarnitine, 2 mM malate, 10 μ M cytochrome c to test for mitochondrial membrane integrity, 5 mM pyruvate, 10 mM glutamate, 50 mM succinate, two additions of 0.25 μ M carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) followed by a titration of 0.5 μ M FCCP to obtain maximal ETS capacity, 10 mM glycerol-3-phosphate, 0.5 μ M rotenone, and 5 μ M antimycin-A.

For the SUIT protocol without fatty acids, high-resolution O₂ flux measurements were conducted either in 2 mL of buffer Z containing 20 mM creatine and 25 µM blebbistatin to inhibit contraction (28). This protocol was completed as follows: 2 mM malate, 4 mM ADP, 20 mM pyruvate, 10 mM glutamate, 10 mM succinate, 10 µM cytochrome c to test for mitochondrial membrane integrity, two additions of 0.25 µM FCCP followed by a titration of 0.5 µM FCCP to obtain maximal ETS capacity, 0.5 µM rotenone, and 5 µM antimycin-A.

Respiration parameters measured in these SUIT protocols are summarized in **Supplemental Table 3**.

To further examine the effects of diet on mitochondrial bioenergetics we also calculated flux control ratios. Flux control ratios were calculated by dividing each respiration parameter by maximum respiration (27).

Citrate synthase activity assay

Citrate synthase (CS) activity was determined according to manufacturer's instructions (Citrate synthase assay kit, Sigma CS0720, St. Louis, MO). Briefly, skeletal muscle (*Vastus lateralis*) was homogenized in cold CelLytic MT (Sigma C3228) at pH 7.4 and protease inhibitor cocktail (Sigma P8340). The homogenized sample was centrifuged at 12,000 g for 10 min and the supernatant containing the protein was collected. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce; Rockford, IL). CS activity was measured by continuous spectrophotometric rate determination at 412 nm. Each sample was run in triplicate.

Western blotting

Skeletal muscle (*Vastus lateralis*) homogenate was loaded at a concentration of 30 µg total protein per well for separation by SDS-PAGE and transferred to PVDF membranes.

The membranes were blocked at room temperature for 1 hour in 5% nonfat dry milk in TBS containing 0.1% Tween-20. Blots were probed overnight at 4°C with primary antibodies (VDAC/Porin, Abcam, ab15895; GAPDH, Abcam, ab9484; CPT1b, Invitrogen, PA5-12218) and incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody. Antibody-bound protein was detected by enhanced chemiluminescence and quantified by densitometry with ImageJ.

Statistical analysis

Distributions of all variables were examined before any further analysis. Normality was assessed by Shapiro-Wilk tests and any variables that were not normally distributed were log transformed to achieve a normal distribution (FAO + complex I, FAO max ETS capacity, FAO ETS rot sensitive, body weight, fasting glucose, fasting insulin, glucose AUC, and insulin AUC). Statistical significance between groups was evaluated by unpaired two-tailed Student's t-tests using Microsoft Excel software. Significance between groups was defined as $P \leq 0.05$. Pearson correlations were assessed between all variables and partial correlations were adjusted for age and weight. Analysis was performed using SAS Enterprise Guide 7.12 (SAS Institute Inc., Cary, NC, USA).

Results

Demographic and bioenergetic characteristics of non-human primate subjects

Age, weight, BMI, homeostatic model assessment of insulin resistance (HOMA-IR), fasting blood glucose, fasting blood insulin, glucose area under the curve, and insulin area under the curve for the sub-cohort of animals utilized for this ancillary study are summarized in **Table 1**. Cross-sectionally, there were no statistically significant differences between groups for any of these characteristics. However, this ancillary study was not adequately powered for between-group differences in these characteristics. In the larger parent study,

body weight was significantly increased in the Western diet group at all time points after 6 months on the diet; these changes were not observed in the Mediterranean diet group (18). Additionally, the parent study found that Western diet resulted in increased body fat, activity, energy expenditure, triglyceride concentrations, insulin resistance, and hepatosteatosis when compared to the Mediterranean diet (18).

Overall energy intake for animals in this ancillary study was not significantly different between the Western or Mediterranean diet groups, both in mean calories consumed ($P = 0.30$) and mean calories consumed per kilogram of body weight ($P = 0.83$) (**Supplemental Figure 1**).

Representative bioenergetic profiles from a single animal are shown in **Figure 1**. All bioenergetic measurements of permeabilized muscle fiber respiration and citrate synthase activity for both diet groups are summarized in **Supplemental Table 4**.

Diet induces bioenergetic changes in permeabilized fibers

Western diet-fed primates exhibited higher oxygen flux across all respirometry measurements in permeabilized fibers when compared to primates fed a Mediterranean diet (**Figure 2A**). There was significantly higher respiration with FAO, FAO + complex I, complex I + II, max ETS, and ETS Rot sensitive. There were also trends for higher FAO + complex I + II, complex I, and ETS Rot insensitive.

To examine potential mechanisms of action, we measured citrate synthase activity, a marker of mitochondrial content (29,30). Mean CS activities were similar for the Western and Mediterranean diet groups (**Figure 2B**). This finding was confirmed by performing western blots for VDAC/Porin, a mitochondrial structural protein. Mean VDAC/Porin protein expression relative to GAPDH was similar for the Western and Mediterranean diet groups (**Supplemental Figure 3**). To examine differences in the relative contributions of

substrates to mitochondrial respiration, we calculated flux control ratios for key respiratory parameters. We found trends for higher FAO FCR ($P=0.08$) and FAO + complex I FCR ($P=0.10$) in the Western diet group compared to the Mediterranean diet group. To further examine these differences in FAO capacity, we performed western blots for CPT1, the rate-limiting step in the transport of fatty acids into mitochondria and β -oxidation (Supplemental Figure 3). While there was no significant difference between the two groups, we found a trend for increased expression of CPT1 in the Mediterranean diet group ($P = 0.09$).

Correlations between permeabilized muscle fiber bioenergetics and insulin sensitivity, glucose, and insulin measurements

Pearson correlations were used to assess relationships between bioenergetic parameters and insulin sensitivity, glucose, and insulin measurements by diet group and are shown in **Table 2** (Western diet), **Table 3** (Mediterranean diet); relationships with both groups combined are shown in **Supplemental Table 5**. For correlation analysis, our primary metabolic outcome was insulin sensitivity calculated by HOMA-IR.

Regression plots illustrating the statistically significant relationships with HOMA-IR are shown in **Figure 3A-E** for both groups combined. HOMA-IR was significantly positively correlated with FAO (Figure 3A), FAO + complex I (Figure 3B), FAO + complex I + II (Figure 3C), FAO max ETS (Figure 3D), and FAO ETS Rot sensitive (Figure 3E). These relationships between measures of FAO and HOMA-IR were strongest in the Western diet group and are shown in **Figure 3F-K**. HOMA-IR was positively correlated with FAO (Figure 3F), FAO + complex I (Figure 3G), and FAO + complex I + II (Figure 3H), FAO max ETS (Figure 3I), ETS Rot sensitive (Figure 3J), and ETS Rot insensitive (Figure 3K).

Additionally, with both groups combined, fasting blood insulin was positively correlated with FAO + complex I + II (**Supplemental Figure 2A**) and FAO ETS Rot sensitive (**Supplemental Figure 2B**). Insulin AUC was positively correlated with FAO + complex I + II (**Supplemental Figure 2C**). In the Western diet group, fasting blood insulin was positively correlated with FAO + complex I (**Supplemental Figure 2D**) and FAO + complex I + II (**Supplemental Figure 2E**). Insulin AUC was significantly positively correlated with FAO (**Supplemental Figure 2F**), FAO + complex I (**Supplemental Figure 2G**), and FAO + complex I + II (**Supplemental Figure 2H**), FAO max ETS (**Supplemental Figure 2I**), and FAO ETS Rot sensitive (**Supplemental Figure 2J**).

Discussion

We observed significantly higher respiration both with and without fatty acid oxidation in permeabilized skeletal muscle fibers from animals fed a Western compared to a Mediterranean diet. These changes were independent of citrate synthase activity, a marker of mitochondrial volume or content (29,30), and expression of VDAC/Porin. These data suggest that the increase in respiration with a Western diet is likely not due to differences in mitochondrial content and could be the result of alterations in other factors such as mitochondrial morphology and composition, mitochondrial quality control, or other intracellular interactions. For example, differences in mitochondrial morphology have been linked to differences in respiration: more fragmented mitochondria are associated with nutrient-rich environments and inefficient energy production, whereas elongated mitochondria are associated with periods of starvation and more efficient production of ATP (31).

We also observed a trend for increased FAO and FAO + complex I flux control ratios in the Western diet group. To examine a potential mechanism for this observation, we looked at the expression of CPT1 by Western blot. Interestingly, we found a trend for increased

CPT1 expression in the Mediterranean diet group. For the measurement of FAO, we used a combination of octanoylcarnitine and malate to induce respiration from fatty acid oxidation. This combination bypasses the rate-limiting step of conversion of acyl-CoAs to acylcarnitines by CPT1, as octanoylcarnitine can freely cross the mitochondrial membrane (32). If we had used octanoyl-CoA and carnitine separately as substrates to induce fatty acid oxidation, we may have observed different results. It is important to consider that this study was not able to examine the stimulation of fatty acid oxidation by longer fatty acids such as palmitate, monounsaturated fatty acids such as oleate, or polyunsaturated fatty acids such as linoleic acid. These limitations may account for lower FAO observed in the Mediterranean diet group despite marginally higher CPT1 expression in skeletal muscle. Indeed, muscle has different capacities for the oxidation of various types of fatty acids (33). Notably, other studies have reported that oleate increases both gene and protein expression of CPT1 (34,35) which is consistent with our observation that a Mediterranean diet rich in monounsaturated fats increased expression of CPT1 in skeletal muscle.

Previous studies of mitochondrial bioenergetics have reported that high-fat diets in rodents are associated with increased mitochondrial respiration in response to both fatty acid and carbohydrate substrates, increased mitochondrial biogenesis, and increased mitochondrial content, despite increased insulin resistance (36–39). However, these studies include very high fat diets with 45% to 60% of total calories from fat and are compared to control diets that contain 8-12% of calories from fat. Additionally, the composition of fats in these studies vary. Some studies include flax seed oil and olive oil as sources of fat, while others have lard as the major source. It should be noted that the study presented here does not compare high-fat and low-fat diets. Both the Western and Mediterranean experimental diets contain 31% of calories from fat. Indeed, we matched the experimental diets on protein, carbohydrate, and total fat content. The Western

experimental diet has a larger amount of saturated fat, while the Mediterranean experimental diet has a larger percentage of monounsaturated and polyunsaturated fats. Our results suggest that manipulations of the types of fats consumed can alter mitochondrial bioenergetics, independent of other factors, such as macronutrient composition, energy intake, and obesity.

Previous studies have linked western diets to chronic low-grade inflammation with elevated levels of biomarkers, such as C-reactive protein, interleukins 6 and 18, and fibrinogen (40). In the context of obesity and insulin resistance, increased levels of tumor necrosis factor- α , interleukin 6, and C-reactive protein have also been reported in both animal models and humans (41). Inflammation is associated with increased mitochondrial respiration in brown adipose tissue, adipocytes, and fibroblasts (42–44). Lark et al. have suggested that high-fat diets that increase mitochondrial respiration and partially oxidized lipid metabolites also increase production of reactive oxygen species and H_2O_2 that could lead to the induction of proinflammatory cascades and prolonged inflammation (41). In the present study, the lower mitochondrial respiration we observed with Mediterranean diet may be linked to reduced reactive oxygen species, with the potential to lower systemic inflammatory burden. This diet is high in n-3 fatty acids and has a lower n-6:n-3 fatty acid ratio, both of these have been associated with anti-inflammatory effects and could play a role in the differences in mitochondrial bioenergetics observed (45,46). Future studies designed to investigate the effects of Western and Mediterranean dietary patterns on inflammation will need to be conducted in order to determine how these relate to differences in mitochondrial metabolism.

We observed strong positive correlations between fatty acid oxidation and HOMA-IR and fasting blood insulin. These results suggest that there is a potential link between increased fatty acid oxidation of octanoylcarnitine and the development of insulin resistance.

Interestingly, we did not observe significant correlations between these parameters when respiration was examined in the absence of octanoylcarnitine. Within group relationships were strongest in the Western diet group and generally not significant in the Mediterranean diet group, perhaps reflecting greater heterogeneity in responses to diet in the Western diet group. However, when both diet groups are combined, similar relationships to the Western diet group persist. Partial correlations controlling for body weight and age suggest that insulin AUC was positively associated with FAO and FAO max ETS capacity in the Western diet group and negatively associated in the Mediterranean diet group, suggesting a complex diet by body weight interaction. In light of these correlations, further studies are necessary to understand the link between fatty acid oxidation and insulin resistance. It should also be noted that we made multiple comparisons in this study, which increases the risk of type I errors and may lead to significant correlations that are false or incorrect. To mitigate these risks, we chose insulin sensitivity calculated by HOMA-IR as our primary metabolic outcome for correlation analysis. If we reduce the significance threshold to $P \leq 0.01$, many of these relationships are still significant including the relationships between measures of fatty acid oxidation and HOMA-IR. Additionally, these results are consistent, not only do measures of fatty acid oxidation significantly and positively correlate with HOMA-IR, but other surrogate measurements of insulin resistance: fasting blood insulin and insulin AUC.

It should be noted that human studies of diabetics and obese individuals have reported reductions in skeletal muscle mitochondrial bioenergetics (12–17). In this study, metabolic perturbations associated with the Western diet may be related to a prediabetic state in humans. A significantly longer study would be required to determine the temporal relationships between bioenergetic changes associated with diet and the development of

overt diabetes. Our results may reflect an early adaptation to insulin resistance which may be followed by bioenergetic decline upon disease progression.

Major strengths of this study include the use of high-resolution mitochondrial respirometric profiling with and without fatty acid substrate, the comparison of whole dietary patterns rather than manipulation of a single macronutrient, and the use of a well-established non-human primate model highly translatable to humans that allowed for tight experimental control that cannot otherwise be achieved. This study utilized similarly aged premenopausal female cynomolgus monkeys, so additional studies would be required to determine the effects of age, sex, and menopause. A major limitation of this study is the absence of a baseline measurement of mitochondrial respiration or a “control” diet. Muscle samples were obtained only after necropsy. Pre-post diet effects would require multiple biopsies and would, therefore, require an experimental design that is different from the parent study utilized here. While baseline differences are less likely in animal studies compared to human trials; the animals were monitored at baseline and assigned to diet groups with stratified randomization that ensured the groups were balanced based on pretreatment characteristics (body weight, body mass index, basal cortisol, and plasma lipid concentrations). The experimental diets were also carefully matched on macronutrient composition (16% protein, 54% carbohydrate, and 31% total fat) and cholesterol so that the only variables were fat composition and the sources of diet components: more animal sources for the Western diet and more plant sources for the Mediterranean diet.

We report that the consumption of a Western diet resulted in higher skeletal muscle mitochondrial respiration when compared to Mediterranean diet and that fatty acid oxidation significantly and positively correlated with insulin resistance, particularly in the Western diet group. While future investigation is warranted, including analysis of

mitochondrial function pre- and post-dietary manipulation, these studies suggest a potential link between high skeletal muscle mitochondrial respiration and the consumption of a Western diet and the development of insulin resistance. Additionally, these results highlight the importance of dietary composition in the study of mitochondrial bioenergetics. We have shown that changes in the types of fat consumed can influence skeletal muscle mitochondrial respiration, independent of other factors, such as macronutrient composition.

Acknowledgments and Author Contributions

J.L.G-A. played a key role in the conceptualization and development of this ancillary study, performed all respirometric analyses of permeabilized muscle fibers, and played a lead role in data analysis and manuscript preparation. Z.G. performed citrate synthase activity analyses. S.E.A. participated in the design of the project, designed the diets in collaboration with the other investigators, supervised the diet laboratory that formulated them, and participated in the monitoring of animal health. M.Z.V. participated in the design of the project and advised on the translation of human to non-human primate diets during the diet development process. K.T.M. collected samples and participated in the monitoring of animal health. T.C.R. participated in the design and conduct of the project, diet development, and supervised the Clinical Chemistry and Endocrinology Laboratory that performed the insulin, glucose, and insulin sensitivity measurements and manuscript preparation. C.A.S. was the PI of the parent study and played a key role in the development of this ancillary study, data analyses, and manuscript preparation. A.J.A.M. was responsible for the development of this study and provided oversight for all mitochondrial assessments, worked directly with the study team to coordinate the experimental plan, and supervised data analyses and manuscript preparation. All authors have read and approved the final manuscript.

References

1. Heidemann C, Schulze MB, Franco OH, Van Dam RM, Mantzoros CS, Hu FB. Dietary patterns and risk of mortality from cardiovascular disease, cancer, and all causes in a prospective cohort of women. *Circulation* [Internet]. 2008 [cited 2018 Apr 17];118:230–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18574045>
2. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, O’Keefe JH, Brand-Miller J. Origins and evolution of the Western diet: Health implications for the 21st century. *Am J Clin Nutr* [Internet]. 2005 [cited 2018 Mar 26];81:341–54. Review. Available from: <https://academic.oup.com/ajcn/article/81/2/341/4607411>
3. Odermatt A. The Western-style diet: a major risk factor for impaired kidney function and chronic kidney disease. *Am J Physiol Ren Physiol* [Internet]. 2011;301:919–31. Review. Available from: <https://doi.org/10.1152/ajprenal.00068.2011>
4. Martínez-González MÁ, De La Fuente-Arrillaga C, Nunez-Cordoba JM, Basterra-Gortari FJ, Beunza JJ, Vazquez Z, Benito S, Tortosa A, Bes-Rastrollo M. Adherence to Mediterranean diet and risk of developing diabetes: Prospective cohort study. *BMJ* [Internet]. British Medical Journal Publishing Group; 2008 [cited 2018 Apr 17];336:1348–51. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18511765>
5. Knoops KTB, De Groot LCPGM, Kromhout D, Perrin AE, Moreiras-Varela O, Menotti A, Van Staveren WA. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: The HALE project. *J Am Med*

- Assoc [Internet]. American Medical Association; 2004 [cited 2019 Jan 28];292:1433–9. Available from:
<http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.292.12.1433>
6. De Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: Final report of the Lyon Diet Heart Study. *Circulation* [Internet]. 1999 [cited 2019 Jan 28];99:779–85. Available from:
<http://www.ncbi.nlm.nih.gov/pubmed/9989963>
 7. Scarmeas N, Luchsinger JA, Mayeux R, Stern Y. Mediterranean diet and Alzheimer disease mortality. *Neurology* [Internet]. NIH Public Access; 2007 [cited 2018 Apr 17];69:1084–93. Available from:
<http://www.ncbi.nlm.nih.gov/pubmed/17846408>
 8. Cole MA, Murray AJ, Cochlin LE, Heather LC, McAleese S, Knight NS, Sutton E, Jamil AA, Parassol N, Clarke K. A high fat diet increases mitochondrial fatty acid oxidation and uncoupling to decrease efficiency in rat heart. *Basic Res Cardiol* [Internet]. Springer-Verlag; 2011 [cited 2018 Mar 13];106:447–57. Available from:
<http://link.springer.com/10.1007/s00395-011-0156-1>
 9. Hoeks J, de Wilde J, Hulshof MFM, van den Berg SAA, Schaart G, van Dijk KW, Smit E, Mariman ECM. High fat diet-induced changes in mouse muscle mitochondrial phospholipids do not impair mitochondrial respiration despite insulin resistance. Shirihaï OS, editor. *PLoS One* [Internet]. Public Library of Science; 2011 [cited 2018 Mar 19];6:e27274. Available from:
<http://dx.plos.org/10.1371/journal.pone.0027274>
 10. Jørgensen T, Grønnet N, Quistorff B. One-year high fat diet affects muscle-but not

brain mitochondria. *J Cereb Blood Flow Metab* [Internet]. SAGE Publications; 2015 [cited 2018 Mar 13];35:943–50. Available from:
<http://www.ncbi.nlm.nih.gov/pubmed/25757754>

11. Stephenson EJ, Camera DM, Jenkins TA, Kosari S, Lee JS, Hawley JA, Stepto NK. Skeletal muscle respiratory capacity is enhanced in rats consuming an obesogenic Western diet. *AJP Endocrinol Metab* [Internet]. 2012 [cited 2018 Mar 13];302:E1541–9. Available from:
<https://www.physiology.org/doi/pdf/10.1152/ajpendo.00590.2011>
12. He J, Watkins S, Kelley DE. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* [Internet]. 2001 [cited 2018 Mar 25];50:817–23. Available from:
<http://www.ncbi.nlm.nih.gov/pubmed/11289047>
13. Simoneau JA, Veerkamp JH, Turcotte LP, Kelley DE. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J* [Internet]. 1999 [cited 2018 Mar 25];13:2051–60. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10544188>
14. Kelley DE, Goodpaster B, Wing RR, Simoneau J-A. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol Metab* [Internet]. American Physiological Society Bethesda, MD; 1999 [cited 2018 Mar 19];277:E1130–41. Available from:
<http://www.physiology.org/doi/10.1152/ajpendo.1999.277.6.E1130>
15. Ara I, Larsen S, Stallknecht B, Guerra B, Morales-Alamo D, Andersen JL, Ponce-González JG, Guadalupe-Grau A, Galbo H, Calbet JAL, et al. Normal mitochondrial function and increased fat oxidation capacity in leg and arm

- muscles in obese humans. *Int J Obes* [Internet]. 2011 [cited 2018 Mar 25];35:99–108. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20548301>
16. Kelley DE, He J, Menshikova E V, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* [Internet]. American Diabetes Association; 2002 [cited 2018 Mar 25];51:2944–50. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12351431>
 17. Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsøe R, Dela F. Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* [Internet]. Springer; 2007 [cited 2018 Mar 25];50:790–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17334651>
 18. Shively CA, Appt SE, Vitolins MZ, Uberseder B, Michalson KT, Silverstein-Metzler MG. Mediterranean Versus Western Diet Effects on Caloric Intake, Obesity, Metabolism, and Hepatosteatosis in Nonhuman Primates. *Obesity*. In Press.
 19. Rhodes, D.G., Clemens, J.C., Goldman, J.D., LaComb, R.P., Moshfegh AJ. 2011-2012 What We Eat in America, NHANES Tables 1-40. Worldw Web Site Food Surv Res Group [Internet]. 2014 [cited 2019 Feb 24]; Available from: www.ars.usda.gov/Services/docs.htm?docid=18349
 20. Bédard A, Riverin M, Dodin S, Corneau L, Lemieux S. Sex differences in the impact of the Mediterranean diet on cardiovascular risk profile. *Br J Nutr* [Internet]. 2012 [cited 2018 Mar 27];108:1428–34. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22221517>
 21. Kafatos A, Verhagen H, Moschandreas J, Apostolaki I, Van Westerop JJM. Mediterranean diet of Crete: Foods and nutrient content. *J Am Diet Assoc* [Internet]. 2000 [cited 2018 Mar 27];100:1487–93. Available from:

<http://www.ncbi.nlm.nih.gov/pubmed/11138441>

22. Estruch R, Ros E, Salas-Salvadó J, Covas M-I, Corella D, Arós F, Gómez-Gracia E, Ruiz-Gutiérrez V, Fiol M, Lapetra J, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. *N Engl J Med* [Internet]. Massachusetts Medical Society; 2013 [cited 2019 Feb 24];368:1279–90. Available from: <http://www.nejm.org/doi/10.1056/NEJMoa1200303>
23. Jayo JM, Shively CA, Kaplan JR, Manuck SB. Effects of exercise and stress on body fat distribution in male cynomolgus monkeys. *Int J Obes Relat Metab Disord* [Internet]. 1993 [cited 2018 May 17];17:597–604. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8242129>
24. Shively CA, Clarkson TB. Regional Obesity and Coronary Artery Atherosclerosis in Females: a Non-human Primate Model. *Acta Med Scand* [Internet]. 1987 [cited 2018 May 17];222:71–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/3164976>
25. Adams MR, Golden DL, Williams JK, Franke AA, Register TC, Kaplan JR. Soy protein containing isoflavones reduces the size of atherosclerotic plaques without affecting coronary artery reactivity in adult male monkeys. *J Nutr* [Internet]. 2005 [cited 2018 May 17];135:2852–6. Available from: <https://academic.oup.com/jn/article/135/12/2852/4669982>
26. Silverstein-Metzler MG, Shively CA, Clarkson TB, Appt SE, Carr JJ, Kritchevsky SB, Jones SR, Register TC. Sertraline inhibits increases in body fat and carbohydrate dysregulation in adult female cynomolgus monkeys. *Psychoneuroendocrinology* [Internet]. 2016 [cited 2018 May 17];68:29–38. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0306453016300427>

27. Pesta D, Gnaiger E. High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. Mitochondrial Bioenergetics: Methods and Protocols [Internet]. 2012. p. 25–58. Available from: http://dx.doi.org/10.1007/978-1-61779-382-0_3

28. Perry CGR, Kane DA, Lin C-T, Kozy R, Cathey BL, Lark DS, Kane CL, Brophy PM, Gavin TP, Anderson EJ, et al. Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle. Biochem J [Internet]. 2011;437:215 LP-222. Available from: <http://www.biochemj.org/content/437/2/215.abstract>

29. Picard M, Ritchie D, Wright KJ, Romestaing C, Thomas MM, Rowan SL, Taivassalo T, Hepple RT. Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging Cell. 2010;9:1032–46.

30. Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol [Internet]. 2012;590:3349–60. Available from: <http://doi.wiley.com/10.1113/jphysiol.2012.230185>

31. Liesa M, Shirihai OS. Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure [Internet]. Cell Metabolism. Cell Press; 2013 [cited 2018 Apr 27]. p. 491–506. Review. Available from: <https://www.sciencedirect.com/science/article/pii/S1550413113001046?via%3Dihub>

32. Ojuka E, Andrew B, Bezuidenhout N, George S, Maarman G, Madlala HP,

- Mendham A, Osiki PO. Measurement of β -oxidation capacity of biological samples by respirometry: a review of principles and substrates. *Am J Physiol Endocrinol Metab* [Internet]. American Physiological Society Bethesda, MD; 2016 [cited 2019 Feb 28];310:E715–23. Review. Available from: <http://www.physiology.org/doi/10.1152/ajpendo.00475.2015>
33. Reubsaet FAG, Veerkamp JH, Trijbels JMF, Monnens LAH. Total and Peroxisomal Oxidation of Various Saturated and Unsaturated Fatty Acids in Rat Liver, Heart and M. Quadriceps. *Lipids* [Internet]. 1989 [cited 2019 Feb 28];24:945–50. Available from: <https://link.springer.com/content/pdf/10.1007%2FBBF02544539.pdf>
 34. Coll T, Eyre E, Rodríguez-Calvo R, Palomer X, Sánchez RM, Merlos M, Laguna JC, Vázquez-Carrera M. Oleate Reverses Palmitate-induced Insulin Resistance and Inflammation in Skeletal Muscle Cells. *J Biol Chem* [Internet]. American Society for Biochemistry and Molecular Biology; 2008 [cited 2019 Feb 28];283:11107–16. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18281277>
 35. Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, Prip-Buus C, Cohen I. Increased Mitochondrial Fatty Acid Oxidation Is Sufficient to Protect Skeletal Muscle Cells from Palmitate-induced Apoptosis. *J Biol Chem* [Internet]. 2010 [cited 2019 Feb 28];285:36818–27. Available from: <http://www.jbc.org/>
 36. Lanza IR, Blachnio-Zabielska A, Johnson ML, Schimke JM, Jakaitis DR, Lebrasseur NK, Jensen MD, Nair KS, Zabielski P. Influence of fish oil on skeletal muscle mitochondrial energetics and lipid metabolites during high-fat diet. *Am J Physiol Metab* [Internet]. 2013 [cited 2019 Feb 25];304:E1391–403. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23632634>

37. Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS, Cooney GJ. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: Evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes* [Internet]. 2007 [cited 2018 Mar 19];56:2085–92. Available from: <http://diabetes.diabetesjournals.org>
38. Hancock CR, Han D-H, Chen M, Terada S, Yasuda T, Wright DC, Holloszy JO. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc Natl Acad Sci* [Internet]. 2008 [cited 2018 Mar 13];105:7815–20. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18509063>
39. Garcia-Roves P, Huss JM, Han D-H, Hancock CR, Iglesias-Gutierrez E, Chen M, Holloszy JO. Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. *Proc Natl Acad Sci* [Internet]. 2007 [cited 2019 Feb 25];104:10709–13. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17548828>
40. Barbaresko J, Koch M, Schulze MB, Nöthlings U. Dietary pattern analysis and biomarkers of low-grade inflammation: A systematic literature review. *Nutr Rev* [Internet]. Oxford University Press; 2013 [cited 2018 May 1];71:511–27. Review. Available from: <https://academic.oup.com/nutritionreviews/article-lookup/doi/10.1111/nure.12035>
41. Lark DS, Fisher-Wellman KH, Neuffer PD. High-fat load: mechanism(s) of insulin resistance in skeletal muscle. *Int J Obes suppl* [Internet]. Nature Publishing Group; 2012 [cited 2018 May 1];2:S31–6. Review. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26052434>
42. Alcalá M, Calderon-Dominguez M, Bustos E, Ramos P, Casals N, Serra D, Viana

- M, Herrero L. Increased inflammation, oxidative stress and mitochondrial respiration in brown adipose tissue from obese mice. *Sci Rep* [Internet]. Nature Publishing Group; 2017 [cited 2018 May 1];7:16082. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29167565>
43. Mauro C, Leow SC, Anso E, Rocha S, Thotakura AK, Tornatore L, Moretti M, De Smaele E, Beg AA, Tergaonkar V, et al. NF- κ B controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. *Nat Cell Biol* [Internet]. 2011 [cited 2018 May 1];13:1272–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21968997>
 44. Lee YS, Kim JW, Osborne O, Oh DY, Sasik R, Schenk S, Chen A, Chung H, Murphy A, Watkins SM, et al. Increased adipocyte O₂ consumption triggers HIF-1 α , causing inflammation and insulin resistance in obesity. *Cell* [Internet]. NIH Public Access; 2014 [cited 2018 May 1];157:1339–52. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24906151>
 45. Esposito K, Marfella R, Ciotola M, Di Palo C, Giugliano F, Giugliano G, D'Armiento M, D'Andrea F, Giugliano D. Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: A randomized trial. *J Am Med Assoc* [Internet]. American Medical Association; 2004 [cited 2019 Jan 28];292:1440–6. Available from: <http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.292.12.1440>
 46. White PJ, Arita M, Taguchi R, Kang JX, Marette A. Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice. *Diabetes* [Internet]. American Diabetes Association; 2010 [cited 2019 Jan

28];59:3066–73. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20841610>

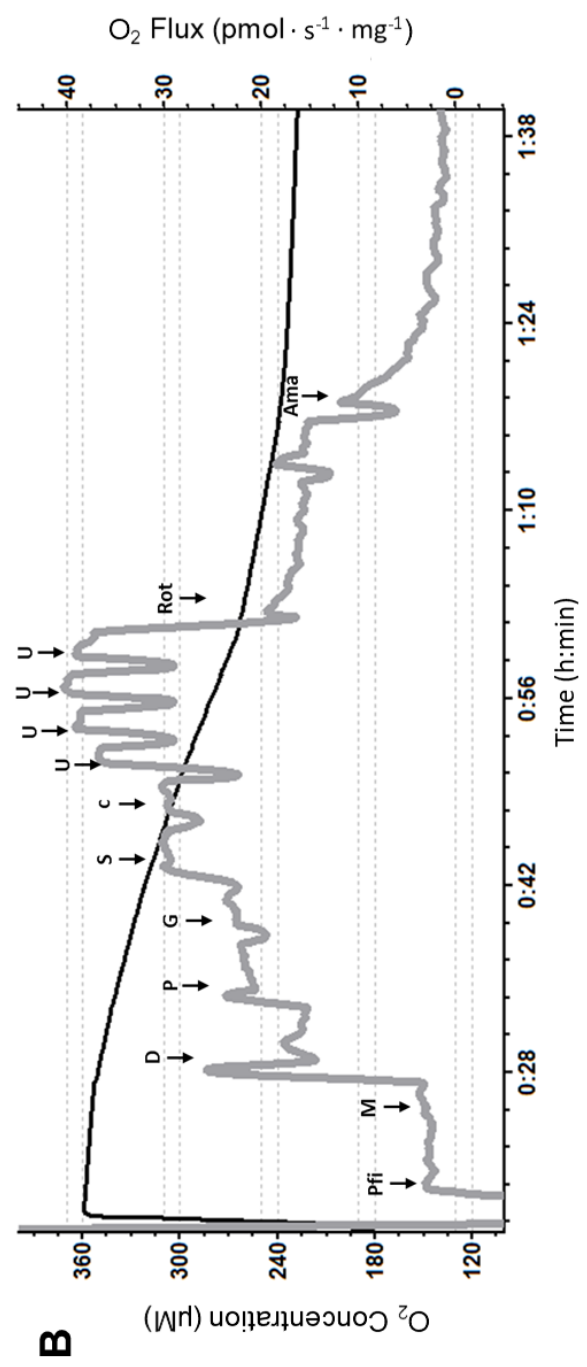
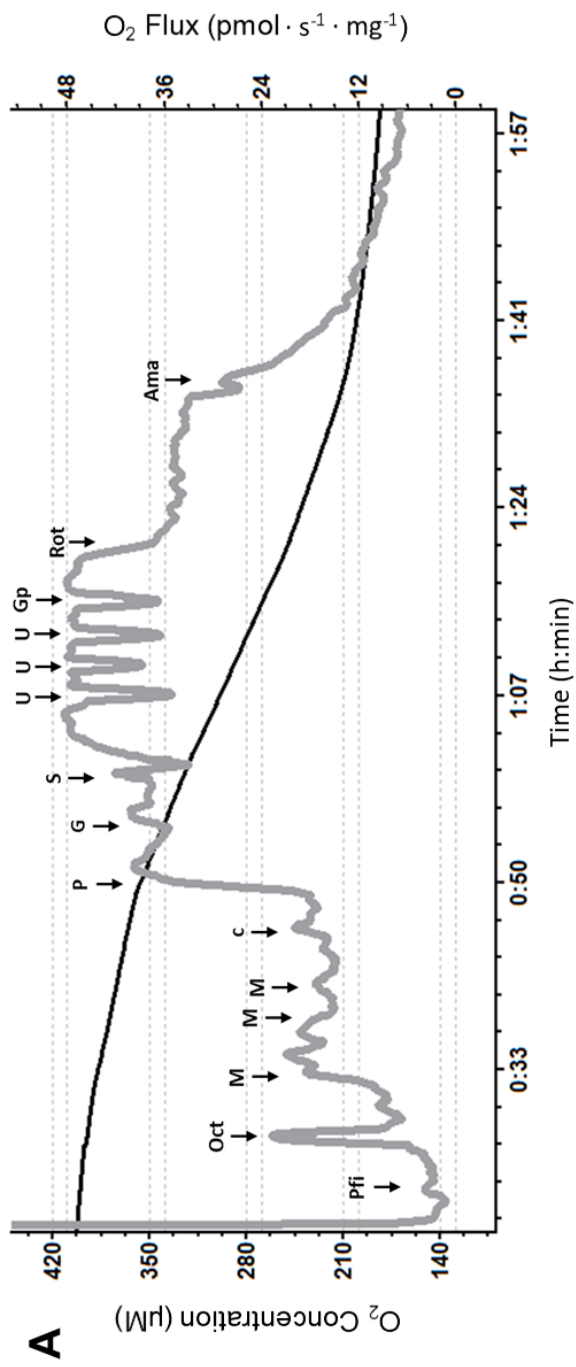
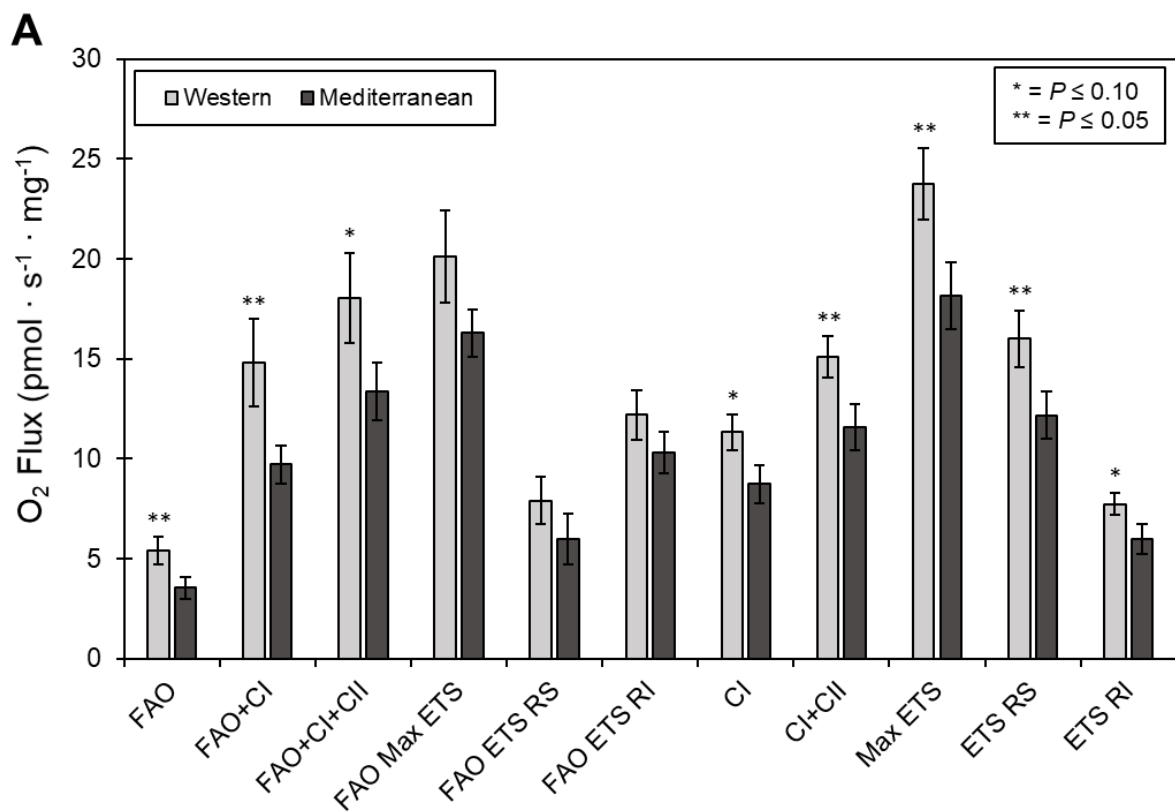


Figure 1. Representative traces of high-resolution respirometry of permeabilized skeletal muscle fibers from a single female cynomolgus macaque

(A) High-resolution respirometry of permeabilized fibers with the SUI protocol with fatty acids.

(B) High-resolution respirometry of permeabilized fibers with the SUI protocol without the addition of fatty acids.

(**Pfi** = permeabilized fibers, **Oct** = octanoylcarnitine, **M** = malate, **D** = ADP, **c** = cytochrome c, **P** = pyruvate, **G** = glutamate, **S** = succinate, **U** = FCCP, **Gp** = glycerol-3-phosphate, **Rot** = rotenone, **Ama** = antimycin-A)



Permeabilized Fiber Respirometry Measurement

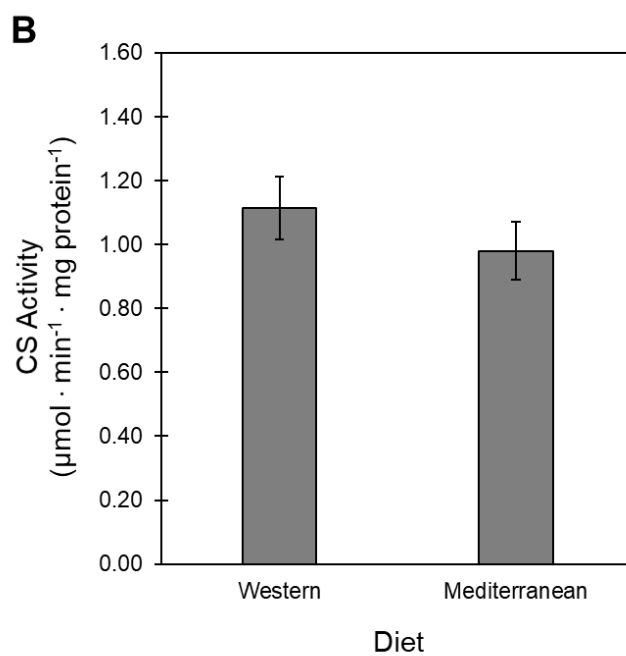


Figure 2. Effects of Western or Mediterranean diet on skeletal muscle bioenergetics of female cynomolgus macaques

(A) High-resolution respirometry of permeabilized muscle fibers. Data are presented as means $\pm$ SEM, $n = 11$. (*different from Mediterranean group, $P \leq 0.10$ and **different from Mediterranean group, $P \leq 0.05$)

(B) Citrate synthase activity. Data are presented as mean $\pm$ SEM, $n = 11$.

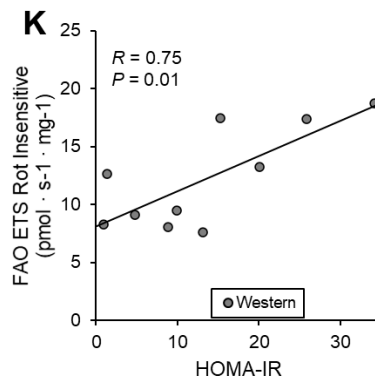
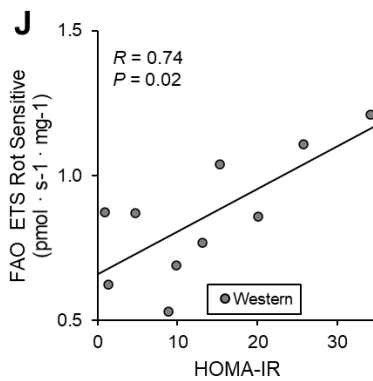
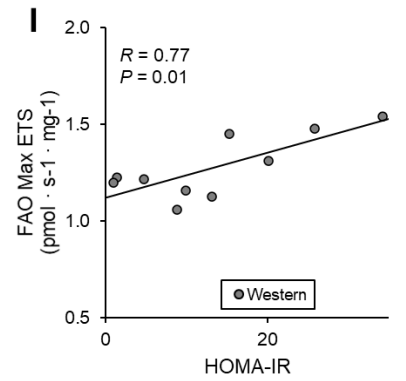
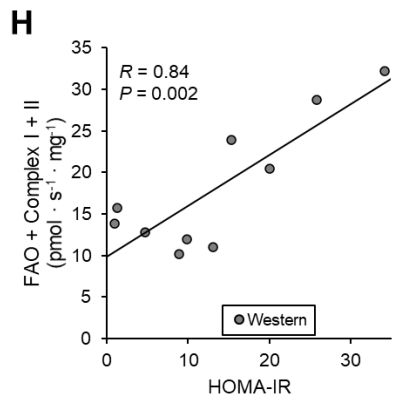
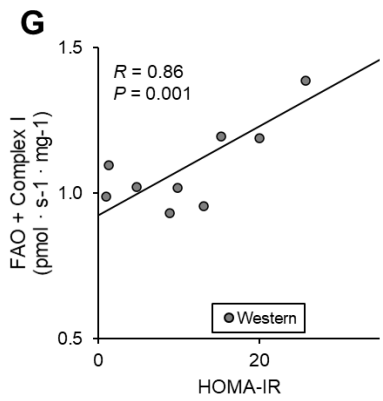
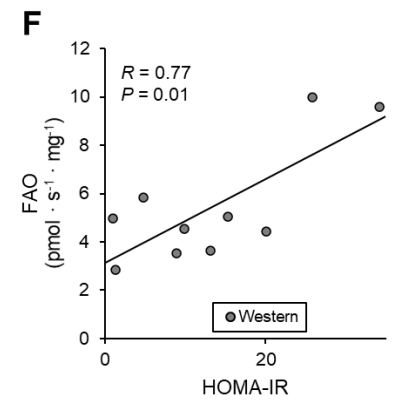
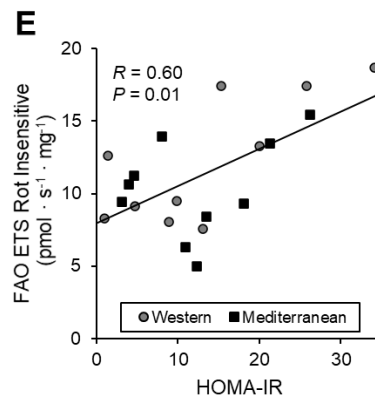
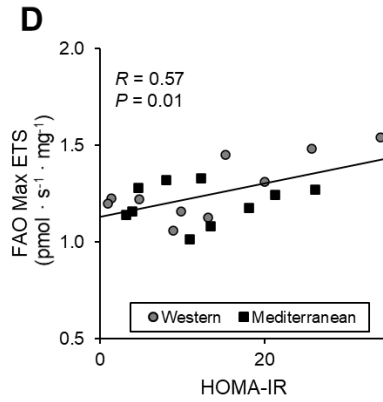
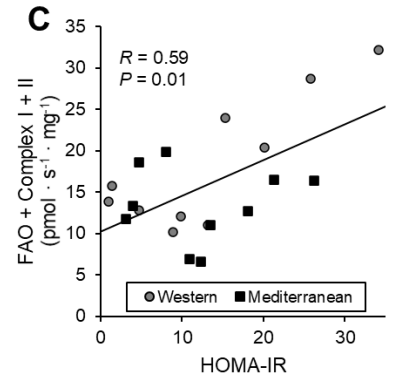
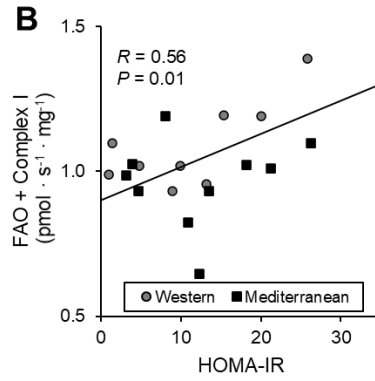
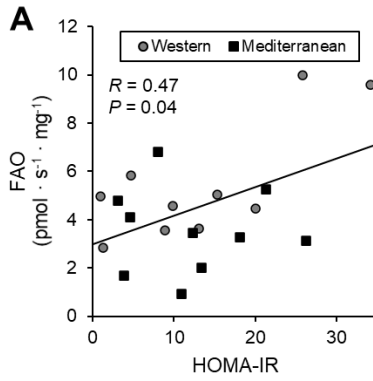


Figure 3. Correlations between permeabilized muscle fiber bioenergetics and insulin sensitivity of female cynomolgus macaques

(A-E) Plots of fatty acid oxidation measures versus HOMA-IR for all animals in the study. Pearson correlation (R) and P -values are shown on each plot.

(F-K) Plots of fatty acid oxidation measures versus HOMA-IR for animals in the Western diet group. Pearson correlation (R) and P -values are shown on each plot.

Table 1. Characteristics of female cynomolgus macaques fed either a Western diet or Mediterranean diet at necropsy¹

	Western diet	Mediterranean diet	<i>P</i> -value
Age (yrs)	12.3 ± 0.2 (11.5 – 13.2)	12.2 ± 0.2 (11.1 – 13.7)	0.75
Weight (kg)	3.65 ± 0.40 (2.59 – 7.10)	3.19 ± 0.33 (2.42 – 5.24)	0.32
BMI (kg/m ²)	46.7 ± 3.4 (36.3 – 73.9)	41.3 ± 2.5 (31.7 – 56.4)	0.18
HOMA-IR	13.4 ± 3.2 (1.0 – 34.2)	11.4 ± 2.4 (2.9 – 26.2)	0.62
Fasting Blood Glucose (mg/dL)	78 ± 3 (61 – 96)	91 ± 7 (64 – 133)	0.12
Fasting Blood Insulin (mIU/L)	66.4 ± 16.7 (6.4 – 175)	48.4 ± 9.5 (16.9 – 117)	0.80
Glucose AUC (mg · dL ⁻¹ · min)	2950 ± 430 (1720 – 6070)	2830 ± 159 (1960 – 3680)	0.84
Insulin AUC (mIU · L ⁻¹ · min)	6820 ± 2450 (1480 – 28900)	4520 ± 1540 (1060 – 18700)	0.43
¹ Values are presented as mean ± SEM (range), <i>n</i> = 11 per group. Differences between groups were evaluated by unpaired two-tailed Student's <i>t</i> -tests and <i>P</i> -values are shown. Glucose and insulin measurements were obtained from plasma samples.			

Table 2. Correlations between carbohydrate metabolism phenotypes and respirometry of permeabilized muscle fibers of female cynomolgus macaques fed a Western diet¹

Western diet	FAO	FAO + Complex I	FAO + Complex I + II	FAO Max ETS Capacity	FAO ETS Rot Sensitive	FAO ETS Rot Insensitive	Complex I	Complex I + II	Max ETS Capacity	ETS Rot Sensitive	ETS Rot Insensitive
<i>Pearson</i>											
HOMA-IR	0.77**	0.86***	0.84**	0.77**	0.74*	0.75**	0.30	0.19	0.32	0.41	-0.01
Fasting Blood Glucose	0.20	0.18	0.11	0.02	0.03	0.05	0.46	0.38	0.53	0.50	0.42
Fasting Blood Insulin	0.57	0.63*	0.63*	0.58	0.58	0.60	0.30	0.15	0.37	0.47	0.02
Glucose AUC	0.25	0.18	0.11	0.09	0.11	0.03	-0.36	-0.15	0.00	0.03	-0.09
Insulin AUC	0.64*	0.68*	0.65*	0.66*	0.66*	0.59	-0.17	-0.03	-0.10	-0.05	-0.17
<i>Partial for Age + Weight</i>											
HOMA-IR	0.81**	0.90**	0.86**	0.81*	0.73*	0.82**	0.30	0.18	0.47	0.62	-0.01
Fasting Blood Glucose	0.09	0.11	0.00	-0.05	-0.11	0.03	0.48	0.36	0.70	0.71	0.46
Fasting Blood Insulin	0.66	0.68	0.66	0.62	0.56	0.65	0.27	0.15	0.53	0.69	0.01
Glucose AUC	0.03	-0.05	-0.05	0.00	0.16	-0.08	0.02	-0.02	0.34	0.42	0.07
Insulin AUC	0.86**	0.83**	0.85**	0.88**	0.92***	0.78**	0.51	0.60	0.71	0.26	-0.12

¹Values are Pearson correlation coefficients (*R*) for relationships between measures of carbohydrate metabolism and measures of respiration of permeabilized skeletal muscle fibers. Partial correlations were controlled for age and weight at time of necropsy.

* Represents a significant correlation with $P \leq 0.05$

** Represents a significant correlation with $P \leq 0.01$

*** Represents a significant correlation with $P \leq 0.001$

Table 3. Correlations between carbohydrate metabolism phenotypes and respirometry of permeabilized muscle fibers of female cynomolgus macaques fed a Mediterranean diet¹

Mediterranean diet	FAO	FAO + Complex I	FAO + Complex I + II	FAO Max ETS Capacity	FAO ETS Rot Sensitive	FAO ETS Rot Insensitive	Complex I	Complex I + II	Max ETS Capacity	ETS Rot Sensitive	ETS Rot Insensitive
Pearson											
HOMA-IR	-0.04	0.13	0.06	0.15	-0.25	0.34	-0.33	-0.32	-0.21	0.02	-0.48
Fasting Blood Glucose	0.44	-0.10	-0.07	0.47	0.41	0.00	-0.11	-0.13	0.03	0.14	-0.14
Fasting Blood Insulin	-0.26	0.04	-0.03	-0.05	-0.30	0.21	-0.43	-0.36	-0.30	-0.07	-0.54
Glucose AUC	0.14	-0.09	0.20	0.24	0.04	0.19	-0.14	-0.27	0.01	0.24	-0.36
Insulin AUC	-0.52	-0.14	-0.12	-0.54	-0.32	-0.14	-0.24	-0.09	-0.38	-0.39	-0.22
Partial for Age + Weight											
HOMA-IR	-0.10	0.07	0.08	0.12	-0.27	0.37	-0.48	-0.43	-0.40	-0.14	-0.65
Fasting Blood Glucose	0.38	-0.39	-0.09	0.45	0.54	-0.12	-0.36	-0.33	-0.24	-0.11	-0.36
Fasting Blood Insulin	-0.26	0.09	0.01	-0.05	-0.36	0.29	-0.43	-0.33	-0.39	-0.17	-0.57
Glucose AUC	0.18	-0.07	0.17	0.31	0.09	0.19	0.03	-0.16	0.22	0.46	-0.29
Insulin AUC	-0.73*	0.19	-0.30	-0.75*	-0.62	-0.16	0.17	0.43	0.05	-0.01	0.13

¹Values are Pearson correlation coefficients (*R*) for relationships between measures of carbohydrate metabolism and measures of respiration of permeabilized skeletal muscle fibers. Partial correlations were controlled for age and weight at time of necropsy.

* Represents a significant correlation with $P \leq 0.05$

Supplemental Table 1. Composition of Western and Mediterranean experimental diets^{1,2}

Western diet		Mediterranean diet	
Ingredient	g/kg	Ingredient	g/kg
Casein, USP	85.0	Casein, USP	17.4
Whey protein – 895	85.0	Whey protein – 895	17.4
		Dried egg white	26.1
		Fishmeal (Menhaden)	26.1
		Walnuts	8.7
		Black bean flour	43.5
		Garbanzo bean flour	17.4
		Wheat flour (all purpose)	243.5
Dextrin	260.0	Dextrin	96.6
Sucrose	180.0	Sucrose	34.8
High fructose corn syrup 55	70.0	Banana	130.4
		Applesauce	38.2
		Tomato paste	17.4
Cellulose (Alphacel) ¹	79.4	Cellulose (Alphacel) ¹	94.8
Lard	41.5	Olive oil (Filippo Berio Extra Virgin)	61.7
Beef tallow	40.0	Menhaden oil (Omegapure)	8.7
Butter, lightly salted	12.5	Butter, lightly salted	8.7
Corn oil	35.0	Corn oil	10.4
Flaxseed oil	3.0	Flaxseed oil	1.7
Dried egg yolk	6.0	Dried egg yolk	14.8
Crystalline cholesterol	0.4		
Complete vitamin mix ³ (Teklad 85529)	25.0	Complete vitamin mix ³ (Teklad 85529)	21.7
Mineral mix ⁴ (without Ca, P, NaCl) (Teklad 140306)	50.0	Mineral mix ⁴ (without Ca, P, NaCl) (Teklad 140306)	43.5
Calcium carbonate	4.3	Calcium carbonate	3.7
Calcium phosphate, monobasic	7.5	Calcium phosphate, monobasic	6.5
NaCl (table salt)	16.0	NaCl (table salt)	6.3
Total	1000	Total	1000

¹Total Fiber (% of diet): Western: 7.94, Mediterranean: 12.7

²Caloric density of diets: Western: 3.42 kcal/g, Mediterranean: 2.64 kcal/g

³Vitamin mix composition: Vitamin A palmitate (500,000 IU/g) (1.8 g/kg), vitamin E DL-alpha tocopheryl acetate (2.5 g/kg), inositol (5.0 g/kg), riboflavin (1.0 g/kg), vitamin K MSB complex (2.3 g/kg), p-aminobenzoic acid (5.0 g/kg), niacin (4.5 g/kg), pyridoxine HCl (1.0 g/kg), thiamin (81%) (1.0 g/kg), calcium pantothenate (3.0 g/kg), vitamin B12 (0.1% in mannitol) (1.4 g/kg), biotin (0.02 g/kg), folic acid (0.1 g/kg), vitamin C, ascorbic acid, coated (97.5%) (90.0 g/kg), choline chloride (75.0 g/kg), vitamin D3, cholecalciferol (500,000 IU/g) (0.2 g/kg), dextrose monohydrate (806.3 g/kg)

⁴Mineral mix composition: Potassium carbonate (313.9 g/kg), magnesium sulfate heptahydrate (143.9 g/kg), dried ferrous sulfate (7.9 g/kg), manganese sulfate monohydrate (1.4 g/kg), zinc chloride (0.9 g/kg), cupric sulfate (0.3 g/kg), potassium iodide (0.08 g/kg), chromium acetate hydroxide (0.05 g/kg), sodium fluoride (0.02 g/kg), sodium selenite pentahydrate (0.004 g/kg), dextrin (531.6 g/kg)

Supplemental Table 2. Macronutrient composition of Western and Mediterranean experimental diets

	Western	Mediterranean
% of Total Calories		
Protein	16	16
Carbohydrate	54	54
Fat	31	31
% of Total Fats		
Saturated	36	21
Monounsaturated	36	57
Polyunsaturated	26	20

Supplemental Table 3. Summary of respiration measurements in each SUIT protocol

Respiration Measurement	Description	Measurement recorded after additions of:
SUIT protocol with fatty acids		
FAO	Respiration mediated by fatty acid oxidation	octanoylcarnitine and malate
FAO + Complex I	Respiration mediated by fatty acid oxidation and complex I	octanoylcarnitine, malate, pyruvate, and glutamate
FAO + Complex I + II	Respiration mediated by fatty acid oxidation and complexes I and II	octanoylcarnitine, malate, pyruvate, glutamate, and succinate
FAO Max ETS Capacity	Maximum electron transport system capacity / maximum respiration	FCCP (titrations until maximum)
FAO ETS Rot Sensitive	Respiration sensitive to inhibition of complex I by rotenone	obtained by subtracting respiration after rotenone from max ETS capacity
FAO ETS Rot Insensitive	Respiration insensitive to inhibition of complex I by rotenone	rotenone
SUIT protocol without fatty acids		
Complex I	Respiration mediated by complex I	pyruvate, malate, and glutamate
Complex I + II	Respiration mediated by complexes I and II	pyruvate, malate, glutamate, and succinate
Max ETS Capacity	Maximum electron transport system capacity / maximum respiration	FCCP (titrations until maximum)
ETS Rot Sensitive	Respiration sensitive to inhibition of complex I by rotenone	obtained by subtracting respiration after rotenone from max ETS capacity
ETS Rot Insensitive	Respiration insensitive to inhibition of complex I by rotenone	rotenone

Supplemental Table 4. Bioenergetic characteristics of female cynomolgus macaques fed either a Western or Mediterranean diet¹

	Western Diet Group	Mediterranean Diet Group	P-value
Respirometry of Permeabilized Muscle Fibers <i>O₂ Flux (pmol · s⁻¹ · mg tissue⁻¹)</i>			
FAO	5.4 ± 0.7 (2.9 – 10.0)	3.5 ± 0.6 (0.9 – 6.8)	0.05*
FAO + Complex I	14.8 ± 2.2 (8.6 – 31.7)	9.7 ± 1.0 (4.4 – 15.5)	0.05*
FAO + Complex I + Complex II	18.0 ± 2.2 (10.2 – 32.2)	13.4 ± 1.4 (6.6 – 19.8)	0.10
FAO Max ETS Capacity	20.1 ± 2.3 (11.6 – 34.9)	16.3 ± 1.2 (10.4 – 21.4)	0.16
FAO ETS Rotenone Sensitive	7.9 ± 1.2 (3.4 – 16.2)	6.0 ± 1.3 (3.2 – 16.4)	0.28
FAO ETS Rotenone Insensitive	12.2 ± 1.2 (7.6 – 18.7)	10.3 ± 1.1 (5.0 – 15.5)	0.26
Complex I	11.3 ± 0.9 (7.2 – 16.4)	8.7 ± 0.9 (4.2 – 12.0)	0.06
Complex I + Complex II	15.1 ± 1.0 (10.0 – 20.5)	11.6 ± 1.1 (5.9 – 15.3)	0.03*
Max ETS Capacity	23.7 ± 1.8 (16.3 – 33.0)	18.2 ± 1.7 (10.3 – 26.7)	0.03*
ETS Rotenone Sensitive	16.0 ± 1.4 (9.0 – 34.7)	12.2 ± 1.2 (6.5 – 19.5)	0.05*
ETS Rotenone Insensitive	7.7 ± 0.5 (4.4 – 11.5)	6.0 ± 0.8 (2.6 – 9.4)	0.08
Citrate Synthase Activity <i>(μmol · min⁻¹ · mg protein⁻¹)</i>	1.11 ± 0.10 (0.72 – 1.84)	0.98 ± 0.09 (0.46 – 1.47)	0.33
¹ Summary of all measurements obtained from respirometry of permeabilized muscle fibers with and without fatty acids and citrate synthase activity. Values are presented as mean ± SEM (range), <i>n</i> = 11 per group. Differences between groups were evaluated by unpaired two-tailed Student's t-tests and <i>P</i> -values are shown. * Represents significant difference between groups with <i>P</i> ≤ 0.05.			

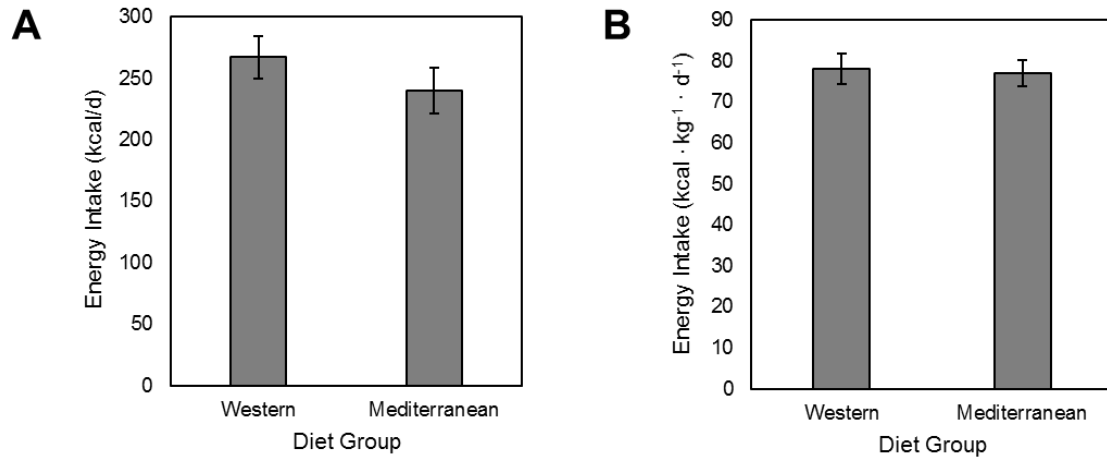
Supplemental Table 5. Correlations between carbohydrate metabolism phenotypes and respirometry of permeabilized muscle fibers of all female cynomolgus macaques in the study¹

	FAO	FAO + Complex I	FAO + Complex I + II	FAO Max ETS Capacity	FAO ETS Rot Sensitive	FAO ETS Rot Insensitive	Complex I	Complex I + II	Max ETS Capacity	ETS Rot Sensitive	ETS Rot Insensitive
Pearson											
HOMA-IR	0.47*	0.56**	0.59**	0.57**	0.32	0.60**	0.06	0.02	0.14	0.27	-0.18
Fasting Blood Glucose	0.08	-0.17	-0.13	0.08	0.09	-0.08	-0.06	-0.11	0.03	0.09	-0.11
Fasting Blood Insulin	0.30	0.40	0.44*	0.40	0.23	0.46*	0.03	-0.03	0.13	0.27	-0.19
Glucose AUC	0.18	0.07	0.10	0.10	0.06	0.06	-0.27	-0.18	-0.02	0.06	-0.17
Insulin AUC	0.27	0.41	0.44*	0.29	0.26	0.34	-0.11	0.03	-0.12	-0.10	-0.11
Partial for Age + Weight											
HOMA-IR	0.48*	0.58**	0.61**	0.57**	0.27**	0.67**	0.05	-0.01	0.12	0.26	-0.20
Fasting Blood Glucose	0.12	-0.17	-0.09	0.09	0.11	-0.06	-0.17	-0.19	-0.05	0.02	-0.18
Fasting Blood Insulin	0.30	0.41	0.44	0.39	0.39	0.50*	0.01	-0.05	0.11	0.25	-0.20
Glucose AUC	0.11	-0.02	-0.01	0.07	0.07	0.04	-0.10	-0.11	0.14	0.25	-0.13
Insulin AUC	0.26	0.48*	0.48*	0.36	0.36	0.44	0.14	0.20	0.02	0.05	-0.03

¹Values are Pearson correlation coefficients (*R*) for relationships between measures of carbohydrate metabolism and measures of respiration of permeabilized skeletal muscle fibers. Partial correlations were controlled for age and weight at time of necropsy.

* Represents significant correlation with $P \leq 0.05$

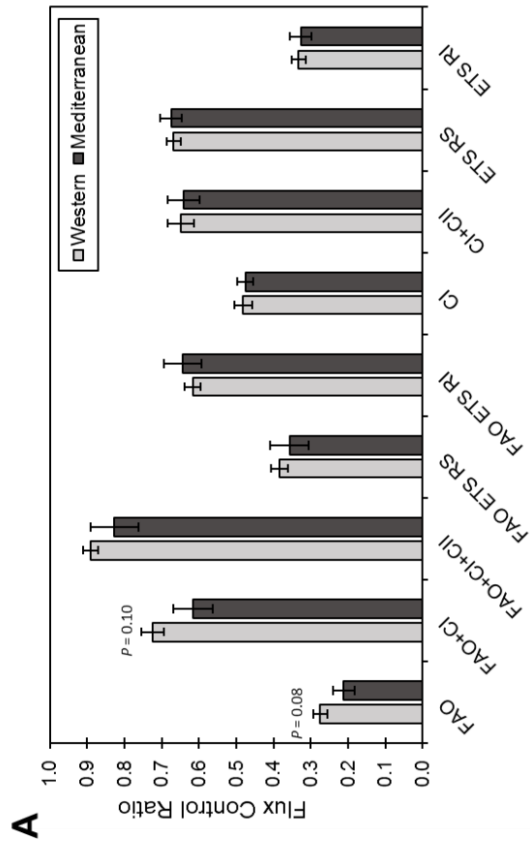
** Represents significant correlation with $P \leq 0.01$



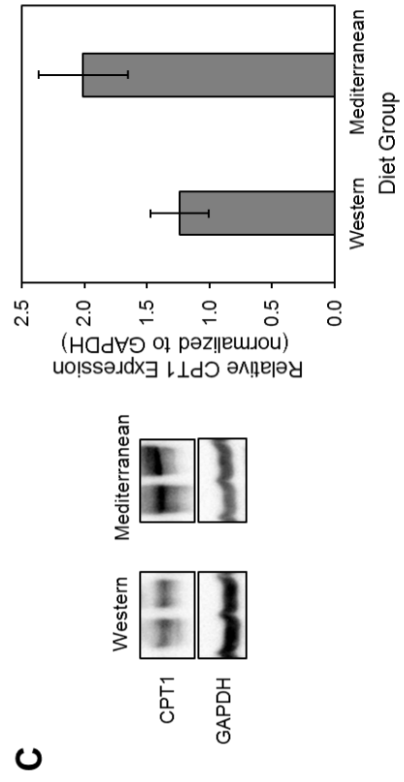
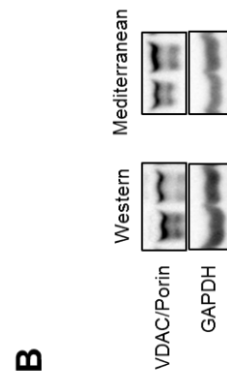
Supplemental Figure 1. Mean energy intake of female cynomolgus macaques fed either a Western or Mediterranean diet for 30 months

(A) Mean calories consumed per day by diet group. Data are presented as means $\pm$ SEM, $n = 11$.

(B) Mean calories consumed per kilogram of body weight per day by diet group. Data are presented as means $\pm$ SEM, $n = 11$.



High Resolution Respirometry Measurement

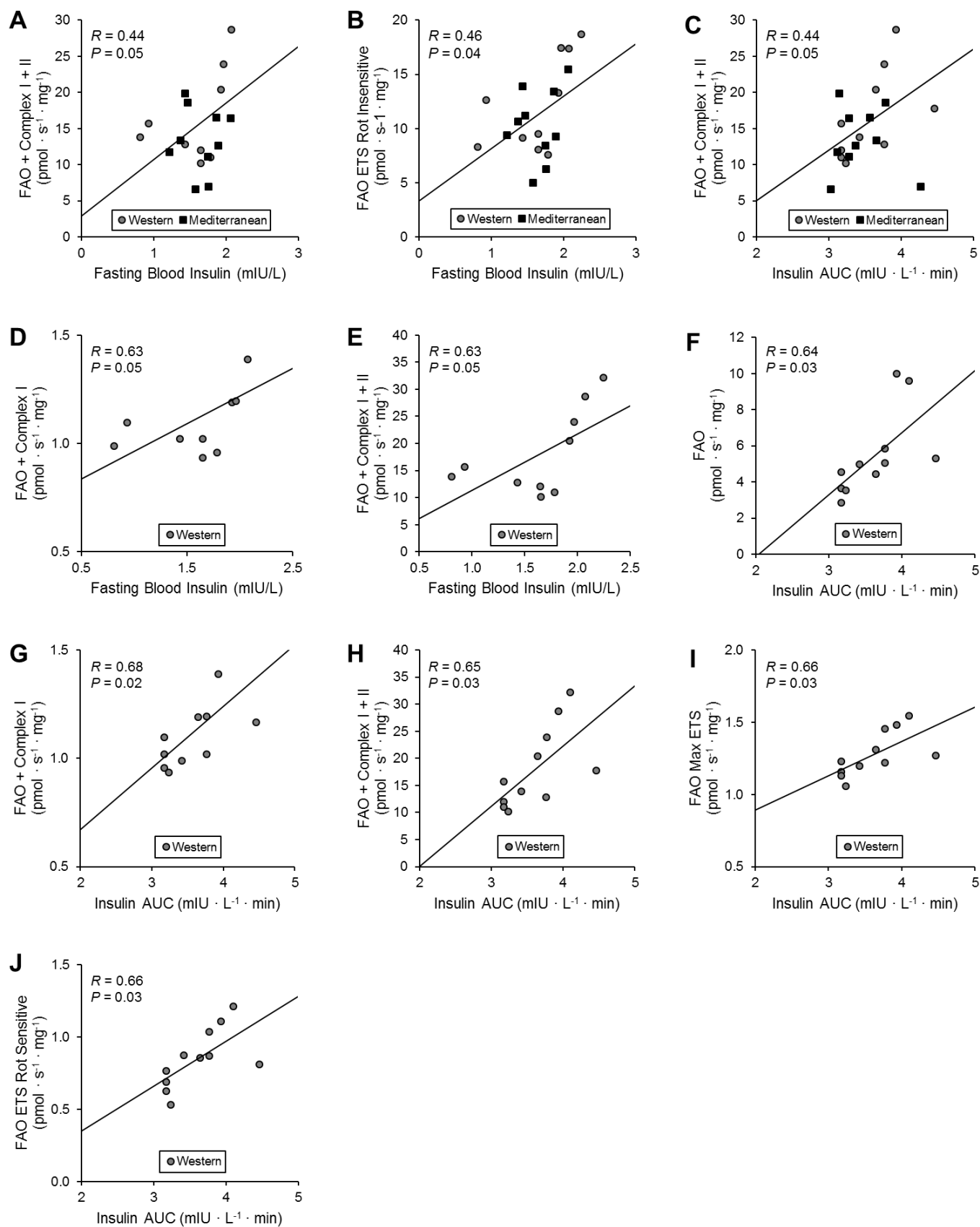


Supplemental Figure 2. Flux control ratios and Western blot analysis of skeletal muscle from female cynomolgus macaques fed a Western or Mediterranean diet

(A) Flux control ratios (FCRs) of high-resolution respirometry of permeabilized skeletal muscle fibers. FCRs were calculated by dividing each respiration parameter by maximum respiration to examine differences in relative contributions of substrates to mitochondrial respiration. Data are presented as means $\pm$ SEM, $n = 11$.

(B) Protein expression of VDAC/Porin in skeletal muscle tissue homogenate from cynomolgus macaques fed either a Western or Mediterranean diet. Data are presented as means $\pm$ SEM, $n = 11$.

(C) Protein expression of CPT1 in skeletal muscle tissue homogenate from cynomolgus macaques fed either a Western or Mediterranean diet. Data are presented as means $\pm$ SEM, $n = 11$.



Supplemental Figure 3. Correlations between female cynomolgus macaque permeabilized muscle fiber bioenergetics and fasting blood insulin and insulin area under the curve

(A-B) Plots of fatty acid oxidation measures versus fasting blood insulin for all animals in the study. Pearson correlation (R) and P -values are shown on each plot.

(C) Plot of FAO + Complex I + II respiration versus insulin area under the curve for all animals in the study. Pearson correlation (R) and P -value are shown on the plot.

(D-E) Plots of fatty acid oxidation measures versus fasting blood insulin for animals in the Western diet group. Pearson correlation (R) and P -values are shown on each plot.

(F-J) Plots of fatty acid oxidation measures versus insulin area under the curve for animals in the Western diet group. Pearson correlation (R) and P -values are shown on each plot.

CHAPTER THREE:

HETEROCHRONIC PARABIOSIS: OLD BLOOD INDUCES CHANGES IN MITOCHONDRIAL STRUCTURE AND FUNCTION OF YOUNG MICE

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manuscript.

Heterochronic Parabiosis: Old Blood Induces Changes in Mitochondrial Structure and Function of Young Mice

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Abstract

Heterochronic parabiosis models have been utilized to demonstrate the role of blood-borne circulating factors in systemic effects of aging. In previous studies, heterochronic parabiosis has shown positive effects across multiple tissues in old mice. More recently, a study demonstrated old blood had a more profound negative effect on muscle performance and neurogenesis of young mice. In this study, we used heterochronic parabiosis to test the hypothesis that circulating factors mediate mitochondrial bioenergetic decline, a well-established biological hallmark of aging. We examined mitochondrial morphology, expression of mitochondrial complexes, and mitochondrial respiration from skeletal muscle of mice connected as heterochronic pairs, as well as young and old isochronic controls. Our results indicate that young heterochronic mice had significantly lower total mitochondrial content and on average had significantly smaller mitochondria compared to young isochronic controls. Expression of complex IV followed a similar pattern: young heterochronic mice had a trend for lower expression compared to young isochronic controls. Additionally, respirometric analyses indicate that young heterochronic mice had significantly lower complex I, complex I + II, and maximal mitochondrial respiration and a trend for lower complex II driven respiration compared to young isochronic controls. Interestingly, we did not observe significant improvements in old heterochronic mice compared to old isochronic controls, demonstrating the profound deleterious effects of circulating factors from old mice on mitochondrial structure and function. We also found no significant differences between the young and old heterochronic mice, demonstrating that circulating factors can be a driver of age-related differences in mitochondrial structure and function.

Keywords: heterochronic parabiosis, mitochondria, mouse model, aging

Introduction

Mitochondrial bioenergetic decline is a hallmark of the aging process. Studies have reported reduced mitochondrial respiratory capacity in older adults, as indicated by reduced phosphocreatine recovery time and reduced mitochondrial respiration in skeletal muscle (1,2). Multiple studies have shown decreased mitochondrial enzyme activities in skeletal muscle, liver, and heart with increasing age, including: succinate dehydrogenase, citrate synthase, cytochrome c oxidase, and β -hydroxyacyl-CoA dehydrogenase (1,3,4). Reduced mitochondrial DNA copy number and increased number of mitochondrial DNA mutations in these tissues have been associated with age in both animal models and human subjects (4–7). Additionally, reduced mitochondrial content and alterations in mitochondrial dynamics are associated with aging (6). Altogether, these studies indicate that mitochondrial changes are systemic and therefore apparent in multiple tissues, including brain, heart, skeletal muscle, and liver (4,8). Yet, little is known about what may mediate the systemic nature of age-related bioenergetic decline.

Heterochronic parabiosis is a surgical technique that involves connecting two mice, in this case one old and one young, so that the circulatory systems of the mice become connected to study the systemic effects of aging. Using parabiosis, researchers have demonstrated that circulating factors in blood play a major role in many age-related processes that occur across a variety of tissues. Heterochronic parabiosis in mice has been reported to have positive effects on muscle, liver, brain, and other tissues for the older parabiont (9–14). Recently, it has been shown that old blood has a greater negative effect on young mice and resulted in decreased muscle performance and neurogenesis (10). While it is apparent that heterochronic parabiosis has the potential to modulate function at the cell and tissue level, the effects of circulating factors on mitochondrial bioenergetics have not been examined.

In this study, we used the heterochronic parabiosis model to test the hypothesis that circulating factors present in blood mediate age-related bioenergetic decline in skeletal muscle. We examined mitochondrial content, expression of mitochondrial electron transport chain complexes, and performed high-resolution respirometry of permeabilized skeletal muscle fibers in pairs of heterochronic and isochronic parabionts. To our knowledge, this study is the first to provide direct evidence that circulating factors alone can mediate age-related changes in mitochondrial structure and function.

Materials and Methods

Heterochronic parabiosis

C57BL/6 young (2 months) and old (18-20 months) female mice were connected as old isochronic controls, young isochronic controls, and heterochronic experimental pairs. Parabiosis surgeries were completed as previously described (15).

All muscle tissues were collected from the free-moving hindlimb opposite of the parabiosis surgery to eliminate any effects of partial immobilization potentially caused by the surgery. For western blot analysis, mice were connected for 35 days before experiments were performed. These mice included 10 old controls (5 pairs), 12 young controls (6 pairs) and 11 young and 11 old heterochronic mice (11 pairs). For high-resolution respirometry analysis, mice were connected for an average of 50 days (range: 40-53 days) before experiments were performed. These experiments included 2 old isochronic controls (1 pair), 4 young isochronic controls (2 pairs), and 6 young and 6 old heterochronic experimental mice (6 pairs). For electron microscopy, mice were connected for an average of 50 days (range: 40-53 days) before experiments were performed and included 2 old isochronic controls (1 pair), 2 young isochronic controls (1 pair), and 2 young and 2 old heterochronic experimental mice (2 pairs). All

procedures and protocols were approved by the Wake Forest School of Medicine Animal Care and Use Committee.

Electron microscopy for mitochondrial morphology

Muscle samples were obtained from a subset of mice and the deep or red *gastrocnemius* of 2 old isochronic controls, 2 young isochronic controls, and 4 heterochronic experimental mice were collected. The tissue was fixed with 2.5% glutaraldehyde in 0.1M Millonig's phosphate buffer (pH 7.3) for a minimum of one hour. Subsequently, the samples were washed 3x in buffer and post-fixed with 1% osmium tetroxide in phosphate buffer for one hour. The samples were washed 3x in buffer, then dehydrated through a graded series of ethanol for 10 minutes each. For preparation of resin infiltration, the samples were incubated in propylene oxide 2x for 15 minutes each. Finally, the samples were gradually infiltrated with 1:1, 1:2, and pure solutions of Spurr's resin and allowed to cure in a 70°C oven overnight. 90 nm longitudinal sections were obtained with a Reichert-Jung Ultracut E ultramicrotome, stained with lead citrate and uranyl acetate, and viewed with a FEI Tecnai Spirit TEM operating at 80 kV. Ten images from each sample were obtained with an AMT 2Vu CCD camera. Mitochondrial morphology parameters (total mitochondrial area, average mitochondrial area, and average mitochondrial length) were measured using ImageJ (National Institutes of Health).

Western blotting

Skeletal muscle samples (*Quadriceps femoris*) were obtained from 10 old isochronic controls, 12 young isochronic controls, and 22 heterochronic experimental mice. Whole tissue homogenate was loaded at a concentration of 20 µg total protein per well for separation by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 1 h in 5% nonfat dry milk in tris-buffered saline containing 0.1%

Tween-20. Blots were probed overnight at 4°C with primary antibodies (VDAC/Porin, Abcam, ab14734; GAPDH, Abcam, ab9484; Total OXPHOS Rodent WB Antibody Cocktail, Abcam, ab110413) and incubated with the appropriate HRP-conjugated anti-IgG antibody. Antibody-bound protein was detected by enhanced chemiluminescence and quantified by densitometry with ImageJ (National Institutes of Health). GAPDH was used as a loading control for whole tissue homogenate and VDAC/Porin was used to control for differences in mitochondrial content.

Preparation of permeabilized skeletal muscle fibers for respirometry

Immediately after animals were euthanized, skeletal muscle tissue (*soleus*) was removed from 2 old isochronic controls, 4 young isochronic controls, and 12 heterochronic experimental mice. Skeletal muscle fibers for high-resolution respirometry were prepared as described previously (16). Briefly, the tissue was placed in ice-cold BIOPS (10 mM Ca-EGTA buffer, 0.1 μ M free calcium, 20 mM imidazole, 20 mM taurine, 50 mM potassium morpholineethanesulfonic acid, 0.5 mM DTT, 6.56 mM MgCl_2 , 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). Any remaining tendon was removed and the tissue fibers were separated mechanically with sharp angular forceps under magnification, permeabilized with saponin (30 mg/mL) for 30 min on ice, and washed with buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4 , 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mg/mL BSA, pH 7.4) for 15 min on ice before analysis.

High-resolution respirometry of permeabilized skeletal muscle fibers

Approximately 0.5-0.9 mg of tissue was added to each chamber and steady-state rate of respiration measurements were obtained after every substrate addition and expressed as picomoles per second per milligram of tissue. Each sample was run in duplicate. High-resolution oxygen flux measurements were measured in 2 mL buffer Z containing 20 mM creatine and 25

μ M blebbistatin to inhibit contraction (17). This injection protocol was completed as follows: 2 mM malate, 4 mM ADP, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate, 10 μ M cytochrome c to test for mitochondrial membrane integrity, 2 additions of 0.25 μ M FCCP followed by a titration of 0.5 μ M FCCP to obtain maximal ETS capacity, 0.5 μ M rotenone, and 5 μ M antimycin-A. Respiration measurements are summarized in (Supplemental Table 1).

Statistical analysis

For analysis of the electron microscopy and high-resolution respirometry data, normality was assessed by Shapiro-Wilk tests and homogeneity of variance was assessed by Levene's test before one-way ANOVAs were performed. Significant ANOVA results were followed by a posthoc analysis to determine which groups were statistically different using Tukey's studentized range test. Significance between groups was defined as $p < 0.05$. All analysis was performed using SAS Enterprise Guide version 7.12 (SAS Institute Inc.).

Results

Old blood induces changes in skeletal muscle mitochondrial morphology

To characterize the effects of heterochronic parabiosis on skeletal muscle mitochondrial structure, we examined overall mitochondrial morphology using electron microscopy. Representative images from each experimental group are shown in Figure 1A. We measured the total mitochondrial area (Figure 1B), average mitochondrial area (Figure 1C), and average length of mitochondria (Figure 1D) in each image field. Our results indicate that young heterochronic mice had significantly lower total mitochondrial area, and significantly lower average mitochondrial area compared to young isochronic controls. We did not observe any significant differences between young heterochronic mice and old heterochronic mice.

Additionally, there were no significant improvement in old heterochronic mice compared to old isochronic controls.

Old blood induces changes in complex IV expression in skeletal muscle

We performed western blots to examine the expression of proteins that are representative of mitochondrial electron transport system complexes in skeletal muscle (Figure 2). We found that VDAC/porin expression was higher in the young isochronic controls compared to old isochronic controls. While not significant, these suggest differences in mitochondrial content. Therefore, we controlled for these differences in mitochondrial content mathematically when comparing differences in mitochondrial complex expression. Our results indicate that the expression of complex IV had a similar pattern as the electron microscopy data: young heterochronic mice had an overall trend for lower expression of complex IV than young isochronic controls, there was no difference between young and old heterochronic mice, and we did not observe a significant improvement in complex IV expression in old heterochronic mice when compared to old isochronic controls.

Old blood induces changes in mitochondrial respiration in permeabilized fibers

Young heterochronic mice exhibited lower oxygen flux across all respirometry measurements in permeabilized fibers when compared to young isochronic controls (Figure 3). Young heterochronic mice had statistically significantly lower mitochondrial respiration with complex I, complex I + II, and max ETS and a trend for lower complex II respiration when compared to young isochronic controls. We did not observe any significant differences in mitochondrial respiration with complex I, complex I + II, max ETS, and complex II between the young and old heterochronic mice groups. Again, we did observe significant improvements in mitochondrial

respiration of skeletal muscle fibers from old heterochronic mice when compared to old isochronic controls.

Discussion

Our results from isochronic parabiosis controls are consistent with previous research that examined mitochondrial morphology, mitochondrial enzyme expression, and mitochondrial respiration across animals of different ages. We found reduced total mitochondrial area, reduced average mitochondrial area, and decreased mitochondrial length in the muscle of older animals compared to young animals. Others have reported similar differences in young and old, non-parabiosis, animals with regard to mitochondrial content reported by electron microscopy (6) and by surrogate measures such as citrate synthase activity (1,3). Moreover, older adults have been reported to have smaller, more fragmented mitochondria accompanied by reduced expression of markers of mitochondrial biogenesis and increased expression of markers of mitochondrial fission (18). We found reduced expression of complex IV in skeletal muscle of the old isochronic controls when compared to the young isochronic controls. Our results are consistent with the decrease in expression of complex IV in skeletal muscle in older individuals that have been reported previously (3,4). Other studies have also reported decreased expression of complex I as well as decreased activities of mitochondrial enzymes, such as: succinate dehydrogenase (complex II), citrate synthase, and cytochrome c oxidase (complex IV) with increasing age (3,4,18,19). We also observed decreased mitochondrial respiration in skeletal muscle from old isochronic controls when compared to young isochronic controls. This observation is consistent with previous studies that found decreased mitochondrial function as measured by both phosphocreatine recovery time and skeletal muscle mitochondrial respiration in older individuals (1,2,20–23). Overall, these studies indicate that the effects of age on mitochondrial structure and function are preserved in our isochronic parabiosis controls.

Our results provide striking evidence of the negative impact of circulating factors in old blood on mitochondrial structure and function. Young heterochronic mice had significantly lower total mitochondrial area, significantly lower average mitochondrial area, and a trend for lower average mitochondrial length in skeletal muscle when compared to young isochronic controls. We found a trend for reduced expression of complex IV in young heterochronic mice when compared to young isochronic controls. We also found significantly reduced mitochondrial respiration with complex I, complex I + II, and max ETS and a trend for lower complex II respiration in young heterochronic mice when compared to young isochronic controls. These mitochondrial alterations may underlie age-related differences in muscle function. Other labs have reported that young heterochronic mice exhibit decreased muscle performance, but no differences in muscle regeneration or fibrosis after muscle injury compared to young isochronic controls (10,24). Notably, lower skeletal muscle mitochondrial respiration has been associated with lower physical function as measured by muscle strength and gait speed (22). Taken together, these results suggest that altered mitochondrial structure and function is not only a major aspect of age-related decline in skeletal muscle, but that circulating factors can mediate these changes. Furthermore, circulating factors may play a role in the link between mitochondrial respiration and physical function.

We did not observe statistically significant increases in mitochondrial area or length, complex IV expression, or mitochondrial respiration in old heterochronic mice when compared to old isochronic controls. Other labs have reported that old heterochronic mice exhibit improved muscle regeneration and reduced fibrosis after muscle injury, but no differences in muscle performance when compared to old isochronic controls (10,24). Taken together, these results suggest that there is a greater negative impact of circulating factors in old blood on mitochondrial structure and function than can be restored by factors in young blood. Another potential explanation for this observation is that intrinsic properties of muscle from older animals

may make these cells resistant to potential benefits of factors found in young blood. It should also be noted that there were a limited number of old isochronic controls and this may have limited our ability to detect differences between these groups.

It is notable that we did not observe any differences in mitochondrial morphology, complex IV expression, or mitochondrial respiration between young heterochronic or old heterochronic mice. When the circulating factors between young and old are mixed by heterochronic parabiosis, mitochondrial structure and function are similar between the parabiosed animals. Other labs have reported a similar responses in heterochronic mice with muscle regeneration and muscle performance (10,24). These results provide a striking demonstration that circulating factors can be a primary driver of mitochondrial structure and function. Completed studies have examined circulating factors as mediators of age-related differences. Some of these have also proposed specific proteins and metabolites that can impact mitochondrial function. For example, previous work from our lab has found that increased levels of IL-6 was associated with lower mitochondrial respiration in older adults (25). Additionally, other studies have also found that alterations in levels of IL-6 (26,27), BCAAs (28,29), and lipid metabolites (30,31) are associated with changes in mitochondrial function.

To our knowledge, this is the first study to examine the effects of age-associated circulating factors on mitochondrial structure and function using the heterochronic parabiosis model. While this study is limited in size, the results are striking and supported by the use of multiple complementary approaches to examine mitochondrial structure and function, including electron microscopy and high-resolution mitochondrial respirometric profiling to examine changes induced by circulating factors.

This study used multiple muscles for analyses: gastrocnemius for electron microscopy, quadriceps femoris for western blotting, and soleus for high-resolution respirometry. This

approach ensured that adequate sample was available for each of our assays. Since we were primarily interested in mitochondrial changes, we chose muscles that were rich in type I muscle fibers that are rich in mitochondria and are highly oxidative (32). A benefit of this approach is that it gives a broader understanding of the mitochondrial changes across muscle tissues; however, there may be region specific effects that we are unable to appreciate. Future studies can include additional tissues for examination to determine if this is muscle-specific or is true across multiple tissue types. A potential limitation of this study is that the tissues were collected from two sets of parabiosis mice and there was variability in time that the animals were connected before analysis. A larger set of experiments would be needed in order to establish if there is a time course to the mitochondrial effects of heterochronic parabiosis. Moreover, this study only included female mice and therefore additional studies would be required to examine the effects of sex on these age-related changes in mitochondria. While equal numbers of parabiosis pairs were surgically created at the beginning of the study, attrition across groups led to a limited sample size, especially for the mitochondrial respiration controls and electron microscopy. This small sample size we were left with upon completion of the study may limit our ability to detect smaller changes. Additionally, the old isochronic controls in the mitochondrial respiration and electron microscopy experiments were from 2 animals from the same parabiosis pair; thus, these may not truly represent independent biological replicates. Outcomes that showed statistical trends for differences in this study can be further examined in future investigations.

The overall impact of this study stems from the finding that circulating factors can mediate alterations in mitochondrial structure and function associated with aging. Additionally, our results demonstrating that the heterochronic pairing of animals leads to similar mitochondrial structure and function between young and old parabionts suggest that circulating factors may be sufficient to mediate age-related bioenergetic differences in skeletal muscle metabolism. These

results support a growing body of evidence that circulating factors are not only potential biomarkers of aging, but may underlie hallmarks of the aging process. These results will support future studies to identify circulating factors mediating age-related bioenergetic decline and their mechanisms of action.

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J.L.G-A. played a key role in the conceptualization and development of this study, performed all experiments, and played a lead role in data analysis and manuscript preparation. N.L. performed all parabiosis surgeries. R-L.L. completed analysis of electron microscopy images. B.L. played a key role in the development of this study, provided the animal model, and supervised the parabiosis surgeries. A.J.A.M. provided oversight for the study and was responsible for its design and development, supervised the work performed by J.L.G-A., provided all of the equipment and reagents for mitochondrial assessments, and worked closely with J.L.G-A. on data analyses and manuscript preparation. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Tonkonogi M, Fernström M, Walsh B, et al. Reduced oxidative power but unchanged antioxidative capacity in skeletal muscle from aged humans. *Pflügers Arch Eur J Physiol.* 2003;446(2):261-269. doi:10.1007/s00424-003-1044-9
2. Johannsen DL, Conley KE, Bajpeyi S, et al. Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *J Clin Endocrinol Metab.* 2012;97(1):242-250. doi:10.1210/jc.2011-1798
3. Coggan AR, Spina RJ, King DS, et al. Histochemical and Enzymatic Comparison of the Gastrocnemius-Muscle of Young and Elderly Men and Women. *Journals Gerontol.* 1992;47(3):B71-B76. doi:10.1093/GERONJ/47.3.B71
4. Barazzoni R, Short KR, Nair KS. Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. *J Biol Chem.* 2000;275(5):3343-3347. doi:10.1074/jbc.275.5.3343
5. Melov S, Shoffner JM, Kaufman A, Wallace DC. Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. *Nucleic Acids Res.* 1995;23(23):4938. doi:10.1093/nar/23.23.4938-a
6. Conley KE, Jubrias SA, Esselman PC. Oxidative capacity and ageing in human muscle. *J Physiol.* 2000;526(1):203-210. doi:10.1111/j.1469-7793.2000.t01-1-00203.x
7. Short KR, Bigelow ML, Kahl J, et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci U S A.* 2005;102(15):5618-5623. doi:10.1073/pnas.0501559102
8. Desler C, Hansen TL, Frederiksen JB, Marcker ML, Singh KK, Juel Rasmussen L. Is there a link between mitochondrial reserve respiratory capacity and aging? *J Aging Res.*

- 2012;2012:1-9. doi:10.1155/2012/192503
9. Conboy IM, Rando TA. Heterochronic parabiosis for the study of the effects of aging on stem cells and their niches. *Cell Cycle*. 2012;11(12):2260-2267. doi:10.4161/cc.20437
 10. Rebo J, Mehdipour M, Gathwala R, et al. A single heterochronic blood exchange reveals rapid inhibition of multiple tissues by old blood. *Nat Commun*. 2016;7. doi:10.1038/ncomms13363
 11. Villeda SA, Plambeck KE, Middeldorp J, et al. Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat Med*. 2014;20(6):659-663. doi:10.1038/nm.3569
 12. Loffredo FS, Steinhauser ML, Jay SM, et al. Growth Differentiation Factor 11 is a Circulating Factor that Reverses Age-Related Cardiac Hypertrophy. *Cell*. 2013;153(4):828-839. doi:10.1016/j.cell.2013.04.015.Growth
 13. Salpeter SJ, Khalaileh A, Weinberg-Corem N, Ziv O, Glaser B, Dor Y. Systemic regulation of the age-Related decline of pancreatic β -Cell replication. *Diabetes*. 2013;62(8):2843-2848. doi:10.2337/db13-0160
 14. Baht GS, Silkstone D, Vi L, et al. Exposure to a youthful circulation rejuvenates bone repair through modulation of β -catenin. *Nat Commun*. 2015;6. doi:10.1038/ncomms8131
 15. Kamran P, Sereti KI, Zhao P, Ali SR, Weissman IL, Ardehali R. Parabiosis in mice: a detailed protocol. *J Vis Exp*. 2013;(80). doi:10.3791/50556
 16. Gonzalez-Armenta JL, Gao Z, Appt SE, et al. Skeletal Muscle Mitochondrial Respiration Is Elevated in Female Cynomolgus Macaques Fed a Western Compared with a Mediterranean Diet. *J Nutr*. 2019;149(9):1493-1502. doi:10.1093/jn/nxz092
 17. Perry CGR, Kane DA, Lin C-T, et al. Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle. *Biochem J*. 2011;437(2):215-222.

doi:10.1042/BJ20110366

18. Boengler K, Kosiol M, Mayr M, Schulz R, Rohrbach S. Mitochondria and ageing: role in heart, skeletal muscle and adipose tissue. 2017. doi:10.1002/jcsm.12178
19. Rooyackers OE, Adey DB, Ades PA, Nair KS. Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proc Natl Acad Sci U S A*. 1996;93(26):15364-15369. doi:10.1073/PNAS.93.26.15364
20. Petersen KF, Befroy D, Dufour S, et al. Mitochondrial dysfunction in the elderly: Possible role in insulin resistance. *Science* (80-). 2003;300(5622):1140-1142. doi:10.1126/science.1082889
21. Picard M, Ritchie D, Wright KJ, et al. Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. *Aging Cell*. 2010;9(6):1032-1046. doi:10.1111/j.1474-9726.2010.00628.x
22. Gonzalez-Freire M, Scalzo | Paul, D'agostino J, et al. Skeletal muscle ex vivo mitochondrial respiration parallels decline in vivo oxidative capacity, cardiorespiratory fitness, and muscle strength: The Baltimore Longitudinal Study of Aging. *Aging Cell*. 2018;17:12725. doi:10.1111/acel.12725
23. Hepple RT. Impact of aging on mitochondrial function in cardiac and skeletal muscle. *Free Radic Biol Med*. 2016;98:177-186. doi:10.1016/j.freeradbiomed.2016.03.017
24. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weismann IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature*. 2005;433(7027):760-764. doi:10.1038/nature03260
25. Tyrrell DJ, Bharadwaj MS, Van Horn CG, Marsh AP, Nicklas BJ, Molina AJA. Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. *Exp Gerontol*. 2015;70:84-91. doi:10.1016/j.exger.2015.07.015

26. VanderVeen BN, Fix DK, Montalvo RN, et al. The regulation of skeletal muscle fatigability and mitochondrial function by chronically elevated interleukin-6. *Exp Physiol*. 2019;104(3):385-397. doi:10.1113/EP087429
27. Tyrrell DJ, Blin MG, Song J, et al. Age-Associated Mitochondrial Dysfunction Accelerates Atherogenesis. *Circ Res*. 2020;126(3):298-314. doi:10.1161/CIRCRESAHA.119.315644
28. Gonzalez-Freire M, Adelnia F, Moaddel R, Ferrucci L. Searching for a mitochondrial root to the decline in muscle function with ageing. *J Cachexia Sarcopenia Muscle*. 2018;9(3):435-440. doi:10.1002/jcsm.12313
29. D'Antona G, Ragni M, Cardile A, et al. Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metab*. 2010;12(4):362-372. doi:10.1016/j.cmet.2010.08.016
30. Zhong Y, Zeng L, Deng J, Duan Y, Li F. β -hydroxy- β -methylbutyrate (HMB) improves mitochondrial function in myocytes through pathways involving PPAR β/δ and CDK4. *Nutrition*. 2019;60:217-226. doi:10.1016/j.nut.2018.09.032
31. Schuster S, Johnson CD, Hennebelle M, et al. Oxidized linoleic acid metabolites induce liver mitochondrial dysfunction, apoptosis, and NLRP3 activation in mice. *J Lipid Res*. 2018;59(9):1597-1609. doi:10.1194/jlr.M083741
32. Picard M, Hepple RT, Burelle Y. Mitochondrial functional specialization in glycolytic and oxidative muscle fibers: tailoring the organelle for optimal function. *Am J Physiol Physiol*. 2012;302(4):C629-C641. doi:10.1152/ajpcell.00368.2011

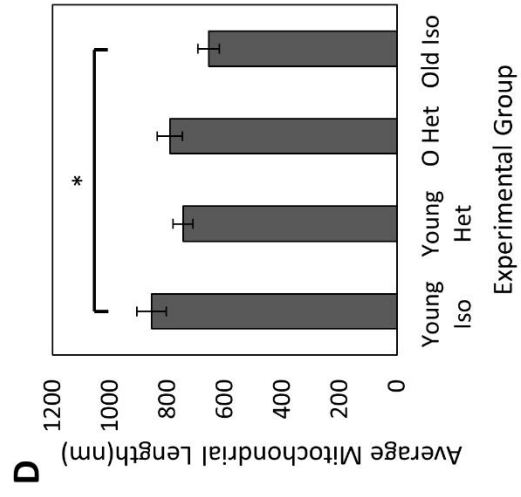
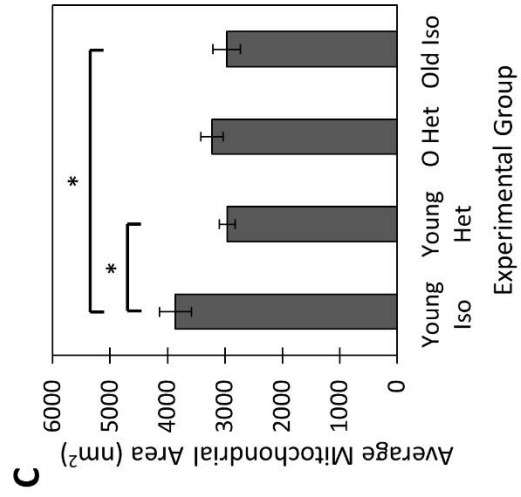
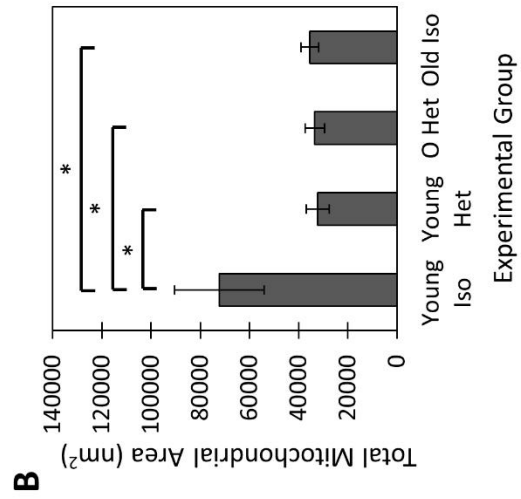
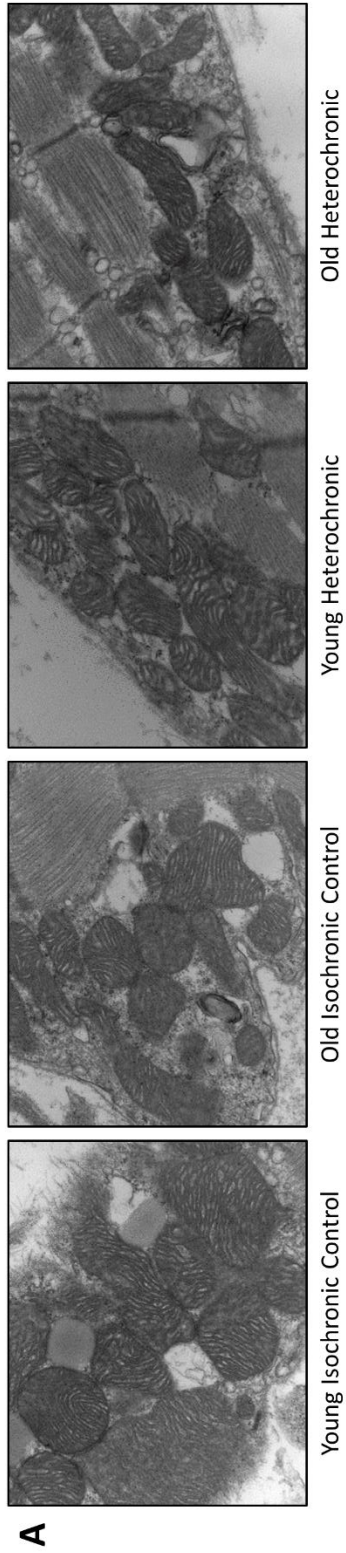


Figure 1. Electron microscopy of skeletal muscle from heterochronic parabiosis mice

A) Representative electron microscopy images from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls.

B) Total mitochondrial area in electron microscopy field. Data are presented as means $\pm$ SEM.

(*, $p < 0.05$)

C) Average mitochondrial area in electron microscopy field. Data are presented as means $\pm$ SEM. (*, $p < 0.05$)

D) Average mitochondrial length in electron microscopy field. Data are presented as means $\pm$ SEM. (*, $p < 0.05$)

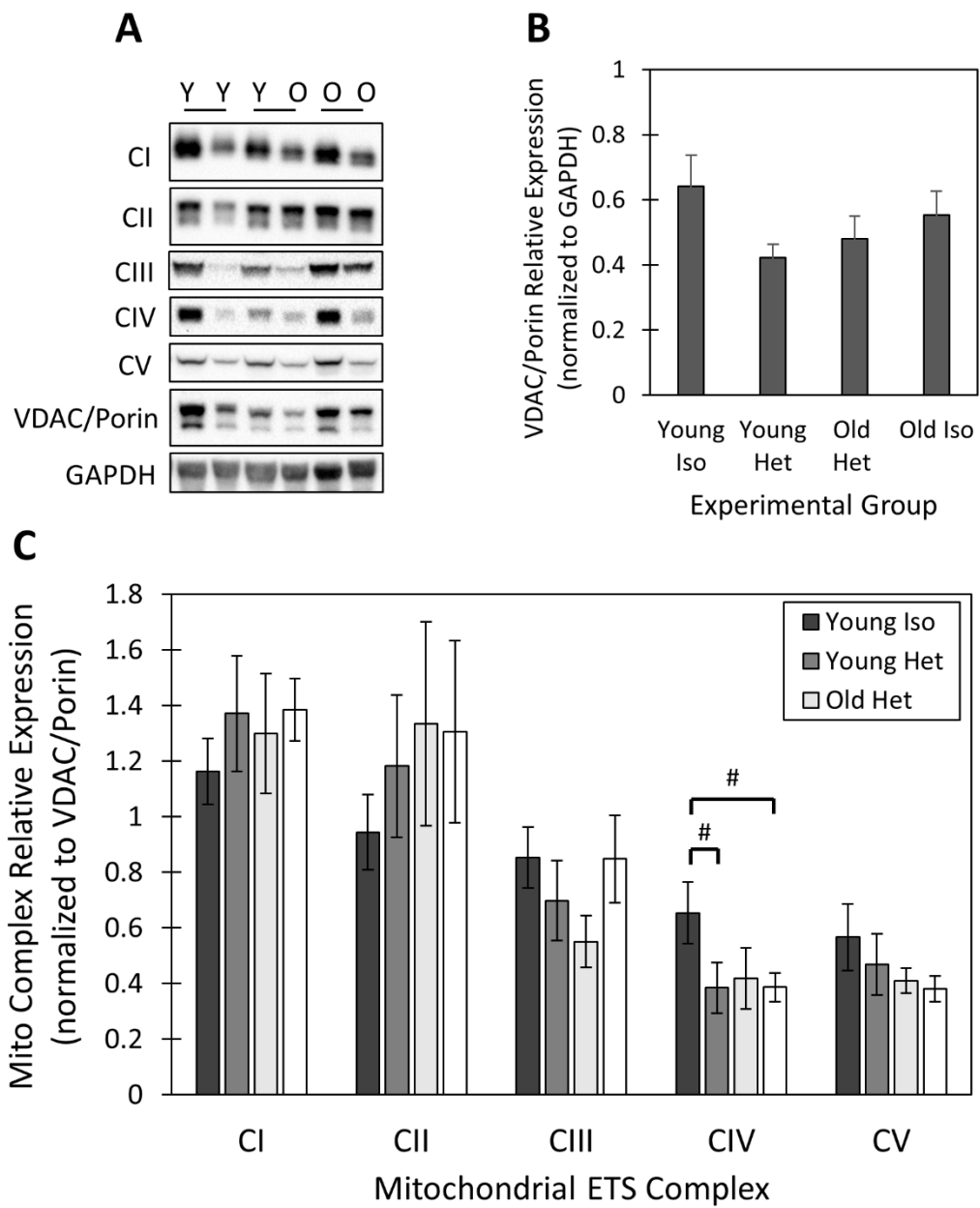


Figure 2. Western blot analysis of skeletal muscle from heterochronic parabiosis mice

A) Representative western blots of mitochondrial complexes in skeletal muscle tissue homogenate images from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls.

B) Relative protein expression of VDAC/Porin in skeletal muscle tissue homogenate from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls. Data are presented as means $\pm$ SEM.

C) Relative protein expression of mitochondrial complexes in skeletal muscle tissue homogenate from young isochronic controls, young mice from heterochronic pairs, old mice from heterochronic pairs, and old isochronic controls. Data are presented as means $\pm$ SEM. (#, $p < 0.10$)

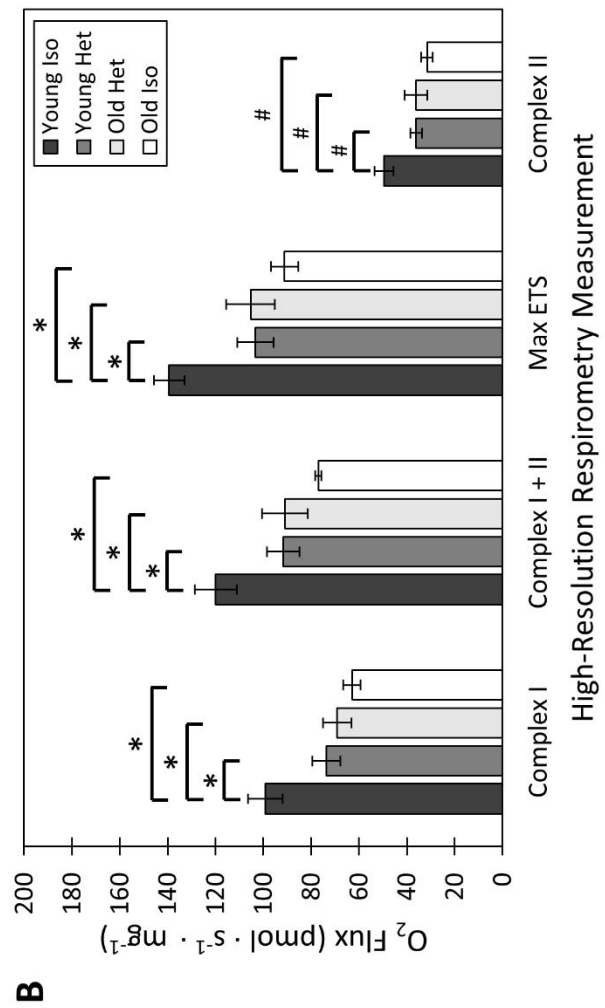
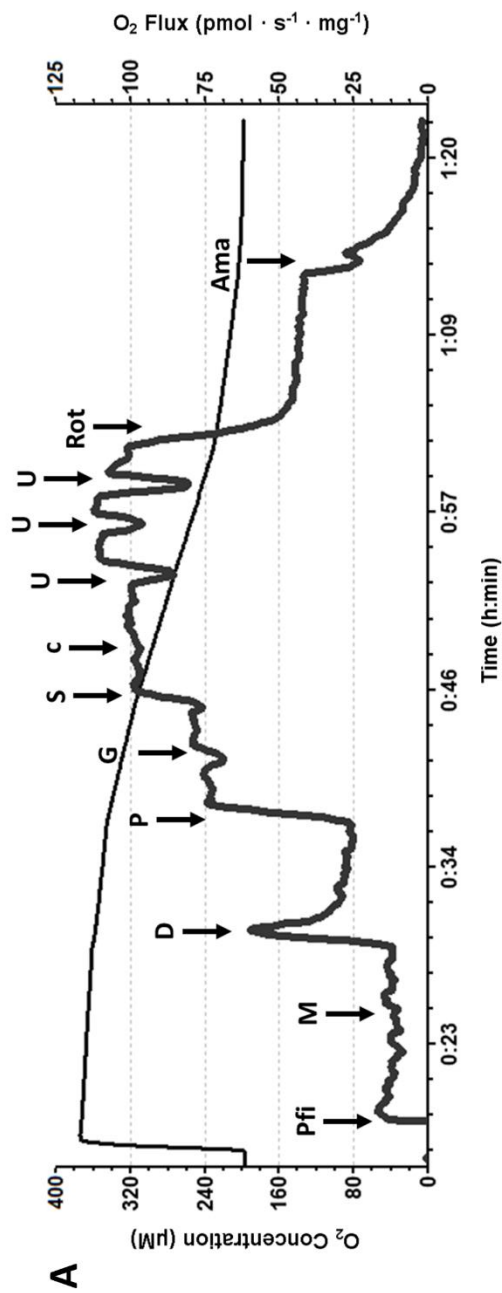


Figure 3. High-resolution respirometry of skeletal muscle from heterochronic parabiosis mice

A) Representative trace of high-resolution respirometry of permeabilized soleus fibers with a SUIT protocol. (**Pfi** = permeabilized fibers, **M** = malate **D** = ADP, **P** = pyruvate, **G** = glutamate, **S** = succinate, **c** = cytochrome c, **U** = FCCP, **Rot** = rotenone, **Ama** = antimycin-A)

B) High-resolution respirometry of permeabilized skeletal muscle fibers. Data are presented as means $\pm$ SEM. (*, $p < 0.05$ and #, $p < 0.10$)

Supplemental Table 1. Summary of respiration measurements

Respiration Measurement	Description	Measurement recorded after additions of:
Complex I	Respiration mediated by complex I	pyruvate, malate, and glutamate
Complex I + II	Respiration mediated by complexes I and II	pyruvate, malate, glutamate, and succinate
Max ETS Capacity	Maximum electron transport system capacity / maximum respiration	FCCP (titrations until maximum)
Complex II	Respiration mediated by complex II, also referred to as rotenone sensitive respiration	obtained by subtracting respiration after rotenone from max ETS capacity

CHAPTER FOUR: SERUM FACTORS MEDIATE THE BIOENERGETIC CHANGES OF DIET AND EXERCISE INTERVENTIONS

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manuscript.

Serum factors mediate the bioenergetic changes of diet and exercise interventions

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Summary

Age-related bioenergetic decline, and the corresponding bioenergetic improvements with interventions, are systemic, suggesting a potential role for blood-borne circulating factors. To determine whether circulating factors can modulate mitochondrial function, we used serum from a clinical trial comparing resistance training (RT) and RT plus caloric restriction (RT+CR) to examine the effects on myoblasts *in vitro*. We report that serum exposure is sufficient to mediate the bioenergetic benefits of these interventions. Additionally, serum-mediated bioenergetics can differentiate between interventions, recapitulate sex differences in bioenergetic responses, and is linked to improvements in physical function and inflammation. Using metabolomics, we identified and tested potential circulating factors responsible for mediating changes in mitochondrial bioenergetics. Specifically, we identified 3-hydroxy-3-methylglutarate and α -hydroxisobutyrate as potent mediators of mitochondrial function and potential therapeutic targets. This study builds upon a growing body of evidence that circulating factors are not only important in aging, but in interventions to improve healthspan as well.

Keywords: mitochondria, bioenergetics, resistance training, caloric restriction, older adults, metabolomics

Introduction

Mitochondrial dysfunction is a hallmark of aging (López-Otín et al., 2013), and is characterized by reduced respiratory capacity, decreased mitochondrial enzyme activities, decreased mtDNA copy number, increased mtDNA mutations, increased ROS production, decreased mitochondrial content, and decreased mitochondrial dynamics (Barazzoni et al., 2000; Coggan et al., 1990; Conley et al., 2000; Johannsen et al., 2012; Melov et al., 1995; Petersen et al., 2003; Rooyackers et al., 1996; Short et al., 2005; Srivastava, 2017; Tonkonogi et al., 2003). Thus far, diet and exercise are some of the most promising interventions to improve healthspan and ameliorate many of the hallmarks of aging, including mitochondrial dysfunction (Barbosa de Queiroz et al., 2017; Drake et al., 2016; Garatachea et al., 2015; Hyatt et al., 2016; Lanza and Nair, 2009; Menshikova et al., 2006; Toledo and Goodpaster, 2013). In addition, age-related mitochondrial dysfunction and the improvements resulting from diet and exercise interventions have been observed in many tissues, including the brain, heart, skeletal muscle, and liver (Barazzoni et al., 2000; Cerqueira et al., 2016; Desler et al., 2012; Forni et al., 2017; Gusdon et al., 2017).

Studies conducted by our lab and others have utilized blood cells as reporters of systemic mitochondrial function and bioenergetic capacity in human studies (Avila et al., 2012; Braganza et al., 2019; Chou et al., 2019; Japiassú et al., 2011; Kramer et al., 2015; Mahapatra et al., 2018; Nguyen et al., 2017, 2019; Sjövall et al., 2010; Tyrrell et al., 2015a, 2015b, 2016, 2017; Willig et al., 2017). Specifically, our lab has demonstrated that blood cell mitochondrial bioenergetics can recapitulate the bioenergetics of the brain, skeletal muscle, and cardiac muscle and that increased circulating IL-6 is associated with decreased mitochondrial bioenergetic capacity in blood cells (Tyrrell et al., 2015a, 2016, 2017). Taken together, these studies support the premise that blood

cells are exposed to circulating factors that mediate mitochondrial function across multiple tissues, and can therefore be used as reliable reporters of human bioenergetic capacity. Importantly, we recently demonstrated that circulating factors in old blood are sufficient to induce age-related changes in mitochondrial structure and function in skeletal muscle of young mice in a heterochronic parabiosis mouse model (Gonzalez-Armenta et al., 2021). Other groups have also shown that circulating factors play a role in many processes, such as aging and exercise interventions, in a variety of tissues: skeletal muscle, liver, brain, skin, and bone (Baht et al., 2015; Bu et al., 2018; Conboy and Rando, 2012; Loffredo et al., 2013; Rebo et al., 2016; Salpeter et al., 2013; Villeda et al., 2011). These multiple lines of evidence suggest that there is a potential role for blood-borne circulating factors in mediating the age-related decline in mitochondrial function and the corresponding improvements from interventions such as diet and exercise.

In this study, we aimed to determine the effects of serum, collected from older adults, before and after intervention, either resistance training or resistance training plus caloric restriction, on muscle cells *in vitro*. We also used metabolomics to identify and test potential circulating factors responsible for mediating changes in mitochondrial bioenergetics in response to resistance training and caloric restriction.

Results

Participant demographic and other characteristics at baseline for this ancillary study

Age, number of female participants, number of white individuals, body mass, height, BMI, waist circumference, hip circumference, and waist-to-hip ratio for the subset of participants included in this ancillary study are summarized in **Table 1**. For this subset of

participants, there were no significant differences between baseline characteristics of the two intervention groups, RT or RT + CR.

Serum-mediated bioenergetics and the effects of intervention

The experimental design and serum-mediated bioenergetics results are summarized in **Figure 1**. The overall effect of intervention on serum-mediated bioenergetics with both intervention groups combined is presented in Figure 1B. Post-intervention serum-mediated bioenergetics were significantly increased compared to pre-intervention serum-mediated bioenergetics as measured by basal, ATP-linked, max, and spare respiratory capacity. The effect of each intervention, RT or RT + CR, on serum-mediated bioenergetics is shown in Figure 1C and the change in OCR from each intervention is shown in Figure 1D. With RT, post-intervention serum-mediated bioenergetics were significantly increased compared to pre-intervention serum-mediated bioenergetics as measured by basal and ATP-linked and there was a trend for increased max respiration. With RT + CR, there were trends for increased post-intervention serum-mediated bioenergetics compared to pre-intervention serum-mediated bioenergetics as measured by max and spare respiratory capacity. The change in max respiration in all participants is shown in Figure 1E, demonstrating the heterogeneity of responses to intervention in terms of serum-mediated bioenergetics. The unadjusted mitochondrial respiration and physical function measurements at baseline and changes with intervention are summarized in **Supplemental Table 1**. The post-intervention mitochondrial respiration and physical function measurement least-square means and adjusted group differences adjusted for age, sex, and baseline value of outcome are also summarized in **Supplemental Table 2**.

Supplemental Figure 1 presents the change in cell count after serum treatment (Supplemental Figure 1A and 1B) and the observed sex differences in these cell counts

(Supplemental Figure 1C and 1D). Cell proliferation upon overnight incubation with participant serum varied depending on intervention group and sex. In particular, RT and female serum appeared to increase overnight proliferation, though these differences were not statistically significant.

Observed sex differences in serum-mediated bioenergetics are shown in **Supplemental Figure 2**. Sex differences in OCR pre- and post-intervention are shown for RT and RT + CR in Supplemental Figures 2A and 2B, respectively. Serum from female participants who completed the RT intervention significantly increased post-intervention basal and ATP-linked respiration and had trends for increased maximal respiration and spare respiratory capacity when compared to pre-intervention. Sex differences in change in OCR in response to intervention are shown for RT and RT + CR in Supplemental Figures 2C and 2D, respectively. Serum from female participants who completed the RT + CR intervention had a trend for increased post-intervention basal respiration and significantly increased leak respiration when compared to pre-intervention. There were no statistically significant differences between pre- and post-intervention serum-mediated bioenergetics with serum from male participants from either intervention, RT or RT + CR.

Correlations between change in serum-mediated bioenergetics and change in physical function and inflammatory cytokines

Spearman correlations were used to assess relationships between bioenergetic parameters and physical function and inflammatory cytokines and are shown in **Supplemental Table 3** (all participants combined), **Supplemental Table 4** (separated by intervention group), and **Supplemental Table 5** (separated by sex).

Regression plots illustrating the relationships between change in serum-mediated max respiration and physical function are shown in **Figure 2A-E**. Change in serum-mediated max respiration was significantly and positively correlated with change in Mat-sf (Figure 2A, $R=0.294$, $p=0.03$), had a trend for a negative correlation with change in 400m walk time (Figure 2B, $R=-0.215$, $p=0.08$), was not significantly associated with change in gait speed (Figure 2C, $R=0.180$, $p=0.14$), was significantly and negatively correlated with change in IL-6 (Figure 2D, $R=-0.255$, $p=0.04$), and had a trend for a negative correlation with change in CRP (Figure 2E, $R=-0.224$, $p=0.08$). Regression plots illustrating the relationships between change in serum-mediated SRC and physical function are shown in **Figure 2F-J**. Change in serum-mediated SRC was significantly and positively correlated with change in Mat-sf (Figure 2F, $R=0.372$, $p=0.004$), significantly and negatively correlated with change in 400m walk time (Figure 2G, $R=-0.246$, $p=0.04$), a trend for a positive correlation with change in gait speed (Figure 2H, $R=0.224$, $p=0.07$), was significantly and negatively correlated with change in IL-6 (Figure 2I, $R=-0.290$, $p=0.02$), and was significantly and negatively correlated with change in CRP (Figure 2J, $R=-0.294$, $p=0.02$).

Metabolomic analysis reveals candidate metabolites that can mediate bioenergetic changes in response to diet and exercise interventions

We sought to determine if metabolites could explain the heterogeneity of serum-mediated bioenergetic response to intervention as shown in Figure 1E. To do this, we first classified the participants as negative responders, non-responders, and positive responders based on the change in serum-mediated maximal respiration and these are shown in **Figure 3A**. A heat map illustrating the relationships of fold change of metabolites and responder status and the scores plot from orthogonal partial least-squares analysis illustrating the separation of responder status based on fold change of

metabolites are shown in **Figure 3B**. We used pattern analysis to determine what metabolites were either positively or negatively associated with the pattern of negative responders to non-responders to positive responders. These metabolites are shown in **Figure 3C**. Additionally, a heat map illustrating relationships of fold change of these top 25 metabolites and negative and positive responders and the scores plot from orthogonal-partial least-squares analysis illustrating the separation of negative and positive responders are shown in **Supplemental Figure 3**.

Correlations between change in serum-mediated bioenergetics and fold change of candidate metabolites

Spearman correlations were used to assess relationships between bioenergetic parameters and the top 25 metabolites identified. Significant relationships are summarized in **Supplemental Table 7**. Regression plots illustrating the relationships between change in serum-mediated max respiration and fold change of metabolites are shown in **Figure 4**. Change in serum-mediated max respiration was significantly and positively correlated with fold change of arabinose (Figure 4A, $R=0.320$, $p= 0.01$), was significantly and positively correlated with fold change of inosine (Figure 4B, $R=0.367$, $p= 0.003$), had a trend for a positive association with fold change of d-gluconic acid γ -lactone (Figure 4C, $R=0.270$, $p= 0.08$), was not significantly associated with fold change of 4-pyridoxate (Figure 4D, $R=0.275$, $p= 0.11$), was not associated with fold change of 10-hydroxydecanoate (Figure 4E, $R=0.060$, $p= 0.64$), was significantly and negatively correlated with fold change of 3-hydroxymethylglutarate (Figure 4F, $R=-0.359$, $p= 0.04$), and had a trend for a negative correlation with fold change of α -hydroxyisobutyrate (Figure 4G, $R=-0.242$, $p= 0.05$).

Candidate metabolite treatment and effects on bioenergetics

To determine if individual metabolites could affect bioenergetics, we tested a selection of top candidates by treating cells with the metabolites directly in **Figure 5**. The treatment effects of various concentrations of inosine, 3-hydroxymethylglutarate, and α -hydroxyisobutyrate on bioenergetics are shown in Figures 5A and 5B, Figures 5C and 5D, and Figures 5E and 5F, respectively. We did not observe an increase in maximal respiration with increasing inosine concentration. However, we did observe decreases in maximal respiration with increasing 3-hydroxymethylglutarate and α -hydroxyisobutyrate concentrations.

Discussion

In this study, we aimed to determine the effects of human serum collected before and after intervention, either resistance training or resistance training plus caloric restriction, on myoblasts *in vitro* and to identify and test potential circulating factors responsible for mediating these changes. We began by examining the overall effect of intervention on serum-mediated bioenergetics. We found that post-intervention serum significantly increased basal, ATP-linked, and maximal mitochondrial respiration when compared to pre-intervention. These results are consistent with observations of increased skeletal muscle mitochondrial function after exercise interventions in older adults (Meex et al., 2010; Menshikova et al., 2005, 2006; Phielix et al., 2010; Porter et al., 2015; Toledo and Goodpaster, 2013).

Next, we examined the effect of intervention group, either RT or RT + CR, on serum-mediated bioenergetics. We found that post-RT serum significantly increased basal and ATP-linked mitochondrial respiration and had a trend for increased maximal mitochondrial respiration, while post-RT + CR serum had trends for increased maximal

mitochondrial respiration and spare respiratory capacity. Previous studies examining the effect of RT on mitochondria report increased mitochondrial function as measured by phosphocreatine recovery time (Meex et al., 2010) and high-resolution respirometry of permeabilized skeletal muscle fibers (Pesta et al., 2011; Porter et al., 2015). Studies examining the effect of CR alone on skeletal muscle mitochondria found no changes in mitochondrial content (Toledo et al., 2008) or mitochondrial function as measured by phosphocreatine recovery time (Sparks et al., 2016). Exercise and CR are rarely combined in studies examining mitochondrial function, but others have reported that aerobic exercise alone increased mitochondrial content and mitochondrial enzyme activities involved in electron transport and fatty acid oxidation while caloric restriction sufficient to induce weight loss did not (Menshikova et al., 2018).

Our results are also consistent with a previous study examining the effects of 10% rat serum on the mitochondrial bioenergetics of cultured β -cells (Cerqueira et al., 2016). They found that serum factors alone from CR rats increased glucose-stimulated insulin secretion, preserved mitochondrial function in response to glucose toxicity, and modified mitochondrial morphology and dynamics. When combined, this information suggests that serum factors alone are sufficient to induce mitochondrial changes that reflect the effects of interventions such as RT and CR.

We observed less robust improvements in serum-mediated bioenergetics after RT + CR serum treatment when compared to RT alone, suggesting that CR may blunt the bioenergetic effects of RT with serum-mediated bioenergetics. Alternatively, RT may alleviate some of the reduced mitochondrial bioenergetics associated with CR. Either of these explanations is consistent with observations that CR decreases overall energy expenditure and increases work efficiency (Hames et al., 2016), RT prevents a substantial amount of lean mass loss associated with CR (Sardeli et al., 2018), and that

lean mass is positively associated with mitochondrial bioenergetics in monocytes (Bellissimo et al., 2019). Therefore, it is likely that CR blunts some of the positive effects of RT, while RT alleviates some of the negative effects of CR on mitochondrial bioenergetics.

Notably, we observed a range of responses to intervention with serum-mediated bioenergetics. This is best exemplified by examining the change in maximal respiration across all participants. We identified three groups of responders: negative responders, non-responders, and positive responders. While there is debate in the literature about whether there are true non-responders or if some of these observations could be attributed to measurement variability that is not adequately controlled for (Dankel and Loenneke, 2020; Sparks, 2017), this observation is also consistent with previous studies that reported heterogeneous responses to a variety of exercise interventions (Baird and Motl, 2019; Bouchard and Rankinen, 2001; Bouchard et al., 1999; Hubal et al., 2005; Kohrt et al., 1991; Pesta et al., 2011; Ross et al., 2019). Indeed, this pattern of heterogeneous response in terms of physical function was also observed in the participants from I'M FIT, the parent study for the samples utilized in this study (Chmelo et al., 2015).

Data on serum-mediated bioenergetics presented in this manuscript are all normalized to cell count. Cell proliferation upon overnight incubation with participant serum varied depending on intervention group and sex. Others have noted that serum source affects cell growth, proliferation, and migration of cultured cells as well as spheroid formation (Heger et al., 2018; Hennig et al., 1989).

We also observed sex differences in serum-mediated bioenergetics. In particular, female participant serum from the RT group significantly increased basal and ATP-linked mitochondrial respiration and had trends for increased maximal respiration and spare

respiratory capacity. We did not observe any significant increases in the male participants. These findings are consistent with observations with high-resolution respirometry that females had greater intrinsic mitochondrial respiratory rates (Cardinale et al., 2018) and exhibit decreased ADP sensitivity and increased sensitivity to malonyl-CoA-mediated respiratory inhibition in skeletal muscle compared to men (Miotto et al., 2018). Furthermore, these observations are supported by other studies, including sex differences in fiber type and fuel utilization during exercise (reviewed in Ansdell et al., 2020).

We found significant correlations between improvements in serum-mediated bioenergetics and improvements in physical function, as measured by Mat-sf, 400-m walk time, and gait speed, and improvements in inflammation, as measured by IL-6 and CRP. These results are consistent with cross-sectional studies completed in a subset of I'M FIT participants, where higher blood cell bioenergetics was associated with faster gait speed, knee extensor strength, and grip strength as well as lower levels of IL-6 (Tyrrell et al., 2015a, 2015b). Taken together, this suggests that improvements in mitochondrial bioenergetics, physical function, and inflammation in older adults are all connected and mediated by blood-borne circulating factors.

We performed metabolomics analyses to identify potential circulating factors that can mediate bioenergetic capacity. Our analyses revealed 25 potential circulating factors that were positively or negatively associated with the pattern of responses that we observed in the change in serum-mediated maximal respiration. We identified 3 metabolites that were significantly and positively associated with serum-mediated maximal respiration (arabinose, inosine, and d-gluconic acid γ -lactone) and 2 metabolites that were significantly and negatively associated with serum-mediated maximal respiration (3-

hydroxymethylglutarate and α -hydroxisobutyrate). Of these, we chose to test inosine, 3-hydroxymethyl-3-glutarate, and α -hydroxisobutyrate as potential circulating factors.

Inosine is a purine nucleoside produced by the catabolism of adenosine and has been examined for potential use in improving exercise performance (McNaughton et al., 1999; Starling et al., 1996; Williams et al., 1990), epilepsy (Kovacs et al., 2015), multiple sclerosis (Muñoz García et al., 2015), and Parkinson's disease (Watanabe et al., 2020) due to potential neuroprotective properties shown in rats (Liu et al., 2006; Zai et al., 2009). We did not find a strong relationship between inosine concentration and increased serum-mediated bioenergetics. This suggests that inosine is not a metabolite that can directly affect mitochondrial bioenergetics and may be downstream of another process or, alternatively, that the mechanism of action involves other cell types.

3-hydroxymethylglutarate is an off-product that is a result of the incomplete conversion of 3-hydroxy-3-methylglutaryl-CoA to acetyl-CoA and acetoacetate by defective or inefficient 3-hydroxy-3-methylglutaryl-CoA lyase. 3-hydroxymethyl-3-glutarate accumulates in the mitochondria and high levels in urine have been associated with inborn errors of metabolism (Dena et al., 1978; Fernandes et al., 2016). We found a strong relationship between increasing concentrations of 3-hydroxymethyl-3-glutarate and decreased serum-mediated bioenergetics, particularly with maximal respiration. This suggests that, at least in older adults, resistance training and caloric restriction can alleviate some of the negative effects of 3-hydroxymethyl-3-glutarate on mitochondrial bioenergetics, through mechanisms that can be explored in future studies.

α -hydroxisobutyrate is an organic acid derived from α -ketobutyrate that is produced by amino acid catabolism and glutathione anabolism (Gall et al., 2010). α -hydroxisobutyrate has been presented as a potential early biomarker for insulin resistance and glucose intolerance (Ferrannini et al., 2013; Gall et al., 2010). In particular, α -hydroxisobutyrate

has been associated with increased insulin resistance and *in vitro* treatment of pancreatic β -cells with α -hydroxisobutyrate inhibited glucose-mediated insulin secretion (Ferrannini et al., 2013). We found a strong relationship between increasing concentrations of α -hydroxisobutyrate and decreased serum-mediated bioenergetics. Taken together, this suggests that α -hydroxisobutyrate may play a key role in the development of insulin resistance and the improvements in insulin sensitivity that are observed with RT and CR interventions.

Interestingly, we observed more robust direct effects of metabolites on mitochondrial bioenergetics that were more negatively associated with serum-mediated bioenergetics. This would be consistent with recent observations of old blood factors being more dominant in old blood (Rebo et al., 2016), including our work (Gonzalez-Armenta et al., 2021). A recent publication demonstrated that dilution of old blood with saline-albumin resulted in improvements in key aspects associated with heterochronic parabiosis, including improved muscle regeneration, reduced liver adiposity and fibrosis, and increased hippocampal neurogenesis (Mehdipour et al., 2020). These results suggest that targeting negative blood factors would have a greater therapeutic benefit and should be the focus of future studies.

Though they were not significantly correlated with serum-mediated bioenergetics, it was interesting that kynurenine, nicotinamide, and nicotinate all were in the top 25 potential circulating factors associated with changes in mitochondrial bioenergetics. These metabolites are important in NAD biosynthesis and recycling pathways (Covarrubias et al., 2021) and suggest that alterations in NAD metabolism could be involved in mediating mitochondrial metabolism as well.

The major strengths of this study include the leveraging of a large, completed randomized controlled clinical trial and the utilization of a method that could be readily applied to other studies and samples. We had to exclude 42 participants from our ancillary study, mainly due to very low mitochondrial respiration and cell death in response to serum treatment, which could be due to immune cross-reactivity between human serum and the mouse cell line or a dominant-negative circulating factor. Further investigation is needed to determine the root cause of cell death and low mitochondrial respiration, but we believe this method is a powerful tool for screening and testing potential mediators of mitochondrial bioenergetics and will help lead to the discovery of new therapeutic targets in a variety of contexts.

In this study, we report that serum, and circulating factors therein, are sufficient to recapitulate the effects of a RT or RT + CR intervention *in vitro*, including differentiating between the interventions, demonstrating sex differences in bioenergetic response to intervention, and is linked to key improvements in human healthspan: physical function and inflammation. We also identified and tested potential circulating factors responsible for mediating mitochondrial bioenergetics. Specifically, we identified 3-hydroxy-3-methylglutarate and α -hydroxisobutyrate as new potential therapeutic targets to improve healthspan. This study further supports a growing body of evidence that circulating factors are not only important for the aging process, but in interventions to improve healthspan as well.

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Author Contributions

Conceptualization – JLGA, BJN, AJAM

Methodology – JLGA, AJAM

Investigation – JLGA, JL, JS

Formal analysis – JLGA, JB

Resources – BJN, CF

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Declaration of Interests

The authors declare no competing interests.

STAR Methods

Parent study: I'M FIT

Samples utilized in this ancillary study were collected during the Improving Muscle for Functional Independence Trial (I'M FIT) ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01049698); NCT01049698). Briefly, I'M FIT was a 5-mo, randomized controlled trial designed to determine whether CR enhances improvements in skeletal muscle function in response to RT in 126 older overweight and obese men and women. Participants were randomly assigned equally to a standardized, progressive RT intervention with CR (RT+CR) or without CR (RT) (Nicklas et al., 2015). The study was approved by the Wake Forest School of Medicine Institutional Review Board, and all participants provided written informed consent to participate.

Description of I'M FIT interventions

All participants in the study underwent 5-mo of RT 3 days/week on weight-stack resistance machines (Cybex International Inc. and Nautilus Inc.) at the Wake Forest University Clinical Research Center exercise facility (Nicklas et al., 2015). Briefly, the resistance training protocol was supervised by two exercise interventionists and included a gradual progression of weight and repetitions during the first month to allow familiarization with the equipment, minimize muscle soreness, and reduce injury potential, then the training goal was to complete 3 sets of 10 repetitions for each exercise at 70% of their one-repetition maximum (1RM) for that specific exercise with 1RM testing every 4 weeks. Participants performed an initial 5-min warm-up by walking or cycling at a slow pace followed by light stretching and concluded each session with a 5-min cool-down and light stretching. The machines used were 1) leg press, 2) leg extension, 3) seated leg curl, 4) seated calf raise, 5) incline press, 6) compound row, 7)

triceps press, and 8) bicep curl. Each participant recorded the weight lifted, the number of repetitions completed, and the number of sets completed for each exercise in a training log.

Participants assigned to RT only were instructed to follow a eucaloric diet, whereas those assigned to RT+CR underwent a dietary weight-loss intervention designed to elicit moderate weight loss (5–10%). Each participant was assigned a daily caloric intake to follow, which was derived from subtracting 600 kcal from his or her estimated daily energy needs for weight maintenance. A maximum of 2 meal replacements per day (shakes and bars; Slim-Fast Inc.) that contained approximately 220 kcal with 7–10 g protein, 33–46 g carbohydrates, 1.5–5 g fat, and 2–5 g fiber were provided to participants for breakfast and lunch. Dinner and snack options were recommended by the RD per each participant's daily caloric goals and tailored to allow for individual preferences for various food items. Participants were asked to keep a diet log of all foods consumed, and the logs were monitored weekly by the RD to verify compliance with the weight-loss intervention.

Measurements completed in the parent study

All assessments took place in the Geriatric Research Center of the Wake Forest School of Medicine J Paul Sticht Center on Aging by examiners blinded to participant treatment assignment (Nicklas et al., 2015). Height, body mass, waist circumference, and hip circumference were measured without shoes and outer garments removed. BMI was calculated as body mass divided by height squared. Waist-to-hip ratio was calculated as waist circumference divided by hip circumference. Grip strength was measured twice on each hand to the nearest kilogram by using an isometric Hydraulic Hand Dynamometer (Jamar), and the maximal value from the right hand was used in analyses. Mobility was assessed using the Mobility Assessment Tool – short form (MAT-sf) (Rejeski et al.,

2010). MAT-sf is a video-animated tool to assess self-perception of mobility that depicts a wooden mannequin performing a wide variety of physical activities (ranging from walking on level ground to carrying bags while climbing stairs) and a question about the participant's ability to perform the task measured on a discrete scale with possible scores of 30-80 (Rejeski et al., 2015). For 400-meter walk time, the participant was instructed to complete the distance (10 laps on a flat indoor surface 20 m in length) as quickly as possible without running. Lower-extremity function was assessed with the short physical performance battery (SPPB) (Guralnik et al., 1994), which consisted of a standing balance test, usual gait speed over a 4-meter course, and time to complete 5 repeated chair rises with arms folded across the chest. Results from each of the 3 tests were scored from 0 (inability to perform the task) to 4. The total SPPB score, which ranged from 0 (lowest function) to 12 (highest function), was used for analyses. Maximal knee extensor strength [in Newton-meters (Nm)] was measured with a dynamometer (Biodex Medical Systems Inc.) at speeds of 608 and 2408/s with the participant sitting and hips and knees flexed at 90°. Participants were asked to extend the knee and push as hard as possible against the resistance pad. The strength of the right leg recorded as the peak torque (in Nm) was used for analyses.

Serum collection and storage

Blood samples were collected in serum separator tubes in the morning after an overnight fast at baseline and post-intervention (at least 24 hours after the last acute bout of exercise). The blood was allowed to clot for 30-60 minutes, separated by centrifugation at 2,000 x g for 10 minutes, divided into aliquots, and stored at -80°C until needed for further analysis. Of the original 126 participants of I'M FIT, 100 participants had pre- and post-intervention serum available for analysis.

Inflammatory cytokines

IL-6 was measured by ELISA using high-sensitivity Quantikine Immunoassay kits from R&D Systems (Cat # SS600C). CRP was measured using high sensitivity assays from Siemens Healthineers USA (Cat # LKCRP1) on an IMMULITE Automated Immunoassay System. To eliminate variability due to reagent conditions, all kits were pre-ordered having the same lot number for all reagents. All samples were measured in duplicate and the mean was used for data analyses. Samples with high variation (CV > 10%) were repeated. Commercial controls and an internal laboratory control were run on each 96-well plate.

Cell culture, serum treatment, and metabolite treatment

C2C12 myoblast cells were obtained from ATCC (CRL-1772) and cultured in DMEM supplemented with 2mM glutamine, 10% FBS, and 1% penicillin/streptomycin. Cells were seeded at a density of 1.5×10^6 in T-75 culture flasks and were split every other day to avoid cultures becoming confluent.

For serum treatment, the experimental design was adapted from Cerqueira et al., 2016. In this study, C2C12 cells were seeded at a density of 15,000/well in a 96-well Seahorse culture plate and allowed to adhere overnight. Media was exchanged for serum-free media and 10% human serum was added to each well. Each participant's pre- and post-serum was used for treatment in quadruplicate.

For metabolite treatment, cells were seeded at a density of 15,000/well in a 96-well Seahorse culture plate and allowed to adhere overnight. Various concentrations of each metabolite were added to each well with 6-8 replicates per plate for a total of $n=10-16$. Each metabolite was added under, within, and over physiological ranges for these experiments. Inosine (Sigma, I4125) was added at concentrations of 0, 0.25, 0.5, 0.75,

1, 1.25, 1.5, 1.75, 2, and 3 μM (Farthing et al., 2015). 3-hydroxy-3-methylglutarate (Sigma, H4392) was added at concentrations of 0, 0.25, 0.5, 0., 1, 1.5, 2, 2.5, 3, 5, and 10 μM (Václavík et al., 2020). α -hydroxyisobutyrate (Sigma, 323594) was added at concentrations of 0, 20, 40, 60, 80, 100, 125, 150, 175, and 200 μM (Tricò et al., 2017).

Cell counting with Gen5

Cell images were obtained pre- and post-treatment with a Cytation 5 Cell Imaging Multi-Mode Reader (Biotek Instruments, Winooski, VT). These images were used to monitor cell health and proliferation during treatment. High-contrast brightfield images were used to count cells and were completed using Gen5 software (Biotek Instruments, Winooski, VT).

High-throughput respirometry

Before each Seahorse run, media was exchanged for Seahorse XF assay media (Agilent, 103575) supplemented with 25mM glucose, 2mM glutamine, and 1mM pyruvate. Additionally, brightfield images were obtained using Seahorse XF Imaging and Cell Counting Software (Agilent Technologies, Santa Clara, CA). Extracellular flux analysis was completed using a Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA) with a standard injection protocol for a mitochondrial stress test: oligomycin (final concentration: 0.75 μM), FCCP (final concentration: 1 μM), and Antimycin-A/Rotenone (final concentration: 1 μM). Hoescht 3342 dye (final concentration: 6 μM) was injected at the end of the assay and incubated at 37°C for 30 mins before fluorescence imaging in the XF Imaging and Cell Counting Software for normalization to cell count. Seahorse assay parameters (basal, ATP-linked, leak, max, and spare respiratory capacity) were corrected for non-mitochondrial respiration and calculated as previously described (Agilent Technologies, 2020).

Seahorse assay exclusion criteria

Seahorse analysis was completed with samples from the 100 participants that had completed the intervention and had pre- and post-serum available. Wells were excluded from Seahorse analysis if: (1) OCR measurements were negative or below 10 pmol/min, (2) oxygen level diverged more than 20 mmHg from background wells (typically a level of ~150 mmHg), (3) automated cell count after nuclei staining did not work due to low cell number, (4) only one or two wells were remaining per sample (i.e. only one well would not be included in the analysis for a pre-intervention sample), or (5) insufficient data for pre- or post-intervention (only full pre- and post-intervention pairs were included in the analysis). After the exclusion of these wells, there was sufficient data for 33 participants that completed the RT intervention and 35 participants that completed the RT + CR intervention, for a total of 68 participants included in this study.

Metabolomics Analysis

LC-MS grade water, acetonitrile, methanol, and formic acid were purchased from ThermoFisher Scientific (Waltham, MA, USA). 2-(N-morpholino)ethanesulfonic acid (MES) and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

50 μ L of I'M FIT serum (total n=68, RT=33, RT+CR=35) was spiked with 10 μ L of MES solution which was freshly prepared in water (20 ng/mL). Metabolites were extracted by adding 200 μ L of cold methanol and incubated on ice for 30 minutes. The supernatant was taken after centrifugation at 18,000 x g for 5 minutes and was dried under vacuum. The residue was reconstituted in 5% methanol for LC-MS analysis.

The LC-MS consisted of a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) and a Vanquish UHPLC system

(Thermo Scientific, Waltham, MA, USA). Samples were analyzed on two different columns, a Hypersil GOLD pentafluorophenyl (PFP) column (2.1 x 100mm, 1.9 μ m, Thermo Scientific, Waltham, MA, USA), and an Accucore Vanquish C18+ (2.1 x 100 mm, 1.5 μ m, Thermo Scientific, Waltham, MA, USA) column. A linear gradient was employed for chromatographic separation using 100% water (mobile phase A) and 90% acetonitrile (mobile phase B) both of which contained 0.1% formic acid and 10mM ammonium formate. For the PFP column, the mobile phase flow rate was 0.25 mL/minutes and the gradient began at 2% B which was held for 2 minutes, then increased to 98% B at 8 minutes. This percentage was held for 2 minutes before being decreased to 2% B at 10.1 minutes and then held at that final percentage until 13 minutes. For the C18 column, the mobile phase flow rate was 0.20 mL/minutes and the gradient began at 0% B which was held for 3 minutes, then increased to 95% B at 6 minutes. This percentage was held for 1 minute before being decreased to 0% B at 7.1 minutes and then held at that final percentage until 10 minutes. Data was acquired by collecting full mass spectra (MS1) using polarity switching (positive/negative) at a resolution of 150K.

To identify metabolites, peak features were detected and integrated by the MSMLS Discovery software (IROA Technologies LLC, Sea Girt, NJ, USA) in combination with in-house compound libraries prepared using a Mass Spectrometry Metabolite Library (Sigma, St. Louis, MO, USA). To eliminate redundancy in compound identification, the most abundant ion in each metabolite was selected, which was then normalized to the total ion current (TIC) for relative quantification.

Statistical analysis

Statistical significance between groups (pre- and post-intervention or RT and RT + CR) was evaluated by paired two-tailed Student's t-tests using Microsoft Excel software. The

significance between groups was defined as $P \leq 0.05$. In addition, we examined the statistical differences between groups for post-intervention outcome values using an ANCOVA and a model adjusted for age, sex, and the baseline value for the outcome.

Spearman correlations were assessed between changes in bioenergetic parameters and physical function and inflammatory cytokines in both groups combined, separated by intervention group, and separated by sex. Partial correlations were adjusted for age, BMI, sex, baseline physical function, and baseline bioenergetics. Significance was defined as $P \leq 0.05$. Analysis was performed using SAS Enterprise Guide 7.12 (SAS Institute Inc., Cary, NC, USA).

To classify participants as negative responders, non-responders, and positive responders based on change in serum-mediated maximal respiration, we modified the method presented in Dankel and Loenneke, 2020 to account for some of the random error associated with measuring outcomes in exercise interventions. Briefly, we used the RT group to calculate a 75% confidence interval (CI) to account for 75% of the random error. For negative responders, we included participants who had a change in maximal respiration below the 75% CI, and for positive responders, we included participants who had a change in maximal respiration above the 75% CI. A summary of these parameters is shown in **Supplemental Table 6**.

Statistical analysis of the metabolomics data was completed in MetaboAnalyst 5.0 (Chong et al., 2019). Spearman correlations were also assessed between change in bioenergetic parameters and fold change of metabolites. Significance was defined as $P \leq 0.05$. Analysis was performed using SAS Enterprise Guide 7.12 (SAS Institute Inc., Cary, NC, USA).

References

Agilent Technologies (2020). Report Generator User Guide - Agilent Seahorse XF Cell Mito Stress Test. 9.

Ansdell, P., Thomas, K., Hicks, K.M., Hunter, S.K., Howatson, G., and Goodall, S. (2020). Physiological sex differences affect the integrative response to exercise: acute and chronic implications. *Experimental Physiology* 105, 2007–2021.

Avila, C., Huang, R.J., Stevens, M.V., Aponte, A.M., Tripodi, D., Kim, K.Y., and Sack, M.N. (2012). Platelet mitochondrial dysfunction is evident in type 2 diabetes in association with modifications of mitochondrial anti-oxidant stress proteins. *Exp Clin Endocrinol Diabetes* 120, 248–251.

Baht, G.S., Silkstone, D., Vi, L., Nadesan, P., Amani, Y., Whetstone, H., Wei, Q., and Alman, B.A. (2015). Exposure to a youthful circulation rejuvenates bone repair through modulation of β -catenin. *Nat Commun* 6, 7131.

Baird, J.F., and Motl, R.W. (2019). Response Heterogeneity with Exercise Training and Physical Activity Interventions among Persons with Multiple Sclerosis. *Neurorehabil Neural Repair* 33, 3–14.

Barazzoni, R., Short, K.R., and Nair, K.S. (2000). Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. *J Biol Chem* 275, 3343–3347.

Barbosa de Queiroz, K., Honorato-Sampaio, K., Rossoni Júnior, J.V., Andrade Leal, D., Pinto, A.B.G., Kappes-Becker, L., Evangelista, E.A., and Guerra-Sá, R. (2017). Physical

activity prevents alterations in mitochondrial ultrastructure and glucometabolic parameters in a high-sugar diet model. *PLoS One* 12, e0172103.

Bellissimo, M., Fleischer, C., Tran, P., Hao, L., Reiter, D., Smith, M., Boebinger, S., Wells, G., Jones, D., Ziegler, T., et al. (2019). Mitochondrial Bioenergetic Metabolism Is Associated with Total Body Composition and Influenced by Normal Weight Obesity (P21-039-19). *Current Developments in Nutrition* 3.

Bouchard, C., and Rankinen, T. (2001). Individual differences in response to regular physical activity. *Med Sci Sports Exerc* 33, S446-451; discussion S452-453.

Bouchard, C., An, P., Rice, T., Skinner, J.S., Wilmore, J.H., Gagnon, J., Pérusse, L., Leon, A.S., and Rao, D.C. (1999). Familial aggregation of VO₂(max) response to exercise training: results from the HERITAGE Family Study. *J Appl Physiol* (1985) 87, 1003–1008.

Braganza, A., Corey, C.G., Santanasto, A.J., Distefano, G., Coen, P.M., Glynn, N.W., Nourai, S.-M., Goodpaster, B.H., Newman, A.B., and Shiva, S. (2019). Platelet bioenergetics correlate with muscle energetics and are altered in older adults. *JCI Insight* 5.

Bu, X.-L., Xiang, Y., Jin, W.-S., Wang, J., Shen, L.-L., Huang, Z.-L., Zhang, K., Liu, Y.-H., Zeng, F., Liu, J.-H., et al. (2018). Blood-derived amyloid- β protein induces Alzheimer's disease pathologies. *Mol Psychiatry* 23, 1948–1956.

Cardinale, D.A., Larsen, F.J., Schiffer, T.A., Morales-Alamo, D., Ekblom, B., Calbet, J.A.L., Holmberg, H.-C., and Boushel, R. (2018). Superior Intrinsic Mitochondrial Respiration in Women Than in Men. *Front Physiol* 9, 1133.

Cerqueira, F.M., Chausse, B., Baranovski, B.M., Liesa, M., Lewis, E.C., Shirihai, O.S., and Kowaltowski, A.J. (2016). Diluted serum from calorie-restricted animals promotes mitochondrial β -cell adaptations and protect against glucolipotoxicity. *FEBS J* 283, 822–833.

Chmelo, E.A., Crotts, C.I., Newman, J.C., Brinkley, T.E., Lyles, M.F., Leng, X., Marsh, A.P., and Nicklas, B.J. (2015). Heterogeneity of physical function responses to exercise training in older adults. *J Am Geriatr Soc* 63, 462–469.

Chong, J., Wishart, D.S., and Xia, J. (2019). Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocols in Bioinformatics* 68, e86.

Chou, C.-H., Fu, T.-C., Tsai, H.-H., Hsu, C.-C., Wang, C.-H., and Wang, J.-S. (2019). High-intensity interval training enhances mitochondrial bioenergetics of platelets in patients with heart failure. *Int J Cardiol* 274, 214–220.

Coggan, A.R., Spina, R.J., Rogers, M.A., King, D.S., Brown, M., Nemeth, P.M., and Holloszy, J.O. (1990). Histochemical and enzymatic characteristics of skeletal muscle in master athletes. *J Appl Physiol* (1985) 68, 1896–1901.

Conboy, I.M., and Rando, T.A. (2012). Heterochronic parabiosis for the study of the effects of aging on stem cells and their niches. *Cell Cycle* 11, 2260–2267.

Conley, K.E., Jubrias, S.A., and Esselman, P.C. (2000). Oxidative capacity and ageing in human muscle. *J Physiol* 526 Pt 1, 203–210.

Covarrubias, A.J., Perrone, R., Grozio, A., and Verdin, E. (2021). NAD + metabolism and its roles in cellular processes during ageing. *Nature Reviews Molecular Cell Biology* 22, 119–141.

Dankel, S.J., and Loenneke, J.P. (2020). A Method to Stop Analyzing Random Error and Start Analyzing Differential Responders to Exercise. *Sports Med* 50, 231–238.

Dena, R., Fabbro, M., and Rigoni, F. (1978). Formation and utilization of 3-hydroxy-3-methylglutarate in liver mitochondria of starved and streptozotocin-diabetic rats. *Biochem J* 172, 371–375.

Desler, C., Hansen, T.L., Frederiksen, J.B., Marcker, M.L., Singh, K.K., and Juel Rasmussen, L. (2012). Is There a Link between Mitochondrial Reserve Respiratory Capacity and Aging? *J Aging Res* 2012, 192503.

Drake, J.C., Wilson, R.J., and Yan, Z. (2016). Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle. *FASEB J* 30, 13–22.

Farthing, D.E., Farthing, C.A., and Xi, L. (2015). Inosine and hypoxanthine as novel biomarkers for cardiac ischemia: From bench to point-of-care. *Exp Biol Med (Maywood)* 240, 821–831.

Fernandes, C.G., Rodrigues, M.D.N., Seminotti, B., Colín-González, A.L., Santamaria, A., Quincozes-Santos, A., and Wajner, M. (2016). Induction of a Proinflammatory Response in Cortical Astrocytes by the Major Metabolites Accumulating in HMG-CoA Lyase Deficiency: the Role of ERK Signaling Pathway in Cytokine Release. *Mol Neurobiol* 53, 3586–3595.

Ferrannini, E., Natali, A., Camastra, S., Nannipieri, M., Mari, A., Adam, K.-P., Milburn, M.V., Kastenmüller, G., Adamski, J., Tuomi, T., et al. (2013). Early Metabolic Markers of the Development of Dysglycemia and Type 2 Diabetes and Their Physiological Significance. *Diabetes* 62, 1730–1737.

Forni, M.F., Peloggia, J., Braga, T.T., Chinchilla, J.E.O., Shinohara, J., Navas, C.A., Camara, N.O.S., and Kowaltowski, A.J. (2017). Caloric Restriction Promotes Structural and Metabolic Changes in the Skin. *Cell Rep* 20, 2678–2692.

Gall, W.E., Beebe, K., Lawton, K.A., Adam, K.-P., Mitchell, M.W., Nakhle, P.J., Ryals, J.A., Milburn, M.V., Nannipieri, M., Camastra, S., et al. (2010). α -Hydroxybutyrate Is an Early Biomarker of Insulin Resistance and Glucose Intolerance in a Nondiabetic Population. *PLoS One* 5.

Garatachea, N., Pareja-Galeano, H., Sanchis-Gomar, F., Santos-Lozano, A., Fiuza-Luces, C., Morán, M., Emanuele, E., Joyner, M.J., and Lucia, A. (2015). Exercise attenuates the major hallmarks of aging. *Rejuvenation Res* 18, 57–89.

Gonzalez-Armenta, J.L., Li, N., Lee, R.-L., Lu, B., and Molina, A.J.A. (2021). Heterochronic Parabiosis: Old Blood Induces Changes in Mitochondrial Structure and Function of Young Mice. *J Gerontol A Biol Sci Med Sci* 76, 434–439.

Guralnik, J.M., Simonsick, E.M., Ferrucci, L., Glynn, R.J., Berkman, L.F., Blazer, D.G., Scherr, P.A., and Wallace, R.B. (1994). A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. *J Gerontol* 49, M85-94.

Gusdon, A.M., Callio, J., Distefano, G., O'Doherty, R.M., Goodpaster, B.H., Coen, P.M., and Chu, C.T. (2017). Exercise increases mitochondrial complex I activity and DRP1 expression in the brains of aged mice. *Exp Gerontol* 90, 1–13.

Hames, K.C., Coen, P.M., King, W.C., Anthony, S.J., Stefanovic-Racic, M., Toledo, F.G.S., Brown, J., Helbling, N., Dubé, J.J., DeLany, J.P., et al. (2016). Resting and Exercise Energy Metabolism in Weight-Reduced Adults with Severe Obesity. *Obesity (Silver Spring)* 24, 1290–1298.

Heger, J.I., Froehlich, K., Pastuschek, J., Schmidt, A., Baer, C., Mrowka, R., Backsch, C., Schleußner, E., Markert, U.R., and Schmidt, A. (2018). Human serum alters cell culture behavior and improves spheroid formation in comparison to fetal bovine serum. *Exp Cell Res* 365, 57–65.

Hennig, B., Boissonneault, G.A., and Glauert, H.P. (1989). Effects of serum type on growth and permeability properties of cultured endothelial cells. *Exp Cell Res* 181, 589–596.

Hubal, M.J., Gordish-Dressman, H., Thompson, P.D., Price, T.B., Hoffman, E.P., Angelopoulos, T.J., Gordon, P.M., Moyna, N.M., Pescatello, L.S., Visich, P.S., et al. (2005). Variability in muscle size and strength gain after unilateral resistance training. *Med Sci Sports Exerc* 37, 964–972.

Hyatt, H.W., Kephart, W.C., Holland, A.M., Mumford, P., Mobley, C.B., Lowery, R.P., Roberts, M.D., Wilson, J.M., and Kavazis, A.N. (2016). A Ketogenic Diet in Rodents Elicits Improved Mitochondrial Adaptations in Response to Resistance Exercise Training Compared to an Isocaloric Western Diet. *Front Physiol* 7, 533.

Japiassú, A.M., Santiago, A.P.S.A., d'Avila, J. da C.P., Garcia-Souza, L.F., Galina, A., Castro Faria-Neto, H.C., Bozza, F.A., and Oliveira, M.F. (2011). Bioenergetic failure of human peripheral blood monocytes in patients with septic shock is mediated by reduced F1Fo adenosine-5'-triphosphate synthase activity. *Crit Care Med* 39, 1056–1063.

Johannsen, D.L., Conley, K.E., Bajpeyi, S., Punyanitya, M., Gallagher, D., Zhang, Z., Covington, J., Smith, S.R., and Ravussin, E. (2012). Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *J Clin Endocrinol Metab* 97, 242–250.

Kohrt, W.M., Malley, M.T., Coggan, A.R., Spina, R.J., Ogawa, T., Ehsani, A.A., Bourey, R.E., Martin, W.H., and Holloszy, J.O. (1991). Effects of gender, age, and fitness level on response of VO₂max to training in 60-71 yr olds. *J Appl Physiol* (1985) 71, 2004–2011.

Kovacs, Z., Kekesi, K.A., Juhasz, G., Barna, J., Heja, L., Lakatos, R., and Dobolyi, A. (2015). Non-adenosine nucleoside inosine, guanosine and uridine as promising antiepileptic drugs: a summary of current literature. *Mini Rev Med Chem* 14, 1033–1042.

Kramer, P.A., Chacko, B.K., George, D.J., Zhi, D., Wei, C.-C., Dell'Italia, L.J., Melby, S.J., George, J.F., and Darley-USmar, V.M. (2015). Decreased Bioenergetic Health Index in monocytes isolated from the pericardial fluid and blood of post-operative cardiac surgery patients. *Biosci Rep* 35.

Lanza, I.R., and Nair, K.S. (2009). Muscle mitochondrial changes with aging and exercise. *Am J Clin Nutr* 89, 467S-71S.

Liu, F., You, S.-W., Yao, L.-P., Liu, H.-L., Jiao, X.-Y., Shi, M., Zhao, Q.-B., and Ju, G. (2006). Secondary degeneration reduced by inosine after spinal cord injury in rats. *Spinal Cord* 44, 421–426.

Loffredo, F.S., Steinhauser, M.L., Jay, S.M., Gannon, J., Pancoast, J.R., Yalamanchi, P., Sinha, M., Dall'Osso, C., Khong, D., Shadrach, J.L., et al. (2013). Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell* 153, 828–839.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The Hallmarks of Aging. *Cell* 153, 1194–1217.

Mahapatra, G., Smith, S.C., Hughes, T.M., Wagner, B., Maldjian, J.A., Freedman, B.I., and Molina, A.J.A. (2018). Blood-based bioenergetic profiling is related to differences in brain morphology in African Americans with Type 2 diabetes. *Clin Sci (Lond)* 132, 2509–2518.

McNaughton, L., Dalton, B., and Tarr, J. (1999). Inosine supplementation has no effect on aerobic or anaerobic cycling performance. *Int J Sport Nutr* 9, 333–344.

Meex, R.C.R., Schrauwen-Hinderling, V.B., Moonen-Kornips, E., Schaart, G., Mensink, M., Phielix, E., van de Weijer, T., Sels, J.-P., Schrauwen, P., and Hesselink, M.K.C. (2010). Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes* 59, 572–579.

Mehdipour, M., Skinner, C., Wong, N., Lieb, M., Liu, C., Etienne, J., Kato, C., Kiprov, D., Conboy, M.J., and Conboy, I.M. (2020). Rejuvenation of three germ layers tissues by exchanging old blood plasma with saline-albumin. *Aging* 12, 8790–8819.

Melov, S., Shoffner, J.M., Kaufman, A., and Wallace, D.C. (1995). Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. *Nucleic Acids Res* 23, 4122–4126.

Menshikova, E.V., Ritov, V.B., Toledo, F.G.S., Ferrell, R.E., Goodpaster, B.H., and Kelley, D.E. (2005). Effects of weight loss and physical activity on skeletal muscle mitochondrial function in obesity. *Am J Physiol Endocrinol Metab* 288, E818-825.

Menshikova, E.V., Ritov, V.B., Fairfull, L., Ferrell, R.E., Kelley, D.E., and Goodpaster, B.H. (2006). Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol A Biol Sci Med Sci* 61, 534–540.

Menshikova, E.V., Ritov, V.B., Dube, J.J., Amati, F., Stefanovic-Racic, M., Toledo, F.G.S., Coen, P.M., and Goodpaster, B.H. (2018). Calorie Restriction-induced Weight Loss and Exercise Have Differential Effects on Skeletal Muscle Mitochondria Despite Similar Effects on Insulin Sensitivity. *The Journals of Gerontology: Series A* 73, 81–87.

Miotto, P.M., McGlory, C., Holloway, T.M., Phillips, S.M., and Holloway, G.P. (2018). Sex differences in mitochondrial respiratory function in human skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 314, R909–R915.

Muñoz García, D., Midaglia, L., Martinez Vilela, J., Marín Sánchez, M., López González, F.J., Arias Gómez, M., Dapena Bolaño, D., Iglesias Castañón, A., Alonso Alonso, M., and Romero López, J. (2015). Associated Inosine to interferon: results of a clinical trial in multiple sclerosis. *Acta Neurol Scand* 131, 405–410.

Nguyen, Q.L., Corey, C., White, P., Watson, A., Gladwin, M.T., Simon, M.A., and Shiva, S. (2017). Platelets from pulmonary hypertension patients show increased mitochondrial reserve capacity. *JCI Insight* 2, e91415.

Nguyen, Q.L., Wang, Y., Helbling, N., Simon, M.A., and Shiva, S. (2019). Alterations in platelet bioenergetics in Group 2 PH-HFpEF patients. *PLoS One* 14, e0220490.

Nicklas, B.J., Chmelo, E., Delbono, O., Carr, J.J., Lyles, M.F., and Marsh, A.P. (2015). Effects of resistance training with and without caloric restriction on physical function and mobility in overweight and obese older adults: a randomized controlled trial. *Am J Clin Nutr* 101, 991–999.

Pesta, D., Hoppel, F., Macek, C., Messner, H., Faulhaber, M., Kobel, C., Parson, W., Bartscher, M., Schocke, M., and Gnaiger, E. (2011). Similar qualitative and quantitative changes of mitochondrial respiration following strength and endurance training in normoxia and hypoxia in sedentary humans. *Am J Physiol Regul Integr Comp Physiol* 301, R1078-1087.

Petersen, K.F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D.L., DiPietro, L., Cline, G.W., and Shulman, G.I. (2003). Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300, 1140–1142.

Phielix, E., Meex, R., Moonen-Kornips, E., Hesselink, M.K.C., and Schrauwen, P. (2010). Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. *Diabetologia* 53, 1714–1721.

- Porter, C., Reidy, P.T., Bhattarai, N., Sidossis, L.S., and Rasmussen, B.B. (2015). Resistance Exercise Training Alters Mitochondrial Function in Human Skeletal Muscle. *Med Sci Sports Exerc* 47, 1922–1931.
- Rebo, J., Mehdipour, M., Gathwala, R., Causey, K., Liu, Y., Conboy, M.J., and Conboy, I.M. (2016). A single heterochronic blood exchange reveals rapid inhibition of multiple tissues by old blood. *Nat Commun* 7, 13363.
- Rejeski, W.J., Ip, E.H., Marsh, A.P., and Barnard, R.T. (2010). Development and Validation of a Video-Animated Tool for Assessing Mobility. *The Journals of Gerontology: Series A* 65A, 664–671.
- Rejeski, W.J., Rushing, J., Guralnik, J.M., Ip, E.H., King, A.C., Manini, T.M., Marsh, A.P., McDermott, M.M., Fielding, R.A., Newman, A.B., et al. (2015). The MAT-sf: Identifying Risk for Major Mobility Disability. *The Journals of Gerontology: Series A* 70, 641–646.
- Rooyackers, O.E., Adey, D.B., Ades, P.A., and Nair, K.S. (1996). Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proc Natl Acad Sci U S A* 93, 15364–15369.
- Ross, R., Goodpaster, B.H., Koch, L.G., Sarzynski, M.A., Kohrt, W.M., Johannsen, N.M., Skinner, J.S., Castro, A., Irving, B.A., Noland, R.C., et al. (2019). Precision exercise medicine: understanding exercise response variability. *Br J Sports Med* 53, 1141–1153.
- Salpeter, S., Khalaileh, A., Weinberg-Corem, N., Ziv, O., Glaser, B., and Dor, Y. (2013). Systemic regulation of the age-related decline of pancreatic β -cell replication. *Diabetes* 2013;62:2843–2848. *Diabetes* 62, 3300.

- Sardeli, A., Komatsu, T., Mori, M., Gaspari, A., and Chacon-Mikahil, M. (2018). Resistance Training Prevents Muscle Loss Induced by Caloric Restriction in Obese Elderly Individuals: A Systematic Review and Meta-Analysis. *Nutrients* 10, 423.
- Short, K.R., Bigelow, M.L., Kahl, J., Singh, R., Coenen-Schimke, J., Raghavakaimal, S., and Nair, K.S. (2005). Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci U S A* 102, 5618–5623.
- Sjövall, F., Morota, S., Hansson, M.J., Friberg, H., Gnaiger, E., and Elmér, E. (2010). Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis. *Crit Care* 14, R214.
- Sparks, L.M. (2017). Exercise training response heterogeneity: physiological and molecular insights. *Diabetologia* 60, 2329–2336.
- Sparks, L.M., Redman, L.M., Conley, K.E., Harper, M.-E., Yi, F., Hodges, A., Eroshkin, A., Costford, S.R., Gabriel, M.E., Shook, C., et al. (2016). Effects of 12 Months of Caloric Restriction on Muscle Mitochondrial Function in Healthy Individuals. *J Clin Endocrinol Metab* 102, 111–121.
- Srivastava, S. (2017). The Mitochondrial Basis of Aging and Age-Related Disorders. *Genes (Basel)* 8.
- Starling, R.D., Trappe, T.A., Short, K.R., Sheffield-Moore, M., Jozsi, A.C., Fink, W.J., and Costill, D.L. (1996). Effect of inosine supplementation on aerobic and anaerobic cycling performance. *Med Sci Sports Exerc* 28, 1193–1198.

Toledo, F.G.S., and Goodpaster, B.H. (2013). The role of weight loss and exercise in correcting skeletal muscle mitochondrial abnormalities in obesity, diabetes and aging. *Mol Cell Endocrinol* 379, 30–34.

Toledo, F.G.S., Menshikova, E.V., Azuma, K., Radiková, Z., Kelley, C.A., Ritov, V.B., and Kelley, D.E. (2008). Mitochondrial Capacity in Skeletal Muscle Is Not Stimulated by Weight Loss Despite Increases in Insulin Action and Decreases in Intramyocellular Lipid Content. *Diabetes* 57, 987–994.

Tonkonogi, M., Fernström, M., Walsh, B., Ji, L.L., Rooyackers, O., Hammarqvist, F., Wernerman, J., and Sahlin, K. (2003). Reduced oxidative power but unchanged antioxidative capacity in skeletal muscle from aged humans. *Pflugers Arch* 446, 261–269.

Tricò, D., Prinsen, H., Giannini, C., de Graaf, R., Juchem, C., Li, F., Caprio, S., Santoro, N., and Herzog, R.I. (2017). Elevated α -Hydroxybutyrate and Branched-Chain Amino Acid Levels Predict Deterioration of Glycemic Control in Adolescents. *The Journal of Clinical Endocrinology & Metabolism* 102, 2473–2481.

Tyrrell, D.J., Bharadwaj, M.S., Van Horn, C.G., Marsh, A.P., Nicklas, B.J., and Molina, A.J.A. (2015a). Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. *Exp Gerontol* 70, 84–91.

Tyrrell, D.J., Bharadwaj, M.S., Van Horn, C.G., Kritchevsky, S.B., Nicklas, B.J., and Molina, A.J.A. (2015b). Respirometric Profiling of Muscle Mitochondria and Blood Cells Are Associated With Differences in Gait Speed Among Community-Dwelling Older Adults. *J Gerontol A Biol Sci Med Sci* 70, 1394–1399.

Tyrrell, D.J., Bharadwaj, M.S., Jorgensen, M.J., Register, T.C., and Molina, A.J.A. (2016). Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: Implications for a minimally invasive biomarker of mitochondrial health. *Redox Biol* 10, 65–77.

Tyrrell, D.J., Bharadwaj, M.S., Jorgensen, M.J., Register, T.C., Shively, C., Andrews, R.N., Neth, B., Keene, C.D., Mintz, A., Craft, S., et al. (2017). Blood-Based Bioenergetic Profiling Reflects Differences in Brain Bioenergetics and Metabolism. *Oxid Med Cell Longev* 2017, 7317251.

Václavík, J., Mádrová, L., Kouřil, Š., de Sousa, J., Brumarová, R., Janečková, H., Jáčová, J., Friedecký, D., Knapková, M., Kluijtmans, L.A.J., et al. (2020). A newborn screening approach to diagnose 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. *JIMD Rep* 54, 79–86.

Villeda, S.A., Luo, J., Mosher, K.I., Zou, B., Britschgi, M., Bieri, G., Stan, T.M., Fainberg, N., Ding, Z., Eggel, A., et al. (2011). The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* 477, 90–94.

Watanabe, H., Hattori, T., Kume, A., Misu, K., Ito, T., Koike, Y., Johnson, T.A., Kamitsuji, S., Kamatani, N., and Sobue, G. (2020). Improved Parkinsons disease motor score in a single-arm open-label trial of febuxostat and inosine. *Medicine (Baltimore)* 99, e21576.

Williams, M.H., Kreider, R.B., Hunter, D.W., Somma, C.T., Shall, L.M., Woodhouse, M.L., and Rokitski, L. (1990). Effect of inosine supplementation on 3-mile treadmill run performance and VO₂ peak. *Med Sci Sports Exerc* 22, 517–522.

Willig, A.L., Kramer, P.A., Chacko, B.K., Darley-Usmar, V.M., Heath, S.L., and Overton, E.T. (2017). Monocyte bioenergetic function is associated with body composition in virologically suppressed HIV-infected women. *Redox Biol* 12, 648–656.

Zai, L., Ferrari, C., Subbaiah, S., Havton, L.A., Coppola, G., Strittmatter, S., Irwin, N., Geschwind, D., and Benowitz, L.I. (2009). Inosine Alters Gene Expression and Axonal Projections in Neurons Contralateral to a Cortical Infarct and Improves Skilled Use of the Impaired Limb. *J Neurosci* 29, 8187–8197.

Table 1. Participant demographic and other characteristics [Mean $\pm$ SD, n (%)] at baseline

	RT (n=33)	RT + CR (n=35)	p-value
Age, y	69.4 $\pm$ 3.0	69.0 $\pm$ 3.2	0.63
Female, n (%)	15 (45)	14 (40)	0.65
White, n (%)	27 (82)	31 (89)	0.51
Body mass, kg	87.3 $\pm$ 15.0	86.7 $\pm$ 12.6	0.85
Height, cm	167.5 $\pm$ 12.0	168.2 $\pm$ 9.9	0.78
BMI, kg/cm ²	30.9 $\pm$ 2.5	30.5 $\pm$ 2.4	0.47
Waist, cm	95.7 $\pm$ 9.8	96.5 $\pm$ 10.0	0.72
Hip, cm	108.6 $\pm$ 7.1	108.1 $\pm$ 8.0	0.80
Waist-to-hip ratio	0.88 $\pm$ 0.09	0.90 $\pm$ 0.09	0.59

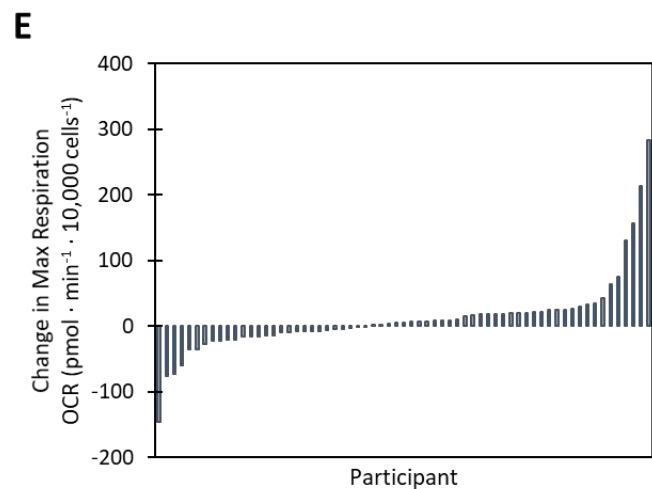
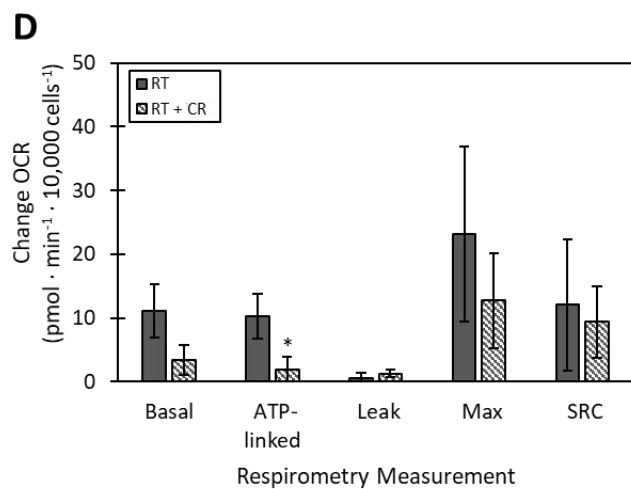
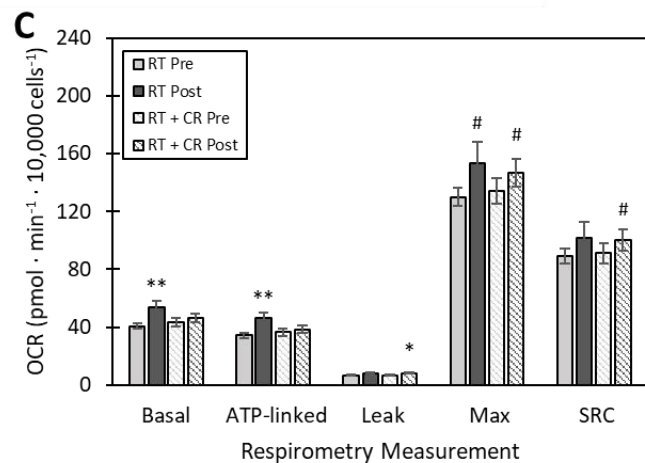
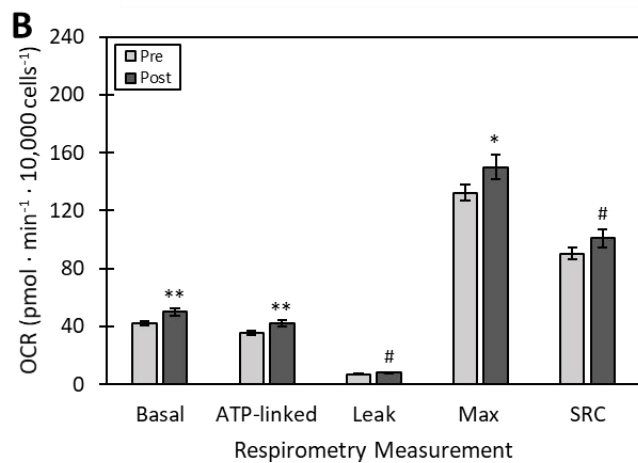
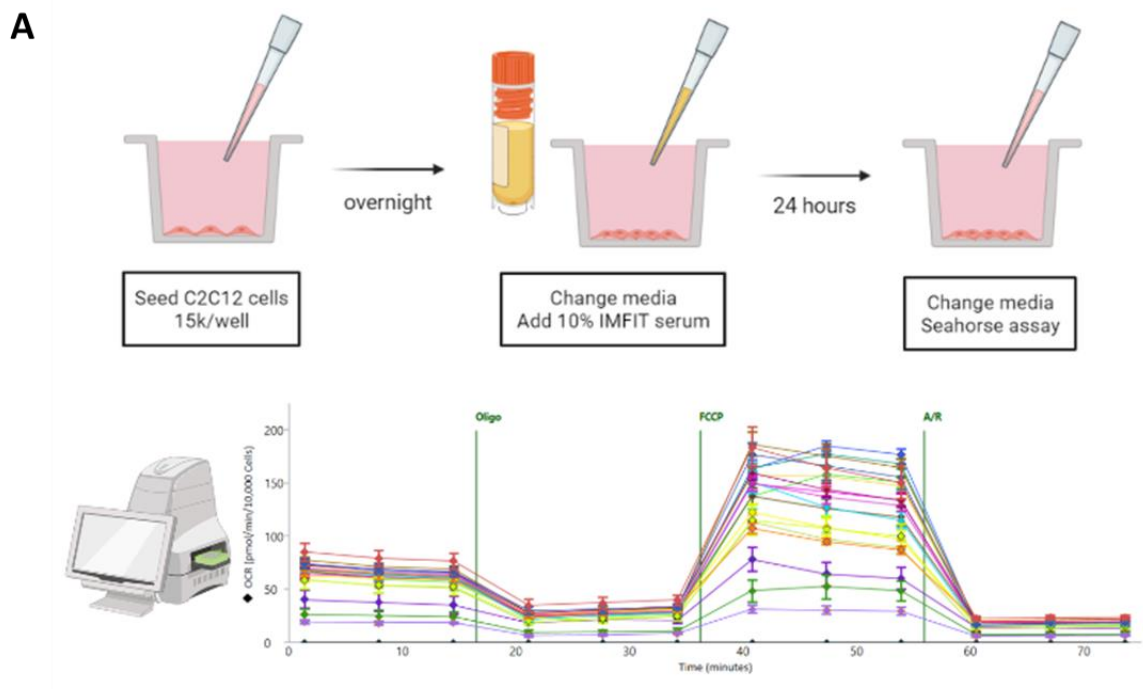


Figure 1. Experimental design and serum-mediated bioenergetics

- A) Overall experimental design
- B) Overall effect of intervention on serum-mediated bioenergetics with both intervention groups combined. Data are represented as mean $\pm$ SEM. Difference from pre, p-value: ** < 0.01, * < 0.05, # < 0.10
- C) Effect of intervention (RT vs RT + CR) on serum-mediated bioenergetics. Data are represented as mean $\pm$ SEM. Difference from pre, p-value: ** < 0.01, * < 0.05, # < 0.10
- D) Effect of intervention (RT vs RT + CR) on change in serum-mediated bioenergetics. Data are represented as mean $\pm$ SEM. Difference between groups, p-value: ** < 0.01, * < 0.05, # < 0.10
- E) Change in max respiration in all participants.

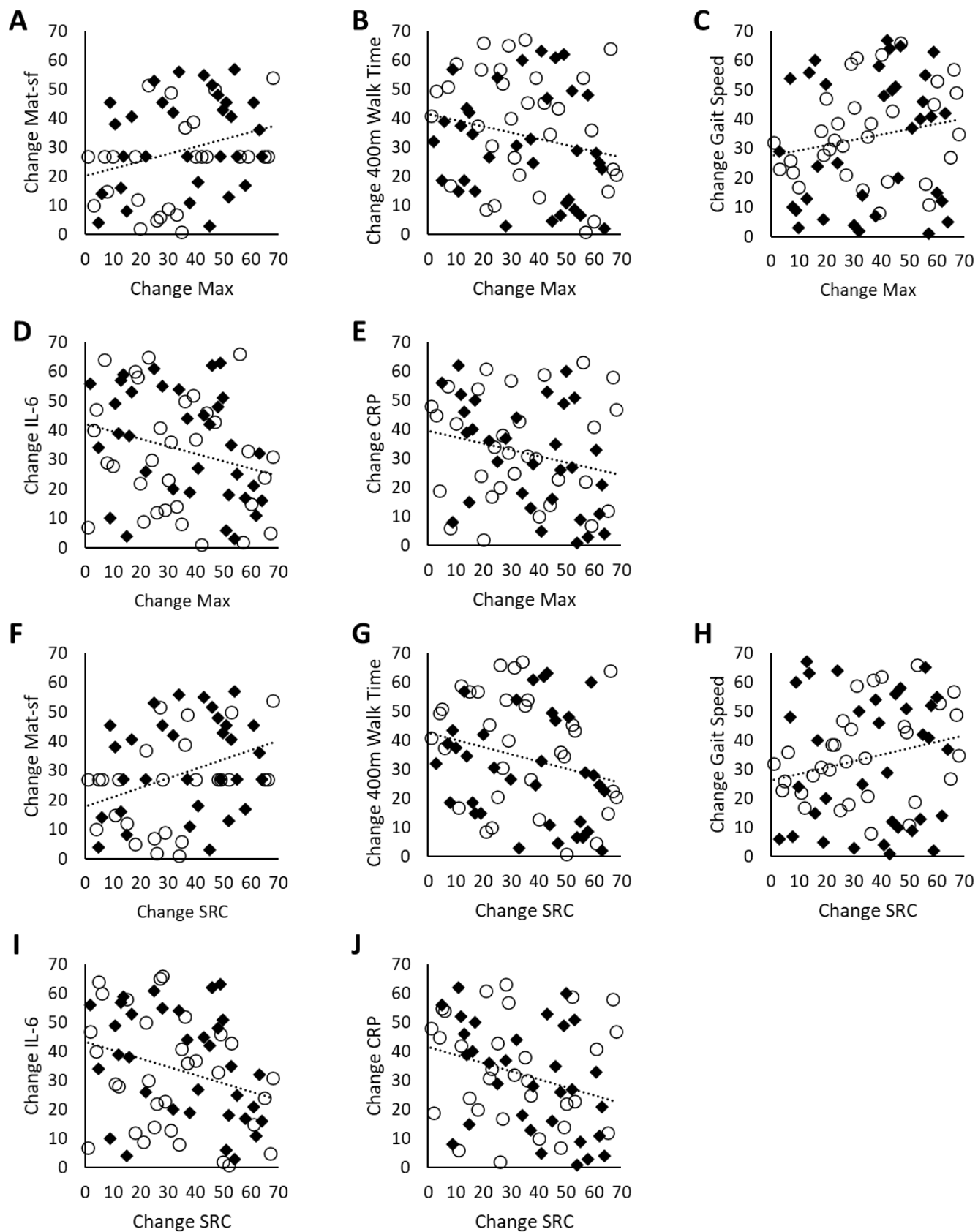


Figure 2. Spearman correlations between change in serum-mediated max respiration and change in physical function and inflammatory cytokines

- A) Spearman correlation between change in max respiration and change in mobility as assessed by Mat-sf. ($R=0.294$, $p= 0.03$)
- B) Spearman correlation between change in max respiration and change in 400m walk time. ($R=-0.215$, $p= 0.08$)
- C) Spearman correlation between change in max respiration and change in gait speed. ($R=0.180$, $p= 0.14$)
- D) Spearman correlation between change in max respiration and change in IL-6. ($R=-0.255$, $p= 0.04$)
- E) Spearman correlation between change in max respiration and change in CRP. ($R=-0.224$, $p= 0.08$)
- F) Spearman correlation between change in spare respiratory capacity and change in mobility as assessed by Mat-sf. ($R=0.372$, $p= 0.004$)
- G) Spearman correlation between change in spare respiratory capacity and change in 400m walk time. ($R=-0.246$, $p= 0.04$)
- H) Spearman correlation between change in spare respiratory capacity and change in gait speed. ($R=0.224$, $p= 0.07$)
- I) Spearman correlation between change in spare respiratory capacity and change in IL-6. ($R=-0.290$, $p= 0.02$)
- J) Spearman correlation between change in spare respiratory capacity and change in CRP. ($R=-0.294$, $p= 0.02$)

Figure 3. Metabolomic analysis reveals potential candidate metabolites that could mediate bioenergetic changes in response to diet and exercise interventions

- A) Using change in maximal respiration, we classified participants as negative responders, non-responders, and positive responders for metabolomics analysis.
- B) Heat map illustrating relationships of fold change of metabolites and responder status and scores plot from orthogonal-partial least squares analysis illustrating the separation of responder status based on fold change of metabolites.
- C) Pattern analysis reveals 25 potential metabolites that are either positively or negatively associated with the pattern of negative responders to non-responders to positive responders.

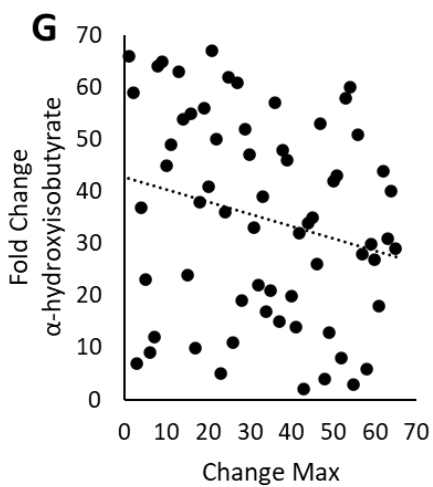
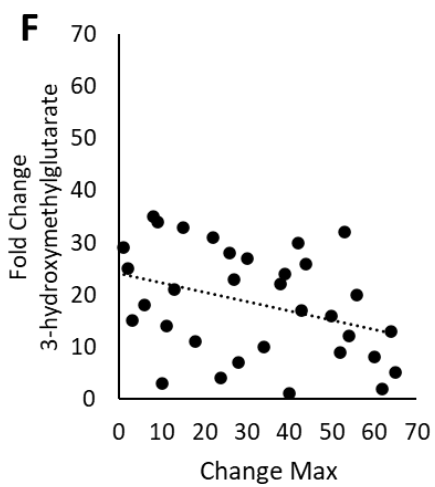
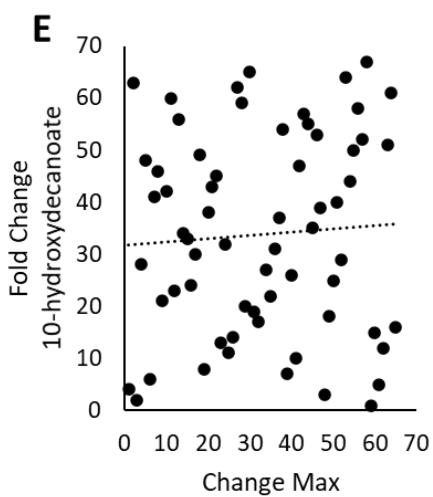
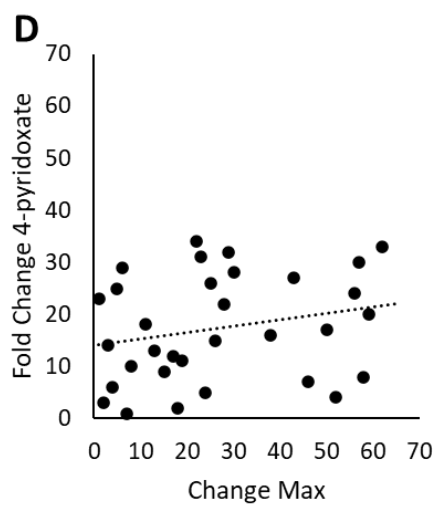
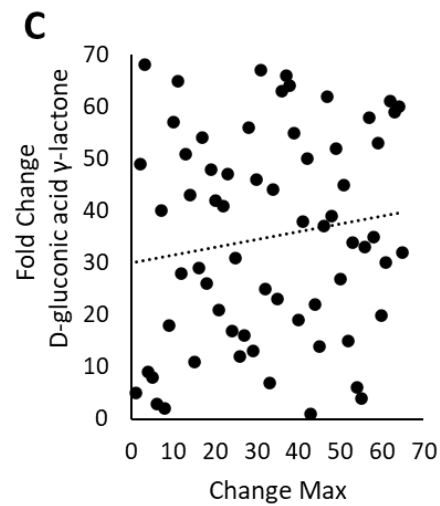
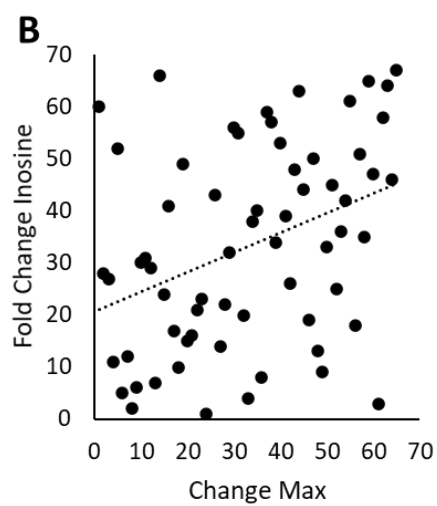
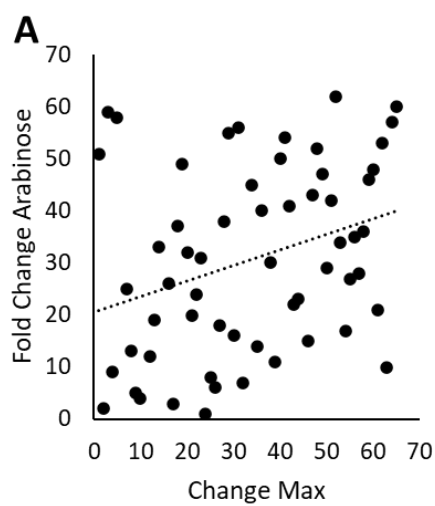


Figure 4. Spearman correlations between change in serum-mediated max respiration and fold change of candidate metabolites

- A) Spearman correlation between change in max respiration and fold change of arabinose. ($R=0.320$, $p= 0.01$)
- B) Spearman correlation between change in max respiration and fold change of inosine. ($R=0.367$, $p= 0.003$)
- C) Spearman correlation between change in max respiration and fold change of D-gluconic acid γ -lactone. ($R=0.270$, $p= 0.08$)
- D) Spearman correlation between change in max respiration and fold change of 4-pyridoxate. ($R=0.275$, $p= 0.11$)
- E) Spearman correlation between change in max respiration and fold change of 10-hydroxydecanoate. ($R=0.060$, $p= 0.64$)
- F) Spearman correlation between change in max respiration and fold change of 3-hydroxymethylglutarate. ($R=-0.359$, $p= 0.04$)
- G) Spearman correlation between change in max respiration and fold change of α -hydroxyisobutyrate. ($R=-0.242$, $p= 0.05$)

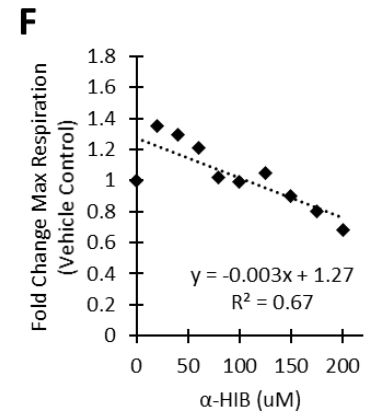
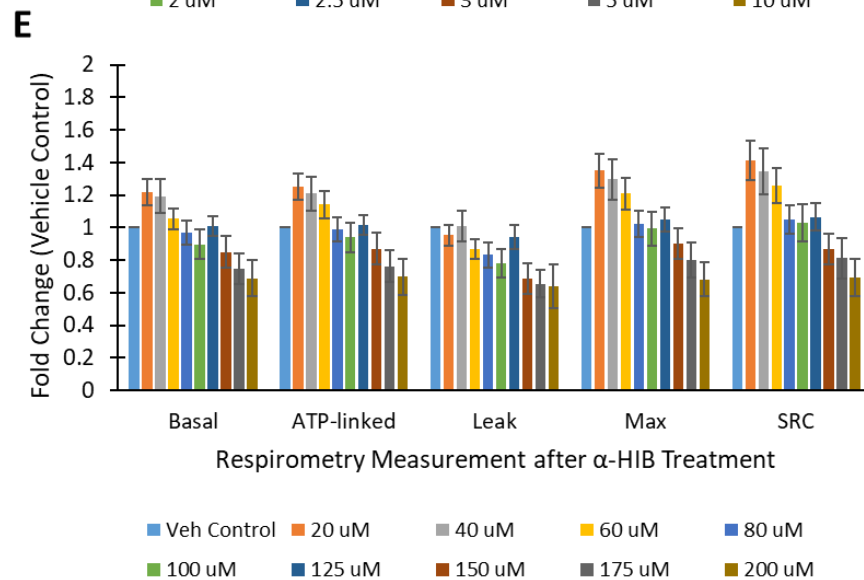
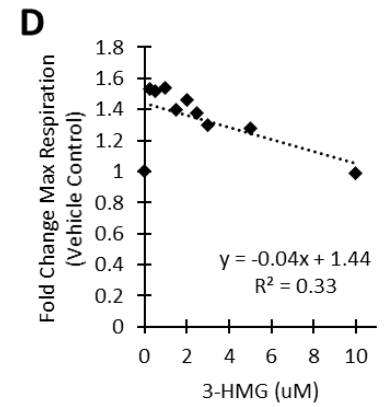
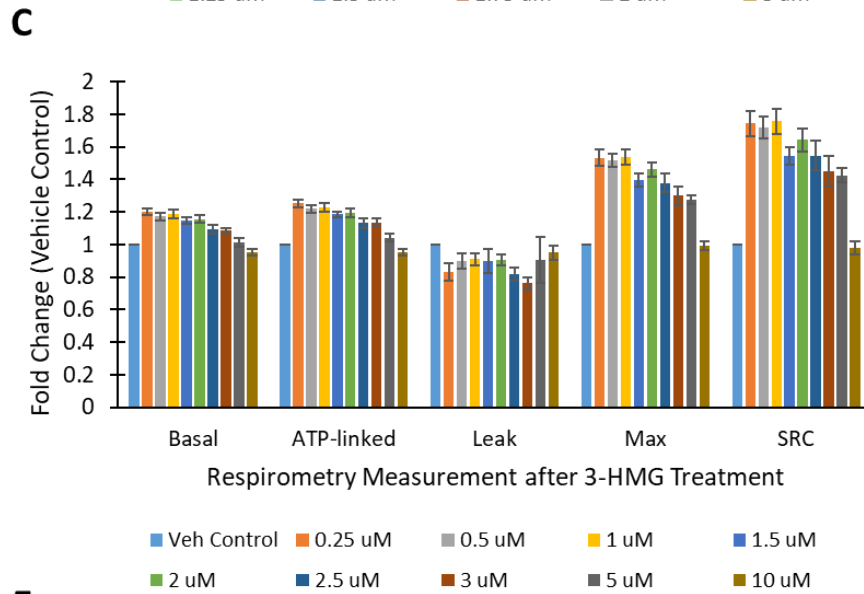
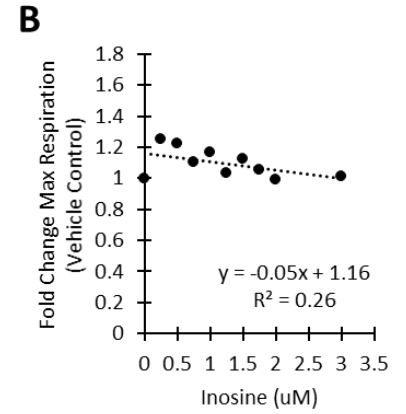
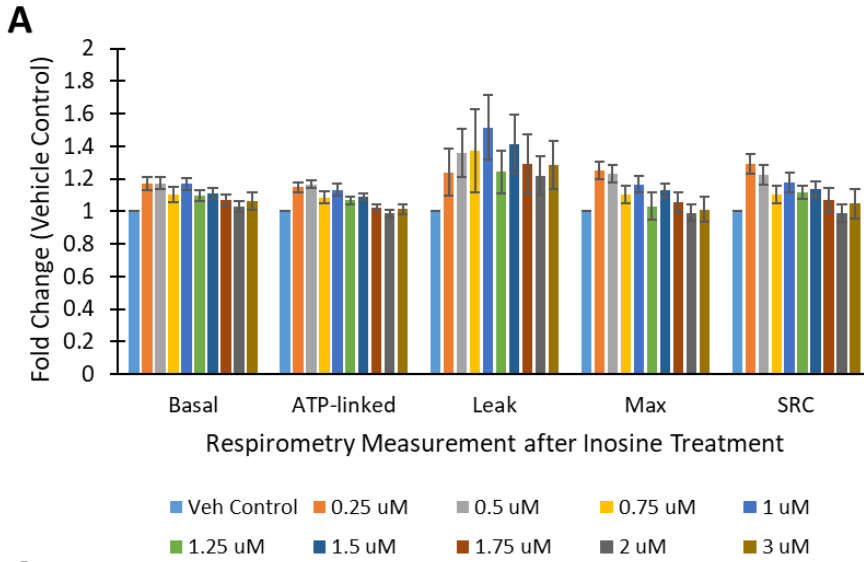


Figure 5. Candidate metabolite treatment and bioenergetics

- A) Fold change over vehicle control of C2C12 cells treated with various concentrations of inosine for 24 hours. Data are represented as mean $\pm$ SEM.
- B) Fold change over vehicle control of max respiration in C2C12 cells treated with increasing concentrations of inosine for 24 hours.
- C) Fold change over vehicle control of C2C12 cells treated with various concentrations of 3-hydroxymethylglutarate for 24 hours. Data are represented as mean $\pm$ SEM.
- D) Fold change over vehicle control of max respiration in C2C12 cells treated with increasing concentrations of 3-hydroxymethylglutarate for 24 hours.
- E) Fold change over vehicle control of C2C12 cells treated with various concentrations of α -hydroxyisobutyrate for 24 hours. Data are represented as mean $\pm$ SEM.
- F) Fold change over vehicle control of max respiration in C2C12 cells treated with increasing concentrations of α -hydroxyisobutyrate for 24 hours.

Supplemental Table 1. Unadjusted mitochondrial respiration and physical function measurements at baseline and changes with intervention (** < 0.01, * < 0.05, # < 0.10)

	RT			RT + CR			Between p-value baseline	Between p-value change
	Baseline (n=29-31)	Change	Within p-value	Baseline (n=32-34)	Change	Within p-value		
Mitochondrial Respiration Measurements								
Basal	40.8 ± 10.6	11.1 ± 23.1	0.01*	43.3 ± 16.8	3.4 ± 13.3	0.15	.4801	0.11
ATP-linked	34.4 ± 10.4	10.2 ± 19.8	0.01*	36.7 ± 15.1	1.9 ± 12.0	0.36	.4704	0.05#
Leak	6.9 ± 2.6	0.5 ± 4.8	0.56	6.8 ± 2.8	1.3 ± 3.0	0.02*	.9167	0.46
Max	130.1 ± 34.8	23.1 ± 76.7	0.10#	134.3 ± 51.7	12.7 ± 43.4	0.10#	.6984	0.51
SRC	89.3 ± 27.6	12.1 ± 57.4	0.25	91.1 ± 38.9	9.3 ± 32.7	0.10#	.8351	0.82
Physical Function Measurements								
Grip Strength	33.3 ± 12.1	1.2 ± 4.0	0.09#	30.4 ± 9.7	3.3 ± 4.5	<0.01*	.2791	0.05#
MAT-sf	62.5 ± 6.4	-1.6 ± 5.9	0.17	62.7 ± 5.7	1.2 ± 5.1	0.18	.8741	0.05#
400m Walk	320.7 ± 52.9	8.0 ± 43.4	0.30	304.3 ± 42.3	-6.2 ± 27.1	0.18	.1628	0.12
Gait Speed	3.6 ± 0.7	-0.1 ± 0.4	0.22	3.6 ± 0.6	-0.2 ± 0.6	0.05#	.8915	0.36
SPPB	10.9 ± 1.3	0.3 ± 1.3	0.29	10.8 ± 1.1	0.5 ± 1.0	<0.01*	.6266	0.35
Knee Ex Strength	115.8 ± 46.1	7.9 ± 17.2	0.02*	115.0 ± 30.7	7.2 ± 15.8	0.01*	.9334	0.87

Supplemental Table 2. Model adjusted¹ postintervention mitochondrial respiration and physical function measurement least-square means and adjusted group differences (95% CIs) (** < 0.01, * < 0.05, # < 0.10)

Postintervention Values	RT	RT + CR	RT – RT+CR differences	p-value
<i>Mitochondrial Respiration Measurement</i>				
Basal	52.8	45.8	7.0 (-1.8, 15.9)	0.12
ATP-linked	45.5	37.8	7.7 (-0.0, 15.4)	0.05[#]
Leak	7.4	8.3	-0.9 (-2.7, 0.9)	0.31
Max	154.7	145.7	9.0 (-21.3, 39.3)	0.56
SRC	101.8	100.1	1.7 (-21.1, 24.6)	0.88
<i>Physical Function Measurements</i>				
Grip Strength	33.2	35.0	-1.8 (-3.9, 0.4)	0.10[#]
MAT-sf	60.7	63.5	-2.8 (-5.8, 0.1)	0.06[#]
400m Walk	321.4	305.9	15.5 (-2.7, 33.7)	0.09[#]
Gait Speed	3.5	3.4	0.1 (-0.2, 0.4)	0.39
SPPB	11.1	11.3	-0.2 (-0.7, 0.3)	0.39
Knee Ex Strength	122.9	122.8	0.1 (-7.7, 8.0)	0.98

¹ model adjusted for age, sex, and baseline value of outcome. P-values were determined using an ANCOVA between-group test of postintervention values.

Supplemental Table 3. Spearman correlations between change in mitochondrial respiration measurements and change in physical function and inflammatory cytokines for all participants (** < 0.01, * < 0.05, # < 0.10)

	Basal	ATP-linked	Leak	Max	SRC
Grip Strength					
<i>Spearman</i>	0.070	0.053	0.283*	0.092	0.064
<i>Partial Age</i>	0.152	0.162	0.219	0.107	0.046
<i>Partial BMI</i>	0.117	0.130	0.220	0.086	0.036
<i>Partial Sex</i>	0.189	0.190	0.294#	0.163	0.081
<i>Partial BL Grip Strength</i>	0.111	0.082	0.341*	0.172	0.140
<i>Partial BL Bioenergetics</i>	0.037	0.036	0.146	0.038	0.002
Mat-sf					
<i>Spearman</i>	0.021	-0.016	0.255#	0.294*	0.372**
<i>Partial Age</i>	-0.199	-0.228	0.052	0.197	0.378*
<i>Partial BMI</i>	-0.206	-0.222	-0.041	0.148	0.332#
<i>Partial Sex</i>	-0.196	-0.227	0.089	0.229	0.403*
<i>Partial BL Mat-sf</i>	0.019	-0.032	0.320*	0.298*	0.380**
<i>Partial BL Bioenergetics</i>	0.100	0.051	0.257#	0.304*	0.373*
400m Walk					
<i>Spearman</i>	-0.020	-0.024	0.066	-0.215#	-0.246*
<i>Partial Age</i>	0.072	0.039	0.080	-0.109	-0.222
<i>Partial BMI</i>	0.087	0.053	0.098	-0.084	-0.201
<i>Partial Sex</i>	0.118	0.079	0.115	-0.071	-0.198
<i>Partial BL 400m Walk Time</i>	-0.025	-0.014	0.011	-0.236#	-0.265*
<i>Partial BL Bioenergetics</i>	-0.019	-0.021	0.032	-0.261#	-0.324*
Gait Speed					
<i>Spearman</i>	0.032	-0.001	0.116	0.180	0.224#
<i>Partial Age</i>	-0.065	-0.099	-0.044	0.089	0.176
<i>Partial BMI</i>	-0.063	-0.116	0.051	0.162	0.275
<i>Partial Sex</i>	-0.029	-0.065	-0.039	0.123	0.199
<i>Partial BL Gait Speed</i>	0.003	-0.013	0.056	0.116	0.154
<i>Partial BL Bioenergetics</i>	0.035	-0.007	0.016	0.179	0.245#
SPPB					
<i>Spearman</i>	0.118	0.056	0.202	0.147	0.129
<i>Partial Age</i>	0.162	0.100	0.259	0.203	0.196
<i>Partial BMI</i>	0.211	0.163	0.180	0.168	0.133
<i>Partial Sex</i>	0.187	0.117	0.317#	0.249	0.225
<i>Partial BL SPPB</i>	0.061	-0.006	0.166	0.143	0.142
<i>Partial BL Bioenergetics</i>	0.315*	0.266#	0.260#	0.254#	0.204
Knee Extensor Strength					
<i>Spearman</i>	-0.019	-0.001	-0.186	-0.057	-0.035
<i>Partial Age</i>	-0.070	-0.014	-0.202	-0.077	0.030
<i>Partial BMI</i>	-0.077	-0.031	-0.157	-0.047	0.074
<i>Partial Sex</i>	-0.153	-0.079	-0.321#	-0.183	-0.035
<i>Partial BL Knee Extensor Strength</i>	-0.009	0.005	-0.179	-0.043	-0.021
<i>Partial BL Bioenergetics</i>	0.061	0.124	-0.242#	0.019	0.061
IL-6					
<i>Spearman</i>	-0.019	-0.077	0.139	-0.255*	-0.290*
<i>Partial Age</i>	0.000	-0.052	0.123	-0.225#	-0.259*
<i>Partial BMI</i>	0.000	-0.054	0.131	-0.213#	-0.251*
<i>Partial Sex</i>	0.049	-0.019	0.173	-0.181	-0.226#
<i>Partial BL IL-6</i>	0.016	-0.014	0.081	-0.278*	-0.312*
<i>Partial BL Bioenergetics</i>	0.035	0.002	0.719	-0.222#	-0.253*
CRP					
<i>Spearman</i>	-0.048	-0.013	-0.024	-0.224#	-0.294*
<i>Partial Age</i>	-0.048	-0.013	-0.024	-0.226#	-0.296*
<i>Partial BMI</i>	-0.056	-0.025	-0.005	-0.205	-0.274*
<i>Partial Sex</i>	-0.018	0.008	0.008	-0.200	-0.277*
<i>Partial BL CRP</i>	-0.046	-0.005	0.004	-0.223#	-0.285*
<i>Partial BL Bioenergetics</i>	-0.041	0.008	-0.046	-0.220#	-0.286*

Supplemental Table 4. Spearman correlations between change in mitochondrial respiration measurements and change in physical function and inflammatory cytokines by intervention group (** < 0.01, * < 0.05, # < 0.10)

	RT					RT + CR				
	Basal	ATP-linked	Leak	Max	SRC	Basal	ATP-linked	Leak	Max	SRC
Grip Strength										
<i>Spearman</i>	0.063	-0.029	0.546**	0.168	0.213	0.189	0.284	-0.159	-0.092	-0.198
<i>Partial Age</i>	0.010	-0.073	0.394	-0.111	-0.045	0.301	0.480*	-0.175	0.080	-0.101
<i>Partial BMI</i>	0.009	-0.079	0.458*	-0.077	-0.034	0.193	0.380	-0.235	0.027	-0.121
<i>Partial Sex</i>	0.023	-0.055	0.401	-0.072	-0.011	0.429*	0.566*	-0.117	0.158	-0.052
<i>Partial BL Grip Strength</i>	0.070	-0.024	0.562**	0.193	0.243	0.265	0.326*	-0.076	0.003	-0.114
<i>Partial BL Bioenergetics</i>	0.143	0.068	0.329	0.142	0.186	0.009	0.159	-0.273	-0.264	-0.403*
Mat-sf										
<i>Spearman</i>	0.165	0.098	0.375*	0.369*	0.363*	-0.073	-0.021	0.039	0.188	0.308*
<i>Partial Age</i>	-0.313	-0.382	0.202	0.227	0.347	-0.195	-0.125	-0.284	0.012	0.196
<i>Partial BMI</i>	-0.310	-0.355	0.091	0.206	0.395	-0.206	-0.163	-0.289	-0.013	0.138
<i>Partial Sex</i>	-0.295	-0.359	0.235	0.315	0.397	-0.201	-0.130	-0.288	0.024	0.210
<i>Partial BL Mat-sf</i>	0.166	0.102	0.388*	0.368*	0.363*	-0.057	-0.048	0.130	0.196	0.295
<i>Partial BL Bioenergetics</i>	0.282	0.220	0.291	0.380*	0.354*	-0.025	0.005	0.074	0.155	0.266
400m Walk										
<i>Spearman</i>	-0.075	-0.106	0.167	-0.259	-0.248	-0.004	-0.032	0.058	-0.219	-0.253
<i>Partial Age</i>	0.266	0.276	0.218	-0.085	-0.295	-0.083	-0.102	0.190	-0.103	-0.116
<i>Partial BMI</i>	0.329	0.385	0.051	-0.157	-0.281	-0.073	-0.078	0.184	-0.089	-0.081
<i>Partial Sex</i>	0.260	0.268	0.211	-0.113	-0.310	0.060	0.008	0.308	-0.010	-0.050
<i>Partial BL 400m Walk Time</i>	-0.090	-0.108	0.134	-0.275	-0.256	0.010	-0.001	0.010	-0.245	-0.294
<i>Partial BL Bioenergetics</i>	-0.127	-0.180	0.197	-0.322	-0.317	-0.022	-0.064	0.142	-0.215	-0.274
Gait Speed										
<i>Spearman</i>	0.040	-0.020	0.322*	0.274	0.355*	0.039	0.004	-0.010	0.066	0.105
<i>Partial Age</i>	-0.237	-0.239	0.054	0.235	0.529*	-0.029	-0.069	-0.054	0.014	0.072
<i>Partial BMI</i>	-0.232	-0.244	0.139	0.337	0.563*	-0.070	-0.077	-0.102	0.008	0.129
<i>Partial Sex</i>	-0.234	-0.226	-0.013	0.250	0.548*	0.021	-0.029	-0.019	0.048	0.097
<i>Partial BL Gait Speed</i>	-0.056	-0.074	0.178	0.206	0.314*	0.058	0.023	0.021	0.018	0.022
<i>Partial BL Bioenergetics</i>	0.141	0.143	0.115	0.402	0.480*	-0.080	-0.110	-0.073	-0.042	-0.028
SPPB										
<i>Spearman</i>	0.179	0.119	0.269	0.113	0.094	0.106	0.051	0.113	0.200	0.126
<i>Partial Age</i>	-0.055	-0.162	0.443*	-0.037	-0.059	0.305	0.327	0.134	0.494*	0.440*
<i>Partial BMI</i>	-0.036	-0.114	0.295	-0.185	-0.044	0.381	0.368	0.209	0.554*	0.441*
<i>Partial Sex</i>	-0.044	-0.154	0.492*	-0.018	-0.061	0.390	0.379	0.184	0.562*	0.484*
<i>Partial BL SPPB</i>	0.031	-0.025	0.184	0.061	0.075	0.109	0.041	0.136	0.240	0.179
<i>Partial BL Bioenergetics</i>	0.414*	0.376*	0.481*	0.080	0.012	0.283	0.296	0.103	0.363*	0.295
Knee Extensor Strength										
<i>Spearman</i>	-0.177	-0.168	-0.151	-0.036	0.048	0.115	0.130	-0.201	-0.010	-0.071
<i>Partial Age</i>	-0.275	-0.231	-0.210	-0.114	0.179	0.179	0.220	-0.297	-0.016	-0.053
<i>Partial BMI</i>	-0.316	-0.301	-0.081	-0.058	0.168	0.154	0.209	-0.306	-0.018	-0.033
<i>Partial Sex</i>	-0.316	-0.276	-0.276	-0.209	0.141	0.054	0.129	-0.425*	-0.118	-0.129
<i>Partial BL Knee Extensor Strength</i>	-0.213	-0.205	-0.163	-0.076	0.011	0.173	0.144	-0.099	0.087	0.008
<i>Partial BL Bioenergetics</i>	-0.057	-0.008	-0.213	0.052	0.176	0.103	0.201	-0.328*	0.034	-0.006
IL-6										
<i>Spearman</i>	0.133	0.052	0.308*	-0.181	-0.245	-0.170	-0.207	-0.082	-0.370*	-0.352*
<i>Partial Age</i>	0.148	0.082	0.280	-0.168	-0.232	-0.182	-0.210	-0.109	-0.313*	-0.284
<i>Partial BMI</i>	0.150	0.082	0.295	-0.174	-0.238	-0.158	-0.179	-0.102	-0.266	-0.232
<i>Partial Sex</i>	0.194	0.110	0.343*	-0.086	-0.166	-0.144	-0.172	-0.083	-0.295	-0.275
<i>Partial BL IL-6</i>	0.113	0.073	0.234	-0.234	-0.213	-0.086	-0.106	-0.087	-0.356*	-0.393*
<i>Partial BL Bioenergetics</i>	0.167	0.113	0.292	-0.166	-0.223	-0.122	-0.136	-0.148	-0.325*	-0.312*
CRP										
<i>Spearman</i>	0.083	0.036	0.230	-0.032	-0.100	-0.223	-0.124	-0.312*	-0.449*	-0.486**
<i>Partial Age</i>	0.076	0.034	0.217	0.004	-0.061	-0.250	-0.150	-0.324*	-0.462*	-0.492**
<i>Partial BMI</i>	0.087	0.042	0.209	-0.070	-0.151	-0.232	-0.122	-0.324*	-0.417*	-0.445*
<i>Partial Sex</i>	0.089	0.040	0.240	-0.017	-0.091	-0.150	-0.078	-0.251	-0.424*	-0.476**
<i>Partial BL CRP</i>	0.061	0.017	0.276	-0.051	-0.062	-0.184	-0.086	-0.279	-0.426*	-0.499**
<i>Partial BL Bioenergetics</i>	0.029	-0.015	0.231	-0.108	-0.163	-0.164	-0.043	-0.328*	-0.397*	-0.416*

Supplemental Table 5. Spearman correlations between change in mitochondrial respiration measurements and change in physical function and inflammatory cytokines by sex (** < 0.01, * < 0.05, # < 0.10)

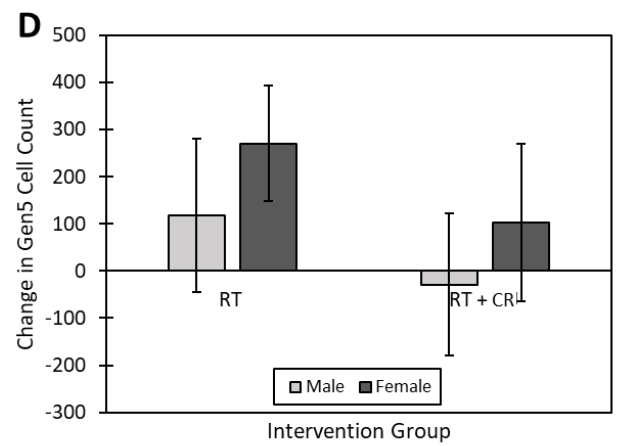
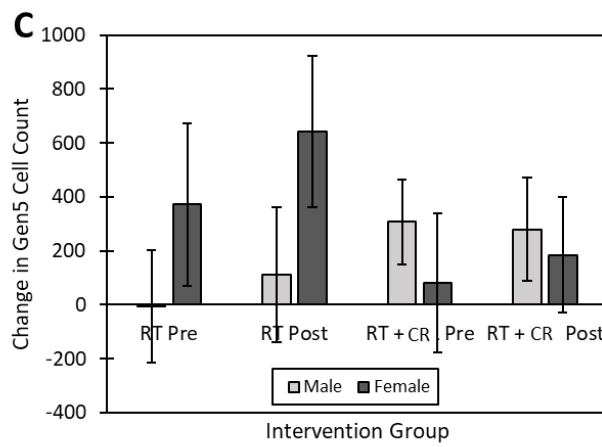
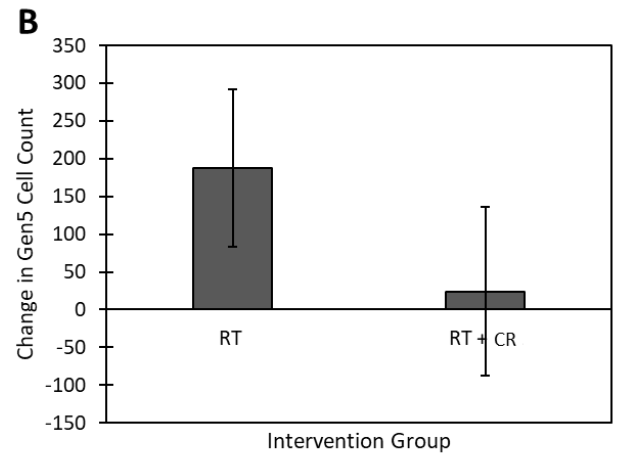
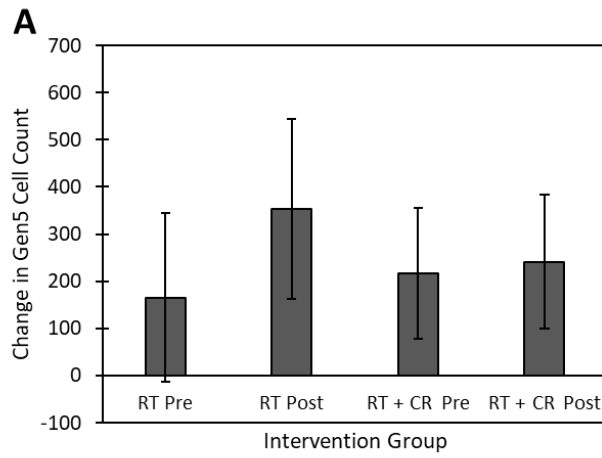
	Male					Female				
	Basal	ATP-linked	Leak	Max	SRC	Basal	ATP-linked	Leak	Max	SRC
Grip Strength										
<i>Spearman</i>	-0.017	-0.045	0.328*	0.006	0.036	0.174	0.166	0.271*	0.141	0.081
<i>Partial Age</i>	-0.021	-0.084	0.286	-0.063	-0.040	0.465	0.610	0.242	0.317	0.130
<i>Partial BMI</i>	-0.024	-0.076	0.322	-0.053	-0.013	0.481	0.620	0.265	0.335	0.160
<i>Partial BL Grip Strength</i>	-0.047	-0.081	0.383*	0.068	0.113	0.187	0.174	0.284*	0.151	0.095
<i>Partial BL Bioenergetics</i>	-0.012	-0.046	0.249	-0.030	-0.015	0.143	0.189	0.004	0.090	0.003
Mat-sf										
<i>Spearman</i>	0.111	0.060	0.338[#]	0.543**	0.628**	0.081	0.016	0.330	0.209	0.231
<i>Partial Age</i>	-0.181	-0.223	0.075	0.489*	0.661**	-0.210	-0.299	0.150	0.019	0.142
<i>Partial BMI</i>	-0.146	-0.157	-0.060	0.463*	0.616**	-0.258	-0.341	0.115	-0.007	0.156
<i>Partial BL Mat-sf</i>	0.086	0.027	0.390	0.535**	0.630**	0.069	-0.001	0.337	0.201	0.227
<i>Partial BL Bioenergetics</i>	0.249	0.157	0.328[#]	0.584**	0.640**	0.103	0.033	0.384[#]	0.222	0.220
400m Walk										
<i>Spearman</i>	0.019	0.016	-0.032	-0.147	-0.131	0.009	-0.031	0.301	-0.236	-0.314[#]
<i>Partial Age</i>	0.320	0.255	0.364	0.171	0.068	-0.168	-0.088	0.088	-0.433	-0.560[#]
<i>Partial BMI</i>	0.361	0.308	0.193	0.091	-0.080	-0.172	-0.106	0.061	-0.482	-0.670*
<i>Partial BL 400m Walk Time</i>	0.025	0.025	-0.071	-0.178	-0.170	-0.016	-0.016	0.222	-0.241	-0.296
<i>Partial BL Bioenergetics</i>	-0.126	-0.094	-0.131	-0.377*	-0.320[#]	0.105	0.137	0.376	-0.116	-0.267[#]
Gait Speed										
<i>Spearman</i>	0.017	-0.017	0.063	0.116	0.148	-0.055	-0.017	0.023	0.103	0.144
<i>Partial Age</i>	-0.085	-0.136	0.042	0.032	0.173	-0.020	0.081	-0.148	0.127	0.212
<i>Partial BMI</i>	-0.112	-0.203	0.075	0.069	0.218	0.008	0.100	-0.167	0.110	0.096
<i>Partial BL Gait Speed</i>	0.007	-0.024	-0.014	0.053	0.078	-0.069	-0.014	-0.003	0.063	0.107
<i>Partial BL Bioenergetics</i>	-0.016	-0.058	0.012	0.119	0.191	-0.076	-0.054	-0.171	0.032	0.101
SPPB										
<i>Spearman</i>	0.037	-0.027	0.335*	0.010	-0.019	0.178	0.142	0.056	0.238	0.265
<i>Partial Age</i>	0.006	-0.038	0.364	0.020	0.020	0.497	0.415	0.291	0.526	0.531[#]
<i>Partial BMI</i>	0.075	0.069	0.255	-0.083	-0.142	0.531[#]	0.449	0.311	0.598*	0.694*
<i>Partial BL SPPB</i>	-0.017	-0.083	0.305[#]	0.027	0.007	0.114	0.074	-0.010	0.228	0.274
<i>Partial BL Bioenergetics</i>	0.330[#]	0.220	0.524**	0.414	0.075	0.314	0.304	-0.008	0.326	0.311
Knee Extensor Strength										
<i>Spearman</i>	-0.133	-0.092	-0.199	-0.221	-0.154	-0.048	-0.003	-0.258	-0.087	-0.048
<i>Partial Age</i>	-0.219	-0.117	-0.262	-0.388#	-0.247	-0.170	-0.098	-0.591*	-0.145[#]	0.073
<i>Partial BMI</i>	-0.283	-0.200	-0.127	-0.333	-0.125	-0.157	-0.089	-0.573[#]	-0.143	0.035
<i>Partial BL Knee Extensor Strength</i>	-0.156	-0.125	-0.165	-0.198	-0.118	0.050	0.096	-0.278	0.009	0.055
<i>Partial BL Bioenergetics</i>	-0.008	0.060	-0.214	-0.042	-0.001	-0.120	-0.010	-0.459*	-0.155	-0.062
IL-6										
<i>Spearman</i>	-0.076	-0.134	0.044	-0.099	-0.036	0.143	0.033	0.371[#]	-0.216	-0.401*
<i>Partial Age</i>	-0.072	-0.132	0.069	-0.083	-0.015	0.194	0.094	0.353[#]	-0.193	-0.396[#]
<i>Partial BMI</i>	-0.076	-0.135	0.044	-0.105	-0.042	0.196	0.090	0.365[#]	-0.180	-0.375[#]
<i>Partial BL IL-6</i>	-0.029	-0.080	0.082	-0.121	-0.054	0.118	0.081	0.206	-0.316	-0.466*
<i>Partial BL Bioenergetics</i>	-0.043	-0.098	0.040	-0.094	-0.021	0.198	0.116	0.238	-0.188	-0.379[#]
CRP										
<i>Spearman</i>	-0.024	0.001	-0.135	-0.219	-0.288[#]	0.005	-0.008	0.128	-0.134	-0.194
<i>Partial Age</i>	-0.024	0.007	-0.135	-0.219	-0.290[#]	0.004	-0.012	0.127	-0.143	-0.210
<i>Partial BMI</i>	-0.035	-0.004	-0.127	-0.210	-0.289[#]	0.010	-0.020	0.154	-0.107	-0.159
<i>Partial BL CRP</i>	0.015	0.054	-0.113	-0.218	-0.301[#]	-0.006	-0.025	0.157	-0.102	-0.119
<i>Partial BL Bioenergetics</i>	0.036	0.079	-0.116	-0.136	-0.220	-0.001	-0.004	0.021	-0.172	-0.228

Supplemental Table 6. Parameters used for classification of responder status (from Dankel, 2019)

Change Mean ± SD	RT	RT + CR	p-value for equality of variance	(RT ΔSD*1.15)	75% CI of random change
Basal	11.1 ± 23.1	3.4 ± 13.3	0.003	26.6	-3.5, 49.7
ATP-linked	10.2 ± 19.8	1.9 ± 12.0	0.006	22.8	-3.0, 42.6
Leak	0.5 ± 4.8	1.3 ± 3.0	0.008	5.5	-0.7, 10.3
Max	23.1 ± 76.7	12.7 ± 43.4	0.002	88.2	-11.5, 164.9
SRC	12.1 ± 57.4	9.3 ± 32.7	0.002	66.0	-8.6, 123.4

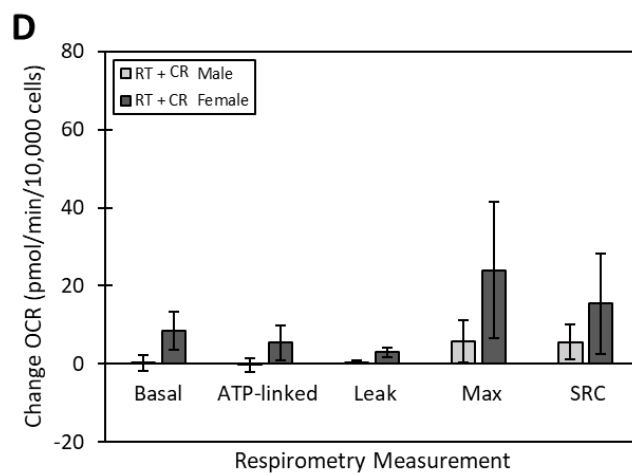
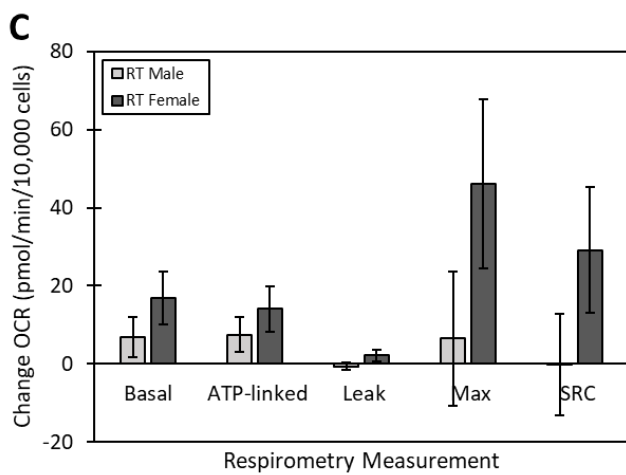
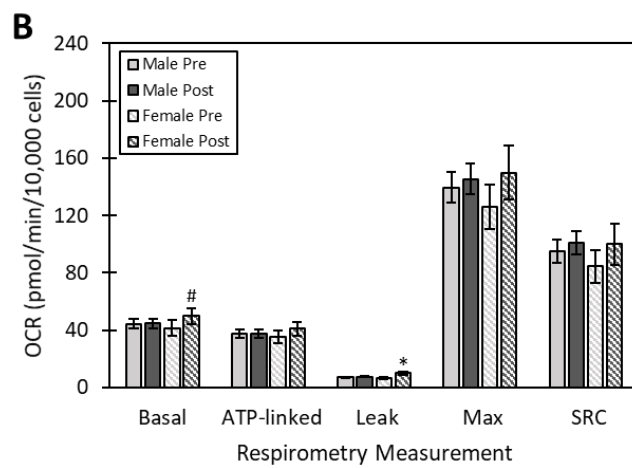
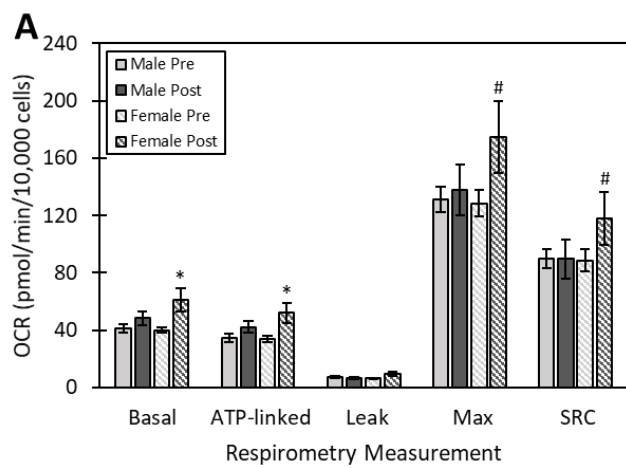
Supplemental Table 7. Spearman correlations between change in mitochondrial respiration measurements and fold change of metabolites (** < 0.01, * < 0.05, # < 0.10)

	Basal	ATP-linked	Leak	Max	SRC
Arabinose	0.282*	0.313*	0.080	0.320*	0.315*
Inosine	0.260*	0.278*	0.087	0.367**	0.304*
D-gluconic acid γ -lactone	0.062	0.038	0.229#	0.270*	0.330*
4-pyridoxate	0.401*	0.386*	0.295	0.275	0.168
10-hydroxydecanoate	-0.201	-0.242#	0.061	0.060	0.078
3-hydroxymethylglutarate	-0.455*	-0.448*	-0.101	-0.359*	-0.334
α -hydroxyisobutyrate	-0.331*	-0.380**	0.001	-0.242#	-0.155



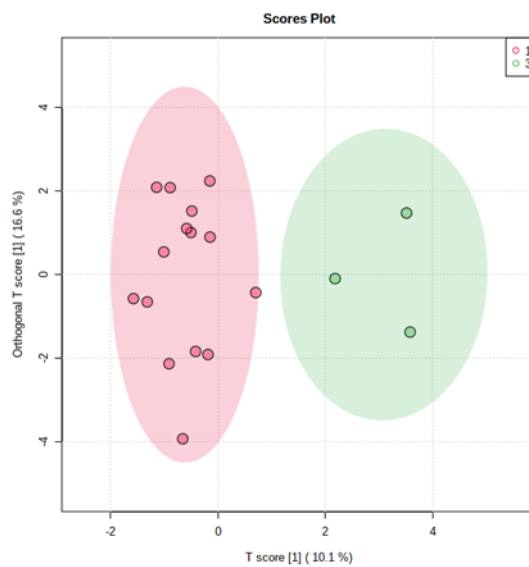
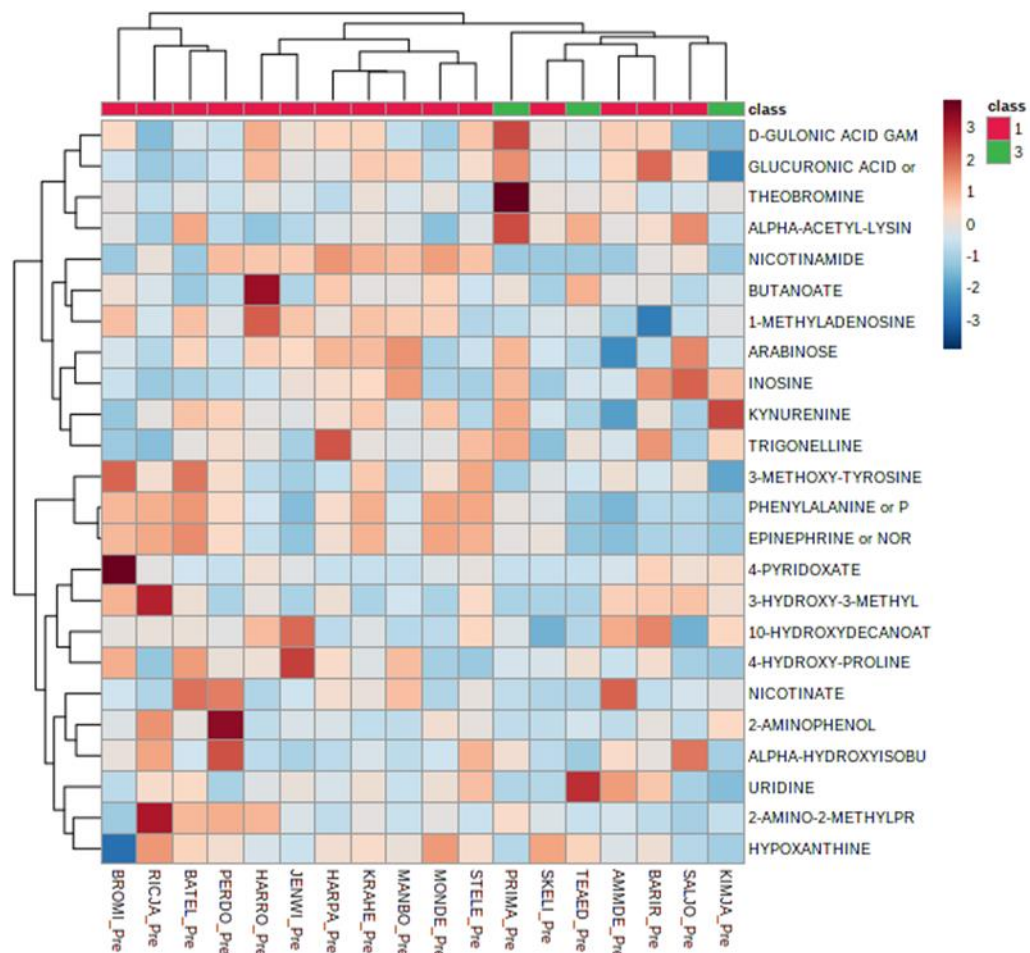
Supplemental Figure 1. Changes in cell count after serum treatment

- A) Change in cell count pre- and post-intervention after serum treatment.
- B) Change in cell count with intervention by intervention group (RT vs RT + CR).
- C) Change in cell count pre- and post-intervention after serum treatment in both treatment groups by sex.
- D) Change in cell count with intervention by intervention group and sex.



Supplemental Figure 2. Sex differences in serum-mediated bioenergetics

- A) Effect of RT intervention on serum-mediated bioenergetics by sex. Data are represented as mean $\pm$ SEM. Difference from pre, p-value: * < 0.05, # < 0.10
- B) Effect of RT + CR intervention on serum-mediated bioenergetics by sex. Data are represented as mean $\pm$ SEM. Difference from pre, p-value: * < 0.05, # < 0.10
- C) Effect of sex (male vs female) on change in serum-mediated bioenergetics in the RT group. Data are represented as mean $\pm$ SEM.
- D) Effect of sex (male vs female) on change in serum-mediated bioenergetics in the RT + CR group. Data are represented as mean $\pm$ SEM.



Supplemental Figure 3. Heat map and scores plot for top 25 metabolites

Heat map illustrating relationships of fold change of top 25 metabolites and responder status and scores plot from orthogonal-partial least squares analysis illustrating the complete separation of negative and positive responders based on fold change of metabolites.

CHAPTER FIVE:

DISCUSSION

Jenny L. Gonzalez-Armenta

I. SUMMARY OF RESULTS

As older adults continue to live longer and, as a result, with more comorbidities, there is a continuing need to understand the cellular and molecular processes, including mitochondrial dysfunction, that are implicated in healthy aging and age-related diseases. Our lab and others have pioneered the use of blood cells as reporters of systemic mitochondrial bioenergetic capacity based on the premise that these circulating cells are continuously exposed to a myriad of factors that mediate bioenergetic capacity across multiple tissues. In support of this theory, our lab has demonstrated that blood cell mitochondrial bioenergetics can recapitulate the bioenergetics of the brain, skeletal muscle, and cardiac muscle and that increased circulating IL-6 is associated with decreased mitochondrial bioenergetic capacity in blood cells. However, direct evidence supporting the effect of circulating factors on bioenergetics is limited. Therefore, the overarching hypothesis of this dissertation was that there are circulating factors in blood associated with aging, and consequently, age-related diseases and interventions to improve healthspan, that can mediate systemic bioenergetic capacity.

In Chapter Two, we examined the effects of whole dietary patterns, either a Mediterranean or a Western diet, on skeletal muscle mitochondrial bioenergetics. We used a cynomolgus macaque model to examine the effects of whole diets administered for 30 months, approximately equivalent to 8 human years, and performed bioenergetic profiling of skeletal muscle comprised of high-resolution respirometry of permeabilized muscle fibers, with and without fatty acid substrates, tissue citrate synthase activity, and western blot analyses. We found higher skeletal muscle mitochondrial respiration with and without fatty acid substrates in female cynomolgus macaques fed a Western versus Mediterranean diet. This was independent of citrate synthase activity, a marker of

mitochondrial volume or content, and VDAC expression. Additionally, we found that measures of respiration in response to fatty acids were significantly and positively correlated with both insulin resistance and plasma insulin levels.

In Chapter Three, we used a heterochronic parabiosis mouse model to test the hypothesis that circulating factors present in blood mediate age-related bioenergetic decline in skeletal muscle. We examined mitochondrial morphology, expression of mitochondrial electron transport chain complexes, and performed high-resolution respirometry of permeabilized skeletal muscle fibers in pairs of heterochronic and isochronic parabionts. When comparing isochronic controls, we found reduced mitochondrial area and length, reduced expression of complex IV in skeletal muscle, and observed decreased mitochondrial respiration in skeletal muscle of older mice compared to young mice. Young heterochronic mice had significantly reduced mitochondrial area and length, a trend for reduced expression of complex IV, and significantly reduced mitochondrial respiration when compared to young isochronic controls. Conversely, when comparing old heterochronic mice to old isochronic controls, we did not observe statistically significant differences in mitochondrial morphology, complex IV expression, or mitochondrial respiration. Further, we did not observe any differences in mitochondrial morphology, complex IV expression, or mitochondrial respiration between young heterochronic or old heterochronic mice.

In Chapter Four, we aimed to determine the effects of human serum, and therefore, blood-borne circulating factors, before and after intervention, either resistance training (RT) or resistance training plus caloric restriction (RT + CR), on naïve muscle cells *in vitro*. We report that serum-mediated bioenergetics can recapitulate the effects of a RT or RT + CR intervention and are consistent with previous studies of mitochondrial

function with these interventions, including: improved mitochondrial bioenergetics after intervention, differential mitochondrial bioenergetic effects of RT versus RT + CR, and the observation that female participants had a more robust bioenergetic response to intervention. We also observed that serum-mediated bioenergetics was associated with key improvements in human healthspan: physical function and reduced inflammation. We also used metabolomics to identify and test potential circulating factors responsible for mediating mitochondrial bioenergetics. Specifically, we identified 3-hydroxy-3-methylglutarate and α -hydroxyisobutyrate as new potential therapeutic targets to improve healthspan.

In summary, this dissertation provides evidence that circulating factors mediate bioenergetic capacity in multiple contexts, including aging, diet, and exercise. We determined that dietary composition, such as a Western diet, directly affects systemic bioenergetic capacity, and increased fatty acid respiration in muscle is associated with early insulin resistance. Using heterochronic parabiosis, we demonstrated that circulating factors modulate age-related bioenergetic decline via changes in mitochondrial structure and function, and circulating factors associated with old blood have a greater negative effect on mitochondrial structure and function when compared to young blood. Finally, we established that serum-mediated bioenergetics can recapitulate the effects of resistance training and caloric restriction *in vitro* and are connected to key features of human health such as physical function and reduced inflammation, and metabolomics analysis revealed new potential mediators of mitochondrial bioenergetics, such as 3-hydroxy-3-methylglutarate and α -hydroxyisobutyrate.

II. DIETARY COMPOSITION AND MITOCHONDRIAL BIOENERGETICS

In Chapter Two, we reported that diet composition and whole dietary patterns influenced skeletal muscle mitochondrial bioenergetics. Previous studies of mitochondrial bioenergetics have focused on the manipulation of a single macronutrient, such as fat, rather than a whole dietary pattern. Others have reported that high-fat diets in rodents are associated with increased mitochondrial respiration in response to both fatty acid and carbohydrate substrates, increased mitochondrial biogenesis, and increased mitochondrial content, despite increased insulin resistance (1–4). However, these studies include very high-fat diets with 45% to 60% of total calories from fat and are compared to control diets that contain 8-12% of calories from fat. Additionally, the composition of fats in these studies vary. Some studies include flaxseed oil and olive oil as sources of fat, while others have lard as the major source. It should be noted that the study presented in this dissertation does not compare high-fat and low-fat diets. Both the Western and Mediterranean experimental diets contain 31% of calories from fat and we matched the experimental diets on protein, carbohydrate, and total fat content. The Western experimental diet has a larger amount of saturated fat, while the Mediterranean experimental diet has a larger percentage of monounsaturated and polyunsaturated fats. Our results suggest that manipulations of the types of fats consumed alone can alter mitochondrial bioenergetics, independent of other factors, such as macronutrient composition, energy intake, and obesity.

We observed significantly higher respiration both with and without fatty acid oxidation in permeabilized skeletal muscle fibers from animals fed a Western compared to a Mediterranean diet. We also observed strong positive correlations between fatty acid oxidation and HOMA-IR and fasting blood insulin. These results suggest that there is a

potential link between increased fatty acid oxidation of octanoylcarnitine and the development of insulin resistance. Within-group relationships were strongest in the Western diet group and generally not significant in the Mediterranean diet group, perhaps reflecting greater heterogeneity in responses to diet in the Western diet group. However, when both diet groups are combined, similar relationships to the Western diet group persist. Partial correlations controlling for body weight and age suggest that insulin AUC was positively associated with FAO and FAO max ETS capacity in the Western diet group and negatively associated in the Mediterranean diet group, suggesting a complex diet by body weight interaction. In light of these correlations, further studies are necessary to understand the link between fatty acid oxidation and insulin resistance.

It should be noted that while animal studies find increased mitochondrial respiration with insulin resistance, human studies of diabetics and obese individuals have reported reductions in skeletal muscle mitochondrial bioenergetics (5–10). In this study, metabolic perturbations associated with the Western diet may be related to a prediabetic state in humans. A significantly longer study would be required to determine the temporal relationships between bioenergetic changes associated with diet and the development of overt diabetes. Our results may reflect an early adaptation to insulin resistance which may be followed by bioenergetic decline upon disease progression.

Previous studies have linked Western diets to chronic low-grade inflammation with elevated levels of biomarkers, such as C-reactive protein, interleukins 6 and 18, and fibrinogen (11). In the context of obesity and insulin resistance, increased levels of tumor necrosis factor- α , interleukin 6, and C-reactive protein have also been reported in both animal models and humans (12). Inflammation is associated with increased mitochondrial respiration in brown adipose tissue, adipocytes, and fibroblasts (13–15).

Lark et al. have suggested that high-fat diets that increase mitochondrial respiration and partially oxidized lipid metabolites also increase the production of reactive oxygen species and H_2O_2 that could lead to the induction of proinflammatory cascades and prolonged inflammation (12). In the present study, the lower mitochondrial respiration we observed with the Mediterranean diet may be linked to reduced reactive oxygen species, with the potential to lower systemic inflammatory burden. This diet is high in n-3 fatty acids and has a lower n-6:n-3 fatty acid ratio, both of these have been associated with anti-inflammatory effects and could play a role in the differences in mitochondrial bioenergetics observed (16, 17). Future studies designed to investigate the effects of Western and Mediterranean dietary patterns on inflammation will need to be conducted to determine how these relate to differences in mitochondrial metabolism.

In light of our findings that circulating factors are involved in age-related bioenergetic decline in Chapter Three and interventions to improve healthspan, such as RT and CR in Chapter Four, the differences in mitochondrial metabolism we observed between Western and Mediterranean diet in this study may be driven by circulating factors. Though further investigation is warranted, this hypothesis is supported by recent discoveries in the field of metabolomics linking unique metabolomic signatures to dietary patterns, such as Western or Mediterranean diets (18, 19). Diet can affect the metabolome in two ways: by affecting the endogenous metabolome (all metabolites present in a biological sample that are derived from genes) or by changing the composition of the food metabolome (metabolites that are derived from food consumption and their metabolism) (20, 21). A Western dietary pattern has been associated with alterations in short-chain acylcarnitines, leucine, phenylalanine (18). On the other hand, a Mediterranean diet has been associated with altered levels of various metabolites derived from carbohydrate, amino acid, and lipid sources, including 3-

hydroxybutyrate, citrate, and cisaconitate, creatine, creatinine, proline, N-acetylglutamine, glycine, branched-chain amino acids, oleic acid, and suberic acid (19). Taken together, this information suggests that both differences in mitochondrial function in skeletal muscle after either a Western or Mediterranean diet and the potential link between high skeletal muscle mitochondrial respiration and the consumption of a Western diet and the development of insulin resistance may be mediated by circulating factors, such as metabolites.

III. CIRCULATING FACTORS, AGING, AND MITOCHONDRIAL BIOENERGETICS

In Chapter Three, our results provided striking evidence of the negative impact of circulating factors in old blood on mitochondrial structure and function. Young heterochronic mice had significantly altered mitochondrial morphology, reduced expression of complex IV, and significantly reduced mitochondrial respiration when compared to young isochronic controls. These mitochondrial alterations may underlie age-related differences in muscle function. Other labs have reported that young heterochronic mice exhibit decreased muscle performance, but no differences in muscle regeneration or fibrosis after muscle injury compared to young isochronic controls (22, 23). Notably, lower skeletal muscle mitochondrial respiration has been associated with lower physical function as measured by muscle strength and gait speed (24). Collectively, these results suggest that altered mitochondrial structure and function is not only a major aspect of age-related decline in skeletal muscle but that circulating factors can mediate these changes. Furthermore, circulating factors may play a role in the link between mitochondrial respiration and physical function.

We did not observe statistically significant increases in mitochondrial area or length, complex IV expression, or mitochondrial respiration in old heterochronic mice when compared to old isochronic controls. Other labs have reported that old heterochronic mice exhibit improved muscle regeneration and reduced fibrosis after muscle injury, but no differences in muscle performance when compared to old isochronic controls (22, 23). Taken together, these results suggest that there is a greater negative impact of circulating factors in old blood on mitochondrial structure and function than can be restored by factors in young blood. Another potential explanation for this observation is

that intrinsic properties of muscle from older animals may make these cells resistant to the potential benefits of factors found in young blood.

Notably, we did not observe any differences in mitochondrial morphology, complex IV expression, or mitochondrial respiration between young heterochronic or old heterochronic mice. When the circulating factors between young and old are mixed by heterochronic parabiosis, mitochondrial structure and function are similar between the parabiosed animals. Other labs have reported a similar response in heterochronic mice with muscle regeneration and muscle performance (22, 25). These results provide a striking demonstration that circulating factors can be a primary driver of mitochondrial structure and function.

Other studies have examined circulating factors as mediators of age-related differences and have also proposed specific proteins and metabolites that can impact mitochondrial function. For example, previous work from our lab has found that increased levels of IL-6 were associated with lower mitochondrial respiration in older adults (26). Additionally, other studies have also found that alterations in levels of IL-6 (27, 28), BCAAs (29, 30), and lipid metabolites (31, 32) are associated with changes in mitochondrial function. While further investigation of the identities of additional circulating factors is warranted, these results support a growing body of evidence that circulating factors are not only potential biomarkers of aging, but may underlie hallmarks of the aging process.

IV. CIRCULATING FACTORS, INTERVENTIONS TO IMPROVE HEALTHSPAN, AND MITOCHONDRIAL BIOENERGETICS

In Chapter Four, we examined the effects of human serum before and after intervention, either resistance training or resistance training plus caloric restriction, on muscle cells in vitro and to identify and test potential circulating factors responsible for mediating these changes. When we examined the overall effect of intervention on serum-mediated bioenergetics, we found mitochondrial respiration was significantly higher post-intervention compared to pre-intervention. These results are consistent with observations of increased skeletal muscle mitochondrial function after exercise interventions in older adults (28–33). Next, we examined the effect of intervention group, either RT or RT + CR, on serum-mediated bioenergetics. We found that post-RT serum significantly increased basal and ATP-linked mitochondrial respiration and had a trend for increased maximal mitochondrial respiration, while post-RT + CR serum had trends for increased maximal mitochondrial respiration and spare respiratory capacity. Previous studies examining the effect of RT on mitochondria report increased mitochondrial function as measured by phosphocreatine recovery time (33) and high-resolution respirometry of permeabilized skeletal muscle fibers (34, 35). Studies examining the effect of CR alone on skeletal muscle mitochondria found no changes in mitochondrial content (36) or mitochondrial function as measured by phosphocreatine recovery time (37). Exercise and CR are rarely combined in studies examining mitochondrial function. Other studies have reported that aerobic exercise alone increased mitochondrial content and mitochondrial enzyme activities involved in electron transport and fatty acid oxidation, while caloric restriction sufficient to induce weight loss did not (38). The results of our study are also consistent with a previous study examining the effects of 10% rat serum on the mitochondrial bioenergetics of cultured β -cells (39). They found that serum

factors alone from CR rats increased glucose-stimulated insulin secretion, preserved mitochondrial function in response to glucose toxicity, and modified mitochondrial morphology and dynamics. These data suggest that serum factors alone are sufficient to induce mitochondrial changes that reflect the effects of interventions such as RT and CR.

Notably, we observed a wide range of responses to intervention with serum-mediated bioenergetics by examining the change in maximal respiration across all participants. There appear to be three groups of responders: negative responders, non-responders, and positive responders. While there is debate in the literature about whether there are true non-responders or if some of these observations could be attributed to measurement variability that is not adequately controlled for (40, 41), this observation is also consistent with previous studies that reported heterogeneous responses to a variety of exercise interventions (34, 42–47). Indeed, this pattern of heterogeneous response in terms of physical function was also observed in the participants from I'M FIT, the parent study for the samples utilized in this study (48). We also observed some sex differences in serum-mediated bioenergetics. In particular, female participant serum from the RT group significantly increased basal and ATP-linked mitochondrial respiration and had trends for increased maximal respiration and spare respiratory capacity. We did not observe any significant increases in the male participants. These findings are consistent with observations of mitochondrial function by high-resolution respirometry that females had greater intrinsic mitochondrial respiratory rates (49) and exhibit decreased ADP sensitivity and increased sensitivity to malonyl-CoA-mediated respiratory inhibition in skeletal muscle compared to men (50). Furthermore, these differences in mitochondrial respiration are supported by previous research, including sex differences in fiber type and fuel utilization during exercise (reviewed in (51)). These findings suggest that

circulating factors are potential mediators of both heterogeneous responses as a whole and sex differences in response to intervention.

We found significant correlations between improvements in serum-mediated bioenergetics and improvements in physical function, as measured by Mat-sf, 400-m walk time, and gait speed, and improvements in inflammation, as measured by IL-6 and CRP. These results are consistent with cross-sectional studies completed in a subset of I'M FIT participants, where higher blood cell bioenergetics was associated with faster gait speed, knee extensor strength, and grip strength as well as lower levels of IL-6 (52, 53). Therefore, the data suggest that improvements in mitochondrial bioenergetics, physical function, and inflammation in older adults are all connected and potentially mediated by blood-borne circulating factors.

We performed metabolomics analyses to identify potential circulating factors that can mediate bioenergetic capacity. Our analyses revealed 25 potential circulating factors that were positively or negatively associated with the pattern of responses that we observed in the change in serum-mediated maximal respiration. We identified 3 metabolites that were significantly and positively associated with serum-mediated maximal respiration (arabinose, inosine, and d-gluconic acid γ -lactone) and 2 metabolites that were significantly and negatively associated with serum-mediated maximal respiration (3-hydroxymethylglutarate and α -hydroxisobutyrate). Of these, we chose to test inosine, 3-hydroxymethyl-3-glutarate, and α -hydroxisobutyrate as potential circulating factors.

Inosine is a purine nucleoside produced by the catabolism of adenosine and has been examined for potential use in improving exercise performance (54–56), epilepsy (57), multiple sclerosis (58), and Parkinson's disease (59) due to potential neuroprotective

properties shown in rats (60, 61). We did not find a significant relationship between inosine concentration and increased serum-mediated bioenergetics. This suggests that inosine is not a metabolite that can directly affect mitochondrial bioenergetics and may be downstream of another process or that the mechanism of action involves other cell types.

3-hydroxy-3-methylglutarate is an off-product that is a result of the incomplete conversion of 3-hydroxy-3-methylglutaryl-CoA to acetyl-CoA and acetoacetate by defective or inefficient 3-hydroxy-3-methylglutaryl-CoA lyase. 3-hydroxymethyl-3-glutarate accumulates in the mitochondria and high levels in urine have been associated with inborn errors of metabolism (62, 63). We found a strong relationship between increasing concentrations of 3-hydroxymethyl-3-glutarate and decreased serum-mediated bioenergetics, particularly with maximal respiration. This suggests that, at least in older adults, resistance training and caloric restriction can alleviate some of the negative effects of 3-hydroxymethyl-3-glutarate on mitochondrial bioenergetics, through mechanisms that can be explored in future studies.

α -hydroxisobutyrate is an organic acid derived from α -ketobutyrate that is produced by amino acid catabolism and glutathione anabolism (64). α -hydroxisobutyrate has been presented as a potential early biomarker for insulin resistance and glucose intolerance (64, 65). In particular, α -hydroxisobutyrate has been associated with increased insulin resistance and in vitro treatment of pancreatic β -cells with α -hydroxisobutyrate inhibited glucose-mediated insulin secretion (65). We found a strong relationship between increasing concentrations of α -hydroxisobutyrate and decreased serum-mediated bioenergetics. This suggests that α -hydroxisobutyrate may play a key role in the development of insulin resistance and the improvements in insulin sensitivity that are

observed with RT and CR interventions. Collectively, these results suggest that individual circulating factors, such as 3-hydroxymethyl-3-glutarate and α -hydroxyisobutyrate, can mediate mitochondrial bioenergetics and are powerful potential therapeutic targets for further investigation.

Interestingly, we observed more robust direct effects of metabolites on mitochondrial bioenergetics that were more negatively associated with serum-mediated bioenergetics, such as 3-hydroxy-3-methylglutarate and α -hydroxyisobutyrate. This is consistent with recent observations of old blood factors being more dominant in old blood (25), including our observations that old blood had a greater negative effect on mitochondrial bioenergetics in Chapter Three. A recent publication demonstrated that dilution of old blood with saline-albumin resulted in improvements in key aspects associated with heterochronic parabiosis, including improved muscle regeneration, reduced liver adiposity and fibrosis, and increased hippocampal neurogenesis (66). These results indicate that targeting negative blood-borne circulating factors would have a greater therapeutic benefit and should be the focus of future studies. Additionally, these results further support a growing body of evidence that circulating factors are not only important for the aging process but in interventions to improve healthspan as well.

V. FUTURE DIRECTIONS FOR RESEARCH

In Chapter Two, we determined that dietary composition, such as a Western or Mediterranean diet, directly affects systemic bioenergetic capacity. Thus far, previous research has been dominated by alterations in single macronutrients, especially fat. Further investigation is needed to determine the mechanistic links between dietary composition, dietary source, and mitochondrial bioenergetics. We also reported that increased mitochondrial respiration in response to fatty acids was significantly and positively correlated with insulin resistance, particularly in the Western diet group. The monkeys included in this study did not yet exhibit overt diabetes, suggesting that this phenomenon may be an early adaptation to insulin resistance. Further research is needed to elucidate the links between the development of insulin resistance and mitochondrial dysfunction. A longitudinal study to determine the time course and trajectories of mitochondrial changes would allow for the exploration of potential early compensation mechanisms that may include changes in mitochondrial structure and function in response to altered glucose metabolism and reduced insulin sensitivity. Given that we found α -hydroxybutyrate, a metabolite that is associated with the development of insulin resistance, could directly affect mitochondrial bioenergetics, the roles of circulating factors should also be explored in this context.

To build upon our preliminary findings with heterochronic parabiosis in Chapter Three, future research should examine other tissues susceptible to age-related bioenergetic decline, such as the brain, heart, and liver. It should be determined if the effects we have observed on mitochondrial structure and function are directly due to circulating factors or simply the dilution of negative circulating factors, as suggested by recent developments in the field (66). Additionally, the identities of the circulating factors involved in mediating

age-related bioenergetic decline should be determined. The parabiosis model would also be useful to answer other outstanding questions in the field of mitochondrial bioenergetics, including the roles of circulating factors in the development of cognitive impairment and Alzheimer's disease, sarcopenia, and other age-related diseases.

The work presented in this dissertation indicates that circulating factors play a major role in mediating mitochondrial bioenergetics. In Chapter Four, we have focused on metabolites, but other types of circulating factors are likely involved in mediating mitochondrial bioenergetics as well. Further research should explore other factors, including, but not limited to: proteins, circulating RNAs, exosomes, damage-associated molecular patterns such as cell-free DNA, or other components of the senescence-associated secretory phenotype.

Serum-mediated bioenergetics can be expanded to many other completed clinical trials or new study cohorts could be created from stored serum samples, allowing many new questions to be explored, including: differences between old and young, the progression of cognitive decline, longitudinal studies of healthy aging, other lifestyle or pharmacological interventions to improve healthspan, and other applications outside of the biology of aging. This method should also be expanded to many other cell types, allowing for the examination of serum-mediated bioenergetics across a wide range of tissues to understand the systemic nature of the effects of circulating factors. Organoids or induced pluripotent stem cells (iPSCs) would also improve the translatability of serum-mediated bioenergetics discoveries in humans.

Additionally, this dissertation highlights the potential to target circulating factors for therapeutic interventions. In particular, we identified 3-hydroxy-3-methylglutarate and α -

hydroxyisobutyrate as potential targets to modify mitochondrial bioenergetics. However, there are likely many targets to be found from RT and CR interventions and healthy aging. One could also imagine there are circulating factors that mediate the negative bioenergetic aspects of Western diet or the positive bioenergetic aspects of Mediterranean diet. Therefore, serum-mediated bioenergetics has the potential to support the development of inhibitors of negative circulating factors or compounds that mimic or enhance the effects of more positive circulating factors. Future studies should explore this potential to develop therapies to improve healthspan in older adults.

VI. CONCLUSIONS

In conclusion, this dissertation provides evidence that circulating factors mediate bioenergetic capacity in multiple contexts, including: aging, diet, and exercise. We determined that dietary composition, such as a Western or Mediterranean diet, directly affects systemic bioenergetic capacity, and increased fatty acid respiration in muscle is associated with early insulin resistance. Using heterochronic parabiosis, we demonstrated that circulating factors modulate age-related bioenergetic decline via changes in mitochondrial structure and function, and circulating factors associated with old blood have a greater negative effect on mitochondrial structure and function when compared to young blood. Finally, we established that serum-mediated bioenergetics can recapitulate the effects of resistance training and caloric restriction *in vitro* and are connected to key features of human health such as physical function and reduced inflammation, and identified new potential mediators of mitochondrial bioenergetics, such as 3-hydroxy-3-methylglutarate and α -hydroxyisobutyrate.

REFERENCES

1. I. R. Lanza, *et al.*, Influence of fish oil on skeletal muscle mitochondrial energetics and lipid metabolites during high-fat diet. *Am J Physiol Endocrinol Metab* **304**, E1391-1403 (2013).
2. N. Turner, *et al.*, Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes* **56**, 2085–2092 (2007).
3. C. R. Hancock, *et al.*, High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc Natl Acad Sci U S A* **105**, 7815–7820 (2008).
4. P. Garcia-Roves, *et al.*, Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. *Proc Natl Acad Sci U S A* **104**, 10709–10713 (2007).
5. J. He, S. Watkins, D. E. Kelley, Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* **50**, 817–823 (2001).
6. J. A. Simoneau, J. H. Veerkamp, L. P. Turcotte, D. E. Kelley, Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J* **13**, 2051–2060 (1999).
7. D. E. Kelley, B. Goodpaster, R. R. Wing, J. A. Simoneau, Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* **277**, E1130-1141 (1999).

8. I. Ara, *et al.*, Normal mitochondrial function and increased fat oxidation capacity in leg and arm muscles in obese humans. *Int J Obes (Lond)* **35**, 99–108 (2011).
9. D. E. Kelley, J. He, E. V. Menshikova, V. B. Ritov, Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* **51**, 2944–2950 (2002).
10. R. Boushel, *et al.*, Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* **50**, 790–796 (2007).
11. J. Barbaresco, M. Koch, M. B. Schulze, U. Nöthlings, Dietary pattern analysis and biomarkers of low-grade inflammation: a systematic literature review. *Nutr Rev* **71**, 511–527 (2013).
12. D. S. Lark, K. H. Fisher-Wellman, P. D. Neuffer, High-fat load: mechanism(s) of insulin resistance in skeletal muscle. *Int J Obes Suppl* **2**, S31–S36 (2012).
13. M. Alcalá, *et al.*, Increased inflammation, oxidative stress and mitochondrial respiration in brown adipose tissue from obese mice. *Sci Rep* **7**, 16082 (2017).
14. C. Mauro, *et al.*, NF- κ B controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. *Nat Cell Biol* **13**, 1272–1279 (2011).
15. Y. S. Lee, *et al.*, Increased adipocyte O₂ consumption triggers HIF-1 α , causing inflammation and insulin resistance in obesity. *Cell* **157**, 1339–1352 (2014).
16. K. Esposito, *et al.*, Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *JAMA* **292**, 1440–1446 (2004).

17. P. J. White, M. Arita, R. Taguchi, J. X. Kang, A. Marette, Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice. *Diabetes* **59**, 3066–3073 (2010).
18. A. Bouchard-Mercier, I. Rudkowska, S. Lemieux, P. Couture, M.-C. Vohl, The metabolic signature associated with the Western dietary pattern: a cross-sectional study. *Nutr J* **12**, 158 (2013).
19. R. Vázquez-Fresno, *et al.*, Metabolomic pattern analysis after mediterranean diet intervention in a nondiabetic population: a 1- and 3-year follow-up in the PREDIMED study. *J Proteome Res* **14**, 531–540 (2015).
20. A. Scalbert, *et al.*, The food metabolome: a window over dietary exposure. *Am J Clin Nutr* **99**, 1286–1308 (2014).
21. D. S. Wishart, Emerging applications of metabolomics in drug discovery and precision medicine. *Nat Rev Drug Discov* **15**, 473–484 (2016).
22. I. M. Conboy, *et al.*, Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* **433**, 760–764 (2005).
23. J. Rebo, *et al.*, A single heterochronic blood exchange reveals rapid inhibition of multiple tissues by old blood. *Nature Communications* **7** (2016).
24. M. Gonzalez-Freire, *et al.*, Skeletal muscle ex vivo mitochondrial respiration parallels decline in vivo oxidative capacity, cardiorespiratory fitness, and muscle strength: The Baltimore Longitudinal Study of Aging. *Aging Cell* **17**, 12725 (2018).

25. J. Rebo, *et al.*, A single heterochronic blood exchange reveals rapid inhibition of multiple tissues by old blood. *Nat Commun* **7**, 13363 (2016).
26. D. J. Tyrrell, *et al.*, Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. *Experimental Gerontology* **70**, 84–91 (2015).
27. B. N. VanderVeen, *et al.*, The regulation of skeletal muscle fatigability and mitochondrial function by chronically elevated interleukin-6. *Experimental Physiology* **104**, 385–397 (2019).
28. D. J. Tyrrell, *et al.*, Age-Associated Mitochondrial Dysfunction Accelerates Atherogenesis. *Circulation Research* **126**, 298–314 (2020).
29. M. Gonzalez-Freire, F. Adelnia, R. Moaddel, L. Ferrucci, Searching for a mitochondrial root to the decline in muscle function with ageing. *Journal of Cachexia, Sarcopenia and Muscle* **9**, 435–440 (2018).
30. G. D'Antona, *et al.*, Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metabolism* **12**, 362–372 (2010).
31. Y. Zhong, L. Zeng, J. Deng, Y. Duan, F. Li, β -hydroxy- β -methylbutyrate (HMB) improves mitochondrial function in myocytes through pathways involving PPAR β/δ and CDK4. *Nutrition* **60**, 217–226 (2019).
32. S. Schuster, *et al.*, Oxidized linoleic acid metabolites induce liver mitochondrial dysfunction, apoptosis, and NLRP3 activation in mice. *Journal of Lipid Research* **59**, 1597–1609 (2018).

33. R. C. R. Meex, *et al.*, Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes* **59**, 572–579 (2010).
34. D. Pesta, *et al.*, Similar qualitative and quantitative changes of mitochondrial respiration following strength and endurance training in normoxia and hypoxia in sedentary humans. *Am J Physiol Regul Integr Comp Physiol* **301**, R1078-1087 (2011).
35. C. Porter, P. T. Reidy, N. Bhattarai, L. S. Sidossis, B. B. Rasmussen, Resistance Exercise Training Alters Mitochondrial Function in Human Skeletal Muscle. *Med Sci Sports Exerc* **47**, 1922–1931 (2015).
36. F. G. S. Toledo, *et al.*, Mitochondrial Capacity in Skeletal Muscle Is Not Stimulated by Weight Loss Despite Increases in Insulin Action and Decreases in Intramyocellular Lipid Content. *Diabetes* **57**, 987–994 (2008).
37. L. M. Sparks, *et al.*, Effects of 12 Months of Caloric Restriction on Muscle Mitochondrial Function in Healthy Individuals. *J Clin Endocrinol Metab* **102**, 111–121 (2016).
38. E. V. Menshikova, *et al.*, Calorie Restriction-induced Weight Loss and Exercise Have Differential Effects on Skeletal Muscle Mitochondria Despite Similar Effects on Insulin Sensitivity. *The Journals of Gerontology: Series A* **73**, 81–87 (2018).
39. F. M. Cerqueira, *et al.*, Diluted serum from calorie-restricted animals promotes mitochondrial β -cell adaptations and protect against glucolipotoxicity. *FEBS J* **283**, 822–833 (2016).

40. S. J. Dankel, J. P. Loenneke, A Method to Stop Analyzing Random Error and Start Analyzing Differential Responders to Exercise. *Sports Med* **50**, 231–238 (2020).
41. L. M. Sparks, Exercise training response heterogeneity: physiological and molecular insights. *Diabetologia* **60**, 2329–2336 (2017).
42. J. F. Baird, R. W. Motl, Response Heterogeneity with Exercise Training and Physical Activity Interventions among Persons with Multiple Sclerosis. *Neurorehabil Neural Repair* **33**, 3–14 (2019).
43. C. Bouchard, T. Rankinen, Individual differences in response to regular physical activity. *Med Sci Sports Exerc* **33**, S446-451; discussion S452-453 (2001).
44. C. Bouchard, *et al.*, Familial aggregation of VO₂max response to exercise training: results from the HERITAGE Family Study. *J Appl Physiol* (1985) **87**, 1003–1008 (1999).
45. M. J. Hubal, *et al.*, Variability in muscle size and strength gain after unilateral resistance training. *Med Sci Sports Exerc* **37**, 964–972 (2005).
46. W. M. Kohrt, *et al.*, Effects of gender, age, and fitness level on response of VO₂max to training in 60-71 yr olds. *J Appl Physiol* (1985) **71**, 2004–2011 (1991).
47. R. Ross, *et al.*, Precision exercise medicine: understanding exercise response variability. *Br J Sports Med* **53**, 1141–1153 (2019).
48. E. A. Chmelo, *et al.*, Heterogeneity of physical function responses to exercise training in older adults. *J Am Geriatr Soc* **63**, 462–469 (2015).

49. D. A. Cardinale, *et al.*, Superior Intrinsic Mitochondrial Respiration in Women Than in Men. *Front Physiol* **9**, 1133 (2018).
50. P. M. Miotto, C. McGlory, T. M. Holloway, S. M. Phillips, G. P. Holloway, Sex differences in mitochondrial respiratory function in human skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **314**, R909–R915 (2018).
51. P. Ansdell, *et al.*, Physiological sex differences affect the integrative response to exercise: acute and chronic implications. *Experimental Physiology* **105**, 2007–2021 (2020).
52. D. J. Tyrrell, *et al.*, Respirometric Profiling of Muscle Mitochondria and Blood Cells Are Associated With Differences in Gait Speed Among Community-Dwelling Older Adults. *J Gerontol A Biol Sci Med Sci* **70**, 1394–1399 (2015).
53. D. J. Tyrrell, *et al.*, Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. *Exp Gerontol* **70**, 84–91 (2015).
54. L. McNaughton, B. Dalton, J. Tarr, Inosine supplementation has no effect on aerobic or anaerobic cycling performance. *Int J Sport Nutr* **9**, 333–344 (1999).
55. R. D. Starling, *et al.*, Effect of inosine supplementation on aerobic and anaerobic cycling performance. *Med Sci Sports Exerc* **28**, 1193–1198 (1996).
56. M. H. Williams, *et al.*, Effect of inosine supplementation on 3-mile treadmill run performance and VO₂ peak. *Med Sci Sports Exerc* **22**, 517–522 (1990).

57. Z. Kovacs, *et al.*, Non-adenosine nucleoside inosine, guanosine and uridine as promising antiepileptic drugs: a summary of current literature. *Mini Rev Med Chem* **14**, 1033–1042 (2015).
58. D. Muñoz García, *et al.*, Associated Inosine to interferon: results of a clinical trial in multiple sclerosis. *Acta Neurol Scand* **131**, 405–410 (2015).
59. H. Watanabe, *et al.*, Improved Parkinsons disease motor score in a single-arm open-label trial of febuxostat and inosine. *Medicine (Baltimore)* **99**, e21576 (2020).
60. F. Liu, *et al.*, Secondary degeneration reduced by inosine after spinal cord injury in rats. *Spinal Cord* **44**, 421–426 (2006).
61. L. Zai, *et al.*, Inosine Alters Gene Expression and Axonal Projections in Neurons Contralateral to a Cortical Infarct and Improves Skilled Use of the Impaired Limb. *J Neurosci* **29**, 8187–8197 (2009).
62. R. Dena, M. Fabbro, F. Rigoni, Formation and utilization of 3-hydroxy-3-methylglutarate in liver mitochondria of starved and streptozotocin-diabetic rats. *Biochem J* **172**, 371–375 (1978).
63. C. G. Fernandes, *et al.*, Induction of a Proinflammatory Response in Cortical Astrocytes by the Major Metabolites Accumulating in HMG-CoA Lyase Deficiency: the Role of ERK Signaling Pathway in Cytokine Release. *Mol Neurobiol* **53**, 3586–3595 (2016).
64. W. E. Gall, *et al.*, α -Hydroxybutyrate Is an Early Biomarker of Insulin Resistance and Glucose Intolerance in a Nondiabetic Population. *PLoS One* **5** (2010).

65. E. Ferrannini, *et al.*, Early Metabolic Markers of the Development of Dysglycemia and Type 2 Diabetes and Their Physiological Significance. *Diabetes* **62**, 1730–1737 (2013).
66. M. Mehdipour, *et al.*, Rejuvenation of three germ layers tissues by exchanging old blood plasma with saline-albumin. *Aging* **12**, 8790–8819 (2020).

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EDUCATION

2016 – present **Ph.D.**, Molecular Medicine and Translational Medicine
Wake Forest School of Medicine, Winston-Salem, NC
Expected graduation: May 2021

2012 – 2016 **B.S.**, Chemistry
Western Carolina University, Cullowhee, NC
American Chemical Society Certified Concentration
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RESEARCH EXPERIENCE

2016 – present **Ph.D. Candidate**
Department of Internal Medicine – Molecular Medicine
Department of Internal Medicine – Gerontology and Geriatric Medicine
Wake Forest School of Medicine
Winston-Salem, NC

Mentor: Anthony Molina, Ph.D.

Project: Effects of circulating factors in mediating changes in systemic mitochondrial bioenergetics in response to aging, diet, and exercise

2015 – 2016 **Undergraduate Research Assistant**
Department of Chemistry and Physics
Western Carolina University
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Mentor: Jamie Wallen, Ph.D.

Project: Studied protein interactions during DNA replication with single-stranded binding protein in T7 bacteriophage

2014 – 2015 **Undergraduate Research Assistant**
Department of Chemistry and Physics
Western Carolina University
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Mentor: Scott Huffman, Ph.D.

Project: Development of printed microfluidic devices for analytical applications

*Received Honors College Undergraduate Research Grant to support this work

2013 – 2014

Undergraduate Research Assistant

Department of Chemistry and Physics
Western Carolina University
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Mentor: Jack Summers, Ph.D.

Project: Development of a storm surge sensor using electrochemistry

PUBLICATIONS

- 2020 **JL Gonzalez-Armenta**, N Li, RL Lee, B Lu, AJA Molina. (published online Dec 2020) Heterochronic Parabiosis: Old Blood Induces Changes in Mitochondrial Structure and Function of Young Mice. *Journals of Gerontology, Series A: Biological Sciences and Medical Sciences*.
- 2019 **JL Gonzalez-Armenta**, Z Gao, SE Appt, MZ Vitolins, KT Michalson, TC Register, CA Shively, AJA Molina. (Sep 2019) Skeletal Muscle Mitochondrial Respiration Is Elevated in Female Cynomolgus Macaques Fed a Western Compared with a Mediterranean Diet. *The Journal of Nutrition*.
- 2018 E Gnaiger, et al. (340 authors, including **JL Gonzalez-Armenta**) (2018) Mitochondrial respiratory states and rates: Building blocks of mitochondrial physiology Part 1, *MitoEAGLE preprint*.

Manuscripts in submission

- 2021 G Mahapatra, Z Gao, AR DeWitt, JE Piloso, JR Bateman III, PA Kramer, **JL Gonzalez-Armenta**, KA Amick, R Casanova, SN Lockhart, S Craft, AJA Molina. Blood-based respirometry reveals differences in systemic bioenergetic capacity associated with cognitive decline and Alzheimer's disease.

Manuscripts in preparation

- 2021 **JL Gonzalez-Armenta**, BJ Nicklas, AJA Molina. Serum-Mediated Mitochondrial Respiration from Weight Loss and Exercise Interventions.

PUBLISHED ABSTRACTS

- 2020 **JL Gonzalez-Armenta**, BJ Nicklas, AJA Molina. (Nov 2020) Serum Factors Mediate the Bioenergetic Benefits of Exercise Training and Caloric Restriction. *Innovation in Aging* 4(Suppl. 1), 127. (Abstract)
- 2018 **JL Gonzalez-Armenta**, G Mahapatra, KA Amick, N Li, B Lu, AJA Molina. (Nov 2018) Heterochronic Parabiosis: Old Blood Attenuates Mitochondrial Bioenergetics of Young Mice. *Innovation in Aging* 2(Suppl. 1), 558. (Abstract)

AWARDS AND HONORS

- 2018 **Emerging Scholars and Professionals Organization/Biological Sciences Symposium Speaker Travel Award from the Gerontological Society of America**
Received funding for travel expenses to present at and attend the Gerontological Society of America National Meeting in Boston, MA in November 2018. This award was given to young investigators who had oral presentations in the ESPO/Biological Sciences Symposium.
- 2018 **Molecular Medicine and Translational Science Travel Award**
Received \$500 for travel expenses to present at and attend the Gerontological Society of America National Meeting in Boston, MA in November 2018. This award is given to two MMTS students in the Fall and one student in the Spring.
- 2017 **MiP Scholarship Recipient**
Received award from the Mitochondrial Physiology Society to cover lodging and meals during MiPSchool 2017 in Obergurgl, Austria.
- 2016 **ACS Outstanding Senior Award**
The American Chemical Society Outstanding Senior Award recognizes a graduating senior chemistry major who has demonstrated excellence in academics, undergraduate research, and service to the department. Students were nominated by faculty and the award recipient is recognized at the ACS Western Carolinas Local Section Meeting.
- 2015 **Honors College Undergraduate Research Grant**
Received \$500 from the Honors College at Western Carolina University to purchase supplies for a research project in Scott Huffman's laboratory.
- 2014 **Frank Brown Sr. Chemistry Scholarship**
This merit scholarship is given to outstanding chemistry students at Western Carolina University. Amount: \$2,000 per year for two years.
- 2012 **Paul A. Reid Scholarship**
This scholarship is given to graduates of Surry County high schools to attend Western Carolina University. Amount: \$1,600 per year for four years.

- 2012 **Edward M. Armfield Sr. Foundation Scholarship**
This scholarship is given to graduates of Surry County high schools to attend undergraduate institutions. Amount: \$5,000 per year for four years.
- 2012 **Honors College Distinguished Achievement Scholarship**
This merit scholarship provides support to high achieving students who are members of the WCU Honors College. Amount: \$2,000 per year for four years.

RESEARCH FUNDING

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PRESENTATIONS

Oral Presentations

- 2018 **JL Gonzalez-Armenta**, G Mahapatra, KA Amick, N Li, B Lu, AJA Molina.
“Heterochronic Parabiosis: Old Blood Attenuates Mitochondrial Bioenergetics of Young Mice.” Gerontological Society of America Annual Scientific Meeting, Boston, MA, USA, November 2018.
* One of top 5 abstracts submitted by young investigators selected for platform presentations during the Emerging Scholars and Professional Organization / Biological Sciences Section Symposium
- 2017 **JL Collins**, J Stone, CA Shively, AJ Molina. “The effects of a Mediterranean versus Western diet on nonhuman primate skeletal muscle bioenergetics.” Mitochondrial Physiology Society MiPSchool, Obergurgl, AU, July 2017.

Poster Presentations

- 2020 **JL Gonzalez-Armenta**, BJ Nicklas, AJA Molina. “Serum Factors Mediate the Bioenergetic Benefits of Exercise Training and Caloric Restriction.” Gerontological Society of America Annual Scientific Meeting, Online, November 2020.
- 2016 **JL Collins**, BM Foster, JR Wallen. “Investigation of Amino Acid Residues in the C-Terminal Tail of Bacteriophage T7 Single-Stranded DNA Binding Protein Predicted to Bind DNA Polymerase.” Western Carolinas ACS Local Section Meeting, Furman University, Greenville, SC, USA, April 2016.
- 2016 **JL Collins**, BM Foster, JR Wallen. “Investigation of Amino Acid Residues in the C-Terminal Tail of Bacteriophage T7 Single-Stranded DNA Binding Protein

Predicted to Bind DNA Polymerase.” National Conference for Undergraduate Research, Asheville, NC, USA, April 2016.

- 2015 BM Foster*, **JL Collins***, M Carver, T Ellenberger, MD Gainey, JR Wallen. “Analysis of amino acid residues predicted to be essential for loading of T7 DNA polymerase on to a primase-helicase ring.” Southeastern Regional Meeting of the American Chemical Society, Memphis, TN, USA, November 2015. (*Co-presenter)
- 2015 JR Wallen, BM Foster, **JL Collins**, T Ellenberger. “Crystal Structure of a bacteriophage T7 primase-helicase DNA polymerase complex provides a molecular snapshot of replisome assembly.” Southeastern Regional Meeting of the American Chemical Society, Memphis, TN, USA, November 2015.

Campus/Departmental Presentations

- 2020 **JL Gonzalez-Armenta**. “Serum-Mediated Mitochondrial Bioenergetics and Exercise and Weight Loss Interventions in Older Adults.” Molecular Medicine and Translational Sciences Seminar Series. Winston-Salem, NC, USA, September 2020.
- 2020 **JL Gonzalez-Armenta**. “Serum-Mediated Mitochondrial Bioenergetics and Exercise and Weight Loss Interventions in Older Adults.” Molecular Medicine and Translational Sciences Seminar Series. Winston-Salem, NC, USA, February 2020.
- 2019 **JL Gonzalez-Armenta**. “Identifying Mediators of Mitochondrial Metabolism Associated with Diet and Exercise Interventions in Older Adults.” Molecular Medicine and Translational Sciences Seminar Series. Winston-Salem, NC, USA, September 2019.
- 2019 **JL Gonzalez-Armenta**. “Identifying Mediators of Mitochondrial Metabolism Associated with Diet and Exercise Interventions in Older Adults.” Molecular Medicine and Translational Sciences Seminar Series. Winston-Salem, NC, USA, March 2019.
- 2018 **JL Gonzalez-Armenta**. “Heterochronic Parabiosis: Effects of Aging on Mitochondrial Bioenergetics.” Molecular Medicine and Translational Sciences Seminar Series. Winston-Salem, NC, USA, November 2018.
- 2018 **JL Gonzalez-Armenta**. “Diet, Aging, and Exercise Are Associated with Changes in Skeletal Muscle Bioenergetics.” Molecular Medicine and Translational Sciences Seminar Series. Winston-Salem, NC, USA, April 2018.

Community Presentations

- 2020 **JL Gonzalez-Armenta**. “We’re Getting Older: What You Should Know About Aging Research.” Table Talk at Thirsty Souls Community Brewing, Mount Airy, NC, USA, January 2020.

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

2018 – Present	Gerontological Society of America, Member
2014 – 2016	American Chemical Society, Member
2013 – 2016	WCU Chemistry Club, Member, Treasurer (2015), President (2016)
2013 – 2016	Alpha Lambda Delta Honors Society, Member
2012 – 2014	WCU Honors College Board of Directors, Member