LINE-1 RNA Expression is Higher in Older Human Skeletal Muscle but Decreases With Endurance Exercise and Physical Activity

by

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Abstract

INTRODUCTION: Long Interspersed Nuclear Element-1 (L1) has the autonomous ability to randomly move about the genome. As a result, L1 has been implicated with disease and aging. We sought to determine if skeletal muscle L1 is expressed differently between young and old humans, if acute cycling exercise alters L1 expression, and if L1 expression is related to daily physical activity. **METHODS**: DNA and RNA expression of L1 measured by PCR was determined on 15 young (23 ± 3 y/o) and 15 old participants (58 ± 8 y/o). Participants reported to the laboratory not having exercised for 48 h, under a 24 h standardized diet, and were 8 h fasted. Participants donated a skeletal muscle biopsy (PRE) directly prior to 1 h of cycling exercise at 71 ± 3% of heart rate reserve (young – 71 ± 4%, old – 71 ± 2%; p>0.05). A second (2 h) and third muscle biopsy (8 h) were donated 2 h and 8 h following exercise, respectively. Physical activity was measured via an accelerometer worn on the hip for 13 ± 4 days. **RESULTS**: PRE L1 DNA was not different between groups (young – 1.00 ± 0.05, old – 1.00 ± 0.05; p>0.050). L1 RNA demonstrated a time effect (p=0.034) and group effect (p=0.014). Old participants expressed more L1 RNA than young participants irrespective of exercise (young – 0.90 ± 0.39, old – 1.19 ± 0.48; p=0.014). Cycling lowered L1 RNA expression 2 h post-exercise regardless of group (PRE – 1.15 ± 0.53, 2 h – 0.89 ± 0.25; p=0.027). Lastly, higher levels of moderate to vigorous physical activity per day is related to lower PRE L1 RNA expression (r=-0.398, p=0.032). **CONCLUSIONS**: PRE L1 RNA expression is higher in older humans; however, expression is reduced with endurance exercise, and higher levels of physical activity are related to lower L1 RNA expression.

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List of Abbreviations

2 h – 2 hours post-exercise skeletal muscle biopsy and blood draw

8 h – 8 hours post-exercise skeletal muscle biopsy and blood draw

1-RM – 1 repetition maximum

A – adenine

A – amp

Ac – Activator locus

AGO3 – Argonaute 3

ANOVA – analysis of variance

B2M – Beta-2-Microglobulin

BCA – bicinchoninic acid

BMI – Body mass index

BRCA2 – Breast Cancer 2 Gene

BSA – Bovine Serum albumin

ºC – Degrees Celsius

C – cytosine

CC – cubic centimeter

cDNA – copy DNA

CFTR - Cystic fibrosis transmembrane conductance regulator

cm – centimeter

CpG – Cytosine, phosphate, Guanine

CRP – c-reactive protein

CT – cycle threshold

DMD – Duchenne's Muscular Dystrophy

DNA – Deoxyribonucleic acid

DNMT – DNA methyltransferases

Ds – Dissociation Locus

EDTA – Ethylenediaminetetraacetic acid

EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (or egtazic acid)

EN – endonuclease

ET- Exercise Trial

G – guanine

g – gram

g – g-force

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

h – hour

HBEC – Human bronchial epithelial cells

HDAC – Histone Deacetylase

HIWI – human PIWI

HR – heart rate

HRP – horseradish peroxidase

HRR – Heart rate reserve

ICAM-1 – intracellular adhesion molecule-1

IgG – Immunoglobulin G

IRB – Institutional Review Board

kb – kilobases

kcal – kilocalorie

kg – kilogram

L – liter

L1 – Long interspersed nuclear element 1

LINE1 – Long interspersed nuclear element 1

LTR – Long terminal repeat

M – Molar

m – meter

m – milli

MeDIP – methylated DNA immunoprecipitation

METs – Metabolic equivalents

min – minute

MIWI – mouse PIWI

mRNA – messenger RNA

MVPA – moderate to vigorous physical activity

NaCl – Sodium Chloride

Na_3VO_4 - Sodium orthovanadate

NF1 – Neurofibromatosis Type 1

ORF – Open reading frame

ORF1p – Open reading frame 1 protein

ORF2p – Open reading frame 2 protein

PCR – polymerase chain reaction

PDHc – Pyruvate Dehydrogenase Complex

PDHX - Pyruvate Dehydrogenase Complex Component X

PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

piRNA – PIWI-interacting RNA

PIWI – p-element induced wimpy testis

PolyA – Polyadenylation

POST – post-exercise blood draw

PRE – Resting skeletal muscle biopsy and blood draw

PTEN – Phosphatase and Tensin Homolog

RER – respiratory exchange ratio

RNA – Ribonucleic acid

RNP – Ribonucleoprotein particle

RPE – rating of perceived exertion

RPM – revolutions per minute

RT – Reverse Transcriptase

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

sec – seconds

Ser – Serine

SINE – Short interspersed nuclear element

SIRT – Sirtuin

SVA – SINE-VNTR-Alu element

T – Thymine

TBST – tris-buffered saline and Tween 20

TE – Transposable element

Thr – Threonine

TPRT – Target-site primed reverse transcription

Tris-HCL – Tris hydrochloride

Tyr - Tyrosine

U – uracil

UTR – Untranslated region

V – volt

VCAM-1 – vascular cell adhesion molecule-1

VNTR – variable number tandem repeats

VO_2 – Volume of oxygen

VO_2max – Volume of oxygen at maximal effort

VO_2R – VO_2Reserve

W - Watt

WBC – white blood cell

Wmax – Watt max

α - alpha

β – beta

Δ – delta

μ - micro

Chapter 1: Introduction

Within our genetic makeup there is a subset of sequences that can randomly move about the genome called transposable elements. These sequences are broken down into retrotransposons and DNA transposons. DNA transposons are suggested to play a role in human evolution but have not been active in the human genome for over 50 million years [1]. Retrotransposons however, are still active within the human genome with the major constituent being Long Interspersed Nuclear Element-1 (L1) [2]. The human genome is estimated to contain over 500,000 L1 copies, and these repeats comprise ~17% of the human genome [3]. L1 is ~6.0 kilobases (kb) in length, contains a 5' untranslated region (UTR) with an internal promoter, contains two open reading frames that encode for an ORF1 protein and an OFR2 protein, and a 3' UTR containing a polyadenylation (polyA) signal and tail [4]. The ORF1 protein is ~40 kDa, and acts to form trimeric complexes with other ORF1 proteins to exhibit chaperone activity [5, 6] The ORF2 protein contains endonuclease and reverse transcriptase activity [7]. The retrotransposition process is termed target-site primed reverse transcription (TPRT) [8]. Following L1 transcription via RNA polymerase II in the nucleus, the biscistronic L1 mRNA is exported to the cytoplasm and will associate with ribosomes. Translated ORF1 and ORF2 proteins show strong cis-preference to bind to their original mRNA intermediate [9]. The resultant molecule, or ribonucleoprotein particle (RNP), enters the nucleus and migrates randomly throughout the nucleus. The endonuclease activity of the ORF2 protein will cleave DNA [10], and the reverse transcriptase activity of the ORF2 protein catalyzes the integration and insertion of a new L1 copy into the genome [11]. Although integration

of new L1 sequences appears to be random, insertion within protein-coding sequences can alter protein structure and function.

Kazazian et al. have reported that over 120 different retrotransposition events are implicated with disease [12, 13]. Regulation of L1 retrotransposition becomes increasingly important in this regard. Epigenetic alterations, specifically L1 promoter methylation, can silence L1 before transcription occurs [14]. In this regard, Baccarelli et al. determined L1 hypomethylation of blood DNA was associated with heart disease and stroke [15]. Furthermore, cardiac ischemia in rats upregulates L1 transcription, and when L1 transcripts were silenced by antisense oligonucleotides targeting the L1 transcript infarct size was significantly lower compared to controls receiving a scrambled oligonucleotide [16]. L1 hypomethylation appears to be related to aging [17, 18] as well as various cancers [19]. L1 transcripts have also been shown to be upregulated in synovial fluid samples obtained from patients suffering from rheumatoid arthritis [20]. These lines of evidence suggest L1 hypomethylation, as well as integration, are detrimental to health.

Several studies have attempted to gain perspective on environmental factors that might affect L1 DNA methylation status and mRNA levels. For instance, women who possessed lower L1 methylation were more likely to have unfavorable body composition [21]. Marques-Rocha et al. observed similar findings in that favorable body composition metrics were associated with higher L1 methylation [22]. To this end, obesity may alter L1 DNA methylation as well as L1 transcript levels across numerous tissues.

While no human muscle aging investigations examining L1 markers exist, De Cecco et al. [17] demonstrated skeletal muscle L1 mRNA expression was 4-fold greater in 36-month old mice versus 5-month old mice. Likewise, skeletal muscle L1 DNA content was ~80% greater in 36-

month old mice versus 5-month old mice. L1 DNA hypomethylation, as well as increased L1 mRNA transcription, in skeletal muscle may be a consequence of aging or physical inactivity.

Physical activity and exercise improve body composition and, importantly, overall health [23-25]. An acute bout of endurance exercise has been shown to alter methylation status in skeletal muscle [26], and chronic general exercise is associated with higher L1 methylation percentage [22]. Resistance exercise training has been shown to reverse the transcriptomic profile associated with aging in skeletal muscle [27]. Our laboratory has shown that acute bouts of resistance exercise, and chronic resistance exercise, increase L1 methylation with an accompanied reduction in L1 mRNA transcripts in human skeletal muscle [28]. Given the metabolic demand differences and cellular signaling disparities between modes of activity, it is possible endurance exercise would promote an alternative response to L1 methylation and transcript expression. The collective evidence above suggests an acute bout of endurance exercise may increase L1 methylation and decrease L1 mRNA expression in both younger and older humans. Moreover, a chronic engagement in higher levels of physical activity, regardless of age, may accomplish a similar molecular phenotype. Therefore, the purpose of this study is to determine baseline differences in skeletal muscle L1 DNA, RNA, and L1 DNA methylation status between young and old humans and determine if an acute bout of endurance exercise alters these markers. A secondary purpose is to determine if physical activity levels are associated with these markers.

Chapter 2: Literature Review

Since the discovery of deoxyribonucleic acid (DNA) in 1953, cellular and molecular biology research has increased drastically [29]. Following the discovery, Francis Crick proposed the "Central Dogma of Molecular Biology" whereby there are "general transfers": DNA to DNA, DNA to ribonucleic acid (RNA), and RNA to protein, and "special transfers": RNA to RNA, RNA to DNA, and, under special circumstances [30, 31], DNA to protein [32]. Prior to characterization of DNA, Barbara McClintock was characterizing the unique machinery, or "special transfer" related to transposable elements (TEs) [33].

Transposable elements are unique pieces of DNA that can move about the genome. Early experiments by Emerson [34] and Rhoades [35, 36] gave way to future experiments by McClintock who observed a dissociation locus (*Ds*) on chromosome 9 in maize that could move about the chromosome. Later, she discovered *Ds* responded to an activator locus (*Ac*), which could also initiate its own movement about the chromosome. When activated by *Ac*, *Ds* (and *Ac*) could transpose within a chromosome or to another chromosome [33]. It was not until 1983 that *Ac* and *Ds* were cloned and isolated to show their structural similarity but key differences [37-39].

Much research has been conducted since to elucidate transposable elements. Transposable elements can be separated into two major classes: retrotransposons (Class I) and DNA transposons (Class II). DNA transposons comprise ~3% of the human genome; however, are not currently active within the genome. Lander et al. suggested there was no evidence for DNA transposition in humans over the past 50 million years [1]. More recently, Pace and Feschotte [40] suggested at least 40 human DNA transposon families have been active in the primate lineage, but found no

evidence of any elements younger than ~37 million years old. DNA transposons can extract themselves from the genome and insert randomly into another portion of the genome – i.e., a cut-and-paste mechanism [41]. Due to DNA transposons not being active within the human genome, this literature review will focus mainly on retrotransposons. Retrotransposons are similar to DNA transposons; however, utilize a copy-and-paste mechanism as opposed to a cut-and-paste mechanism. Retrotransposons utilize an RNA intermediate which is inserted through reverse transcription.

Retrotransposons consist of two main classes: autonomous and nonautonomous. Autonomous retrotransposons consist of long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. Nonautonomous retrotransposons include Short Interspersed Nuclear Elements (SINEs), SINE-variable number tandem repeats (VNTR) -Alu elements (SVA), and processed pseudogenes [42]. Autonomous retrotransposons include endogenous retroviruses (ERVs) and Long Interspersed Nuclear Elements (LINEs). Of these, few are known to be important for human biology: LINEs, SINEs, and SVAs [43]. LINEs are autonomous in that their specific gene is transcribed and translated similar to normal central dogma concepts. Once the proteins are translated, they will bind to the RNA intermediate, translocate back into the nucleus, and then integrate randomly into the genome. SINEs and SVAs lack the protein coding sequences to create the proteins necessary to translocate back into the genome. As a result, SINEs and SVAs utilize the LINE-encoded ORF1 and ORF2 proteins to achieve translocation and retrotransposition back into the genome. This concept denotes the difference in autonomy of retrotransposition but also the importance of LINEs.

LINEs consist of LINE1 (L1) and LINE2 (L2); however, LINE2 is inactive within the human genome [1, 44, 45]. There are over 500,000 L1 copies in the human genome, ~17% of the

genetic code. The L1 gene is ~6.0 kb in length, contains an ~1000 bp 5' untranslated region (UTR) with internal promoter activity, two open reading frames (ORF) that encode for ORF1 protein and ORF2 protein, and an ~200 bp 3' UTR containing a polyadenylation (polyA) signal and tail [4, 46, 47]. The 5' UTR contains an internal RNA polymerase II promoter that promotes transcription of L1 [48]. A Yin Yang 1 (YY1)-binding site on the 5' UTR is crucial for initiating transcription [49]. Additionally, the 5' UTR contains binding sites for certain transcription factors including Runx3, Spl, and SRY-related proteins [3]. ORF1 protein (ORF1p) is an ~40 kDa RNA-binding protein [50, 51] that forms trimeric complexes and possesses chaperone-activity to translocate the RNA intermediate into the nucleus [5, 52]. ORF1p contains three structural domains: a coiled-coil domain that modulates trimerization, an RNA recognition motif domain, and a C-terminal domain [53]. When mutations occur in the RNA recognition motif or C-terminal domain, L1 retrotransposition is adversely affected [54]. The ORF2 protein (ORF2p) is ~150 kDa and contains an endonuclease (EN) domain [7], a reverse transcriptase (RT) domain [55], and a C-terminal domain. ORF2p is critical for retrotransposition as it mediates the nicking of DNA and reverse transcription back into the genome. Not much is known regarding the importance of the 3' UTR; however, it contains a polypurine tract that is not required for retrotransposition but can inhibit L1 RT activity [56, 57]. Interestingly, there is evidence in humans that the 3' UTR contains promoter activity that generates alternative L1 transcripts [58].

The process of the L1 gene being transcribed, translated, and retrotransposed into the genome is a process referred to as target-site primed reverse transcription (TPRT) [8]. First, the internal promoter within the 5' UTR promotes autonomous transcription [48]. Following transcription, a biscistronic L1 messenger RNA (mRNA) is exported into the cytoplasm. The L1 RNA will then associate with ribosomes and will be translated to create ORF1p and ORF2p which

show a strong cis-preference to bind to their original mRNA intermediate [9]. Once the ORF1p and ORF2p bind to the original mRNA, the resultant molecule is termed a ribonucleoprotein particle (RNP) [59, 60]. It has been demonstrated that ORF1p is required for RNP formation which is a necessary step for L1 retrotransposition [61]. Additionally, when mutations alter the C-domain of ORF2p, there is a strong inhibition of L1 retrotransposition [60]. Nonetheless, when the RNP is formed, there is a L1 mRNA, several OFR1p trimers, and at least one, if not more, ORF2ps that are bound to the complex [62]. Furthermore, it has been proposed that several proteins and RNAs may be attached to the RNP, but their function is unknown [63].

Once the RNP has formed it will enter the nucleus. The mechanism behind entrance into the nucleus is not fully understood; however, it has been suggested cell division may assist the process [64, 65]. Notwithstanding, when the RNP migrates into the nucleus, the endonuclease associated with the ORF2p will cleave DNA at random. Cleaving typically occurs between T and A at 5'-TTTTAA-3' consensus sites [10]. Upon cleavage, the liberated 3' hydroxyl is then used to prime reverse transcription of the L1 mRNA by the reverse transcriptase activity of the ORF2p [7, 11]. As one strand is being reverse transcribed, the second strand will be cleaved and reverse transcribed. Mechanisms behind second strand cleavage remain poorly understood. Once reverse transcription occurs, full integration is achieved and the L1 mRNA is now inserted into a new locus.

The location of retrotransposition becomes of utmost importance in determining if cellular function may be altered. Hypothetically, if a new L1 sequence is inserted within an intronic sequence or a non-coding region of the genome then there may be no adverse effects. However, if the insertion of L1 sequences initiates splicing mechanisms, insertional mutagenesis, or deletions genes may become inactive, stimulated, or frame-shifts occur potentially leading to disease.

Kazazian et al. first noted a retrotransposon insertion in the Factor VIII gene of an individual possessing Hemophilia A [12]. It is now understood that over 120 different retrotransposition events are implicated in disease [13]. Some of the diseases implicated with retrotransposition, listed as "disease (gene affected)", include: Breast Cancer (BRCA2), Neurofibromatosis Type 1 (NF1), Duchenne's muscular dystrophy (DMD), PDHc deficiency (PDHX), endometrial carcinoma (PTEN), and Cystic Fibrosis (CFTR) [13]. Given retrotransposition has been implicated in such diseases, regulation of transposable elements becomes increasingly important.

A prominent means of regulating L1 transcription is through DNA methylation by DNA methlytranferases (DNMTs). Methylation of CpG sequences in the 5' UTR of the L1 gene is associated with inhibiting L1 expression [66]. Methylation involves the addition of methyl groups to cytosine bases to form 5-methylcytosine; typically occurring in genomic regions termed CpG sequences or islands [67]. DNA methylation is carried out by the enzymes DNMT3a and DNMT3b [68]. Interestingly, DNMT3-L (similar to DNMT3a; albeit, expressed in germline cells) knockout mice exhibit meiotic catastrophe and overexpression of L1 and TEs suggesting the importance of DNA methylation in silencing L1 [69]. Histone deacetylases (HDAC) act similarly by removing acetyl groups from histone proteins, making DNA less accessible. Likewise, HDAC1 and HDAC2 play a role in silencing L1 expression where the L1 promoter shows increased acetylation (more open DNA) in the absence of HDAC1 and HDAC2 [70].

Another novel mechanism for TE regulation involves the p-element induced wimpy testis, or PIWI, proteins. PIWI proteins are found in mice (i.e. MIWI) or human (i.e. HIWI) [71]. PIWI proteins interact with piRNAs (PIWI-interacting RNAs) which are thought not to arise from double-stranded DNA but from existing cleaved transcripts; however, this mechanism is not fully elucidated. Once associated, the PIWI protein and piRNA sequence will find a complementary

target (e.g. an L1 mRNA) and cleave the transcript. Interestingly, cleavage not only inactivates the target but creates a transcript sequence that will then associate with Argonaute 3 (AGO3), another protein with cleaving capabilities. Once AGO3 associates with this transcript, AGO3 can then cleave transcripts to reproduce the original piRNA. This amplification mechanism, referred to as Ping-Pong Amplification, provides a novel and self-propagating mechanism to defend against TEs [72]. Likewise, studies involving MILI and MIWI2, mouse PIWI-like elements, deficient mice show transposon activation and severe defects to germ cell development [73, 74]. Of note relevant to humans, HIWI proteins are expressed primarily in the testis while the AGO family is ubiquitously expressed across tissues [75].

As noted before, L1 is implicated with several diseases due to deletions and insertions. More recently, associations have been made between L1, environmental factors, behaviors, and clinical diseases. Baccarelli et al. determined from the Boston-are Normative Aging Study that L1 DNA hypomethylation of blood DNA was associated with heart disease and stroke [15]. Through longitudinal analyses (3-5 year follow-up) it was determined that patients with lower L1 methylation were at greater risk for ischemic heart disease, stroke, and mortality [15]. Turunen et al. corroborated these findings by demonstrating L1 DNA hypomethylation was associated with atherosclerosis [76]. L1 methylation appears to be inversely related to vascular cell adhesion molecule-1 (VCAM-1), a vascular inflammatory marker [77]. Of note, the inverse relationship between VCAM-1 and hypomethylation of L1 was only in those free of heart disease or stroke, not those who possessed heart disease or had a stroke [77]. Somewhat contradictory to the prior studies mentioned, Zaina et al. analyzed atherosclerotic and nonatherosclerotic tissue from post-mortem human aorta and found hypomethylation in some LINEs, hypermethylation in Alu elements, and normal methylation of L1 [78]. The most convincing evidence implicating the

involvement of L1 in heart disease is evidenced through use of the ischemia-reperfusion technique in rodents. Lucchinetti et al. injected oligonucleotides into rat hearts to cleave L1 transcripts. The hearts were treated with antisense and scrambled oligonucleotides for 60 min, exposed to ischemia for 40 min, and then reperfused for 60 min. ORF1 and ORF2 transcript levels were significantly lower when treated with the antisense oligonucleotide which cleaved L1 transcripts. Furthermore, infarct size of the hearts was significantly less (~25%) when treated with the antisense oligonucleotide compared to the scrambled oligonucleotide [16]. Clearly, there is a role for L1 and its implication for cardiovascular disease; however, precise associations and mechanisms need to be further elucidated.

A common conspirator to cardiovascular disease is aging. Indeed, when mice were compared at 5, 24, and 36 months old, L1 mRNA expression was higher in liver of 36 month old mice relative to other groups, and higher in skeletal muscle of 24 and 36 month old mice compared to 5 month old mice. Furthermore, L1 DNA copy number was greater in 36 month old mice in both liver and skeletal muscle. When calorie restriction was enforced however, L1 mRNA levels were lower in older mice compared to counterparts that were fed *ad libitum* [17]. This same group also demonstrated that when human diploid fibroblasts become senescent, there is a smoothing of chromatin profiles; meaning chromatin is more closed in promoter and enhancer regions of active genes and more open in genomic regions harboring retrotransposons [79]. This concept has also been demonstrated in primate skeletal muscle [80]. Others have also reported heterochromatin rearrangements in senescent cells which can alter mRNA signatures compared to younger cells [81, 82]. In conjunction with, or perhaps a result of, chromatin changes associated with aging, methylation status alters with aging as well. Fraga et al. utilized monozygous twins to address epigenetic differences over a lifetime [83]. Since monozygous twins share a common genotype,

epigenetic differences could explain differences in twins with aging. These authors found younger twins are nearly identical in their epigenetic makeup; however, with aging the twins become vastly different both in overall methylation as well as distribution of methylation [83]. Furthermore, others have also noted global DNA methylation, and Alu methylation, is lower in centenarians compared to young controls [84]. In another study, 32 healthy male volunteers' white blood cells (WBCs) were analyzed for L1 methylation. These authors reported L1 hypomethylation increased with aging but did not find a relation between L1 methylation status and smoking or drinking [18]. Due to the certainty of epigenetic changes occurring with aging, some have suggested the 'epigenetic clock' whereby certain methylation changes can predict one's age. Garagnani et al. determined methylation in the promoter of 3 genes shows a significant correlation with age [85]. Weidner et al. reported similar findings in aged blood in that only 3 CpG island methylation sites can be used to determine age [86].

While aging is an inevitable facet of life, overall health and quality of life can be manipulated through several lifestyle factors. For instance, smoking has been associated with hypomethylation [87, 88]. Tajuddin et al found a significant B-weight coefficient of -0.7 and -0.6 in smokers of blond tobacco and in smokers of blond and black tobacco, respectively, compared to nonsmokers [89]. *In vitro* models demonstrate similar findings in that human bronchial epithelial cells (HBEC) cultured with cigarette smoke condensate showed hypomethylation of L1 sequences compared to controls [90]. Lastly, when WBCs were analyzed in a cohort of 107 participants, participants were categorized either above or below 83.03% L1 methylation, and it was determined a higher percentage of current smokers fell in the lower L1 methylation cohort. In conjunction, smokers who fell below 83.03% L1 methylation also smoked significantly more cigarettes per day (3.6 ± 4.8 versus 0.9 ± 1.7) [22].

Body mass index (BMI) (weight in kg divided by height in m^2 (kg/m^2)) has been used as an indicator of overall health. Several investigators have attempted to determine the relationship between BMI and L1 expression and methylation. Piyathilake et al. found that when peripheral blood mononuclear cells (PBMC) were analyzed in 470 women for L1 methylation, those who contained lower L1 methylation were more likely to have a BMI higher than 25 kg/m^2, body fat percentage greater than 33%, and waist circumference above 88 cm [21]. On the contrary, Perng et al. found a positive linear relationship between BMI and L1 methylation. It should be noted nearly 1000 people participated in this study, potentially explaining why the 0.30-0.35% higher L1 methylation in those who are obese (>40.0 kg/m^2) compared to those with normal BMI (<25 kg/m^2) was statistically significant [91]; however, this difference may not be biologically significant. Several researchers have determined BMI is not associated with L1 methylation [22, 92, 93]. Interestingly, lower body fat percentage, lower sum of skinfold thickness, and higher fat-free mass were all independently associated with higher L1 methylation in white blood cells [22] suggesting body composition may be more meaningful than BMI with regard to L1 methylation status.

Exercise and physical activity alters DNA methylation status in skeletal muscle [26] and other tissues. Indeed, McCullough et al. demonstrated methylation was related to total non-sedentary time at one genomic locus [94]. Similarly, Marques-Rocha et al. categorized participants above or below median L1 methylation percentage, and found a greater percentage of participants practicing sport were above the median compared to below the median [22]. On the contrary, Zhang et al. demonstrated physical activity is not associated with L1 methylation [95]. Romero et al. provide the most comprehensive L1 and exercise study to date showing resistance exercise alters L1 markers in human skeletal muscle [28]. The authors performed two separate studies to

determine acute (3 days of 10 sets by 5 repetitions at 80% of one-repetition maximum (1-RM)) and chronic (resistance exercise 3 days per week for 12 weeks; total body workout, various set by repetition scheme) effects of resistance exercise on humans. During the acute study, skeletal muscle biopsies were extracted from the vastus lateralis prior to exercise, 2 h following the first exercise session, and 72 h following the last exercise session. During the chronic study, skeletal muscle biopsies were obtained prior to the twelve-week training endeavor and 72 h following the last training session. The acute study indicated L1 mRNA was significantly lower 2 h following the first bout and 72 h following the last exercise session compared to resting conditions. The chronic study indicated a trend for lower L1 mRNA following 12 weeks of resistance training; however, L1 promoter methylation was significantly higher following exercise training. The authors also found a negative relationship between the change in satellite cell number with training and the change in reverse transcriptase (RT) activity whereby lower RT activity correlated with increasing satellite cells [28]. Importantly, this is the first study to show markers of L1 in human skeletal muscle improving with exercise.

Lack of exercise, or physical inactivity, is severely deleterious as it contributes to disease [96], sarcopenia [97], metabolic disease [98], and all-cause mortality [99]. To this end, exercise improves many of these deleterious effects [23, 24, 100]. Resistance exercise is traditionally associated with improving or maintaining muscle mass and activities of daily living, while aerobic exercise is associated with improving cardiovascular outcomes [25]. Molecular signaling specific to the exercise modalities dictates the differing phenotypic responses [101-104]. Given the disparities in molecular signaling between modalities one could hypothesize resistance training would affect L1 expression and associated markers differently than aerobic exercise. As a result, the purpose of this project is to determine differences in L1 DNA expression, L1 RNA expression,

ORF1p expression, and L1 methylation status prior to and in response to endurance exercise in young and old human skeletal muscle. Furthermore, we will examine physical activity levels via accelerometry to draw relationships between L1 markers and physical activity. Lastly, we will measure indices of body composition to draw relationships between L1 associated markers and body composition.

Specific Aims:

1) Determine if baseline differences exist in skeletal muscle L1 DNA expression, methylation status, L1 RNA expression, and ORF1p expression between young and old humans.
2) Determine if methylation status, L1 RNA expression, and ORF1p expression in human skeletal muscle are altered following an acute bout of endurance exercise.
3) Determine if the magnitude of the response to endurance exercise in these markers differs between young and old humans.
4) Determine if there is a relationship between physical activity levels and skeletal muscle L1 DNA expression, methylation status, L1 RNA expression, and ORF1p expression.
5) Determine if there is a relationship between body composition and skeletal muscle L1 DNA expression, methylation status, L1 RNA expression, and ORF1p expression.

Hypotheses:

1) Skeletal muscle from older subjects will express more L1 DNA, exhibit more L1 hypomethylation, express more L1 RNA, and express more ORF1p.
2) L1 DNA methylation status will increase, L1 RNA expression will decrease, and ORF1p expression will decrease in human skeletal muscle following endurance exercise.

3) Skeletal muscle from younger humans will exhibit a more robust response in L1 DNA methylation status, L1 RNA expression, and ORF1p expression compared to old human skeletal muscle following an acute endurance exercise bout.
4) Higher daily physical activity/exercise levels will be associated with lower L1 DNA expression, increased L1 DNA methylation, lower L1 RNA expression, and less ORF1p expression.
5) Lower body fat percentage and higher muscle mass percentage will relate to lower L1 DNA expression, increased L1 DNA methylation, lower L1 RNA expression, and less ORF1p expression.

Chapter 3: Methods

Participants

This study was approved by the Auburn University Institutional Review Board (IRB) and was in compliance with the Helsinki Declaration (IRB protocol #: 18-226 AR 1806). Participants read and signed an informed consent form prior to participating in the study. Inclusion criteria were as follows: a) participants could be male or females between the ages of 18-30 and 50-80 years old, b) be able to cycle for at least 1 h, c) participants had to be apparently healthy and free of any known disease determined by a medical history questionnaire, and d) participants had to abstain from sport or dietary supplements for 1 month prior to participating. A physical activity questionnaire and medical history questionnaire were filled out prior to participation to establish physical activity participation, health, and to identify potential risk factors that could be aggravated by training.

Experimental Design

Participants were initially screened using a medical history questionnaire and physical activity questionnaire. Thereafter participants were split into two groups based on age: "young" (18-30 years old), and "old" (50-80 years old) and given an Actigraph GT3X accelerometer (Actigraph, Pensacola, FL, USA) to monitor physical activity throughout participation in the study. Following 1-2 days, right leg maximum torque was assessed to determine muscle function. During the same session, VO_2max testing occurred on a cycle ergometer to determine each participant's maximum aerobic power and heart rate. Following at least 7 days, but no more than 14 days, the exercise trial began which consisted of: body composition testing, ultrasonography, a resting

biopsy and blood draw (PRE), cycling for 1 h at 70% VO_2max, an immediate post-exercise blood draw (POST), a 2 h post-exercise muscle biopsy and blood draw (2 h), and an 8 h post-exercise muscle biopsy and blood draw (8 h).

Medical History Questionnaire

Participants recruited were apparently healthy and free of medication with the exception of certain hypertensive medications due to difficulty recruiting medication-free older adults. The effect of antihypertensive medications on our measured variables is unknown and is a limitation to the study design.

Muscle Function and VO_2max Testing

Participants first performed maximal isokinetic right leg extensions on an isokinetic dynamometer (System 4 Pro, BioDex Medical Systems, Shirley, NY, USA) at 30° and 120° · sec^{-1}. Participants warmed up prior to exercise by completing 1 set of 4 repetitions at 30° · sec^{-1} at 80% max effort. Participants then performed 2 sets of 4 repetitions for each velocity. The first two repetitions were warm-up while the following 2 repetitions were maximal effort. Sets were separated by 60 sec of rest. The highest value for each velocity was determined as maximal torque.

Following muscle function testing, VO_2max testing was performed to determine maximal aerobic power. Maximal aerobic power (VO_2max) and peak power (Wmax) testing were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Inc., Seattle, WA, USA). Subjects performed a self-selected warm-up for five minutes and began the test at an intensity corresponding to what the subject perceived as comfortable for a 60 min cycling session. Intensity was increased by 30 W every 2 min until voluntary termination or until cadence dropped below 50 RPM. Participants then rested 10 min and performed a validation stage thereafter. The validation stage consisted of participants cycling to exhaustion at the wattage at which they ceased

the continuous test. Using indirect calorimetry (Parvomedics Inc., Sandy, UT, USA), expired air was continuously analyzed for oxygen uptake (VO_2) and respiratory exchange ratio (RER). Values were averaged in 30 sec intervals. Heart rate (HR) was measured using a Polar heart rate monitor (Lake Success, NY, USA). Subjects indicated rating of perceived exertion (RPE) by pointing to a Borg RPE scale (6-20) at the end of each stage. A test was accepted if heart rate was within 10% of age-predicted max HR, RPE $\geq$ 17, RER $\geq$ 1.10, and a plateau in VO_2 occurred (<250 ml increase in VO_2 from the end of the original test, and over the validation stage).

Standardized Meals Prior to the Exercise Trial

As mentioned previously, the exercise trial occurred 7-14 days following muscle function and VO_2max testing. To control for differences in dietary intakes, participants consumed standardized meals 24 h prior to their exercise trial. Standardized meals were based on basal metabolic rate determined by the Harris-Benedict Equation. Each participant's basal metabolic rate was determined and then multiplied by 1.5 to ensure participants were adequately nourished given caloric restriction can alter L1 expression in skeletal muscle [17].

Standardized meals were provided in the form of commercially-prepared sandwiches (Uncrustables™, Smucker's, Orrville, OH, USA). Based on basal metabolic rate, sandwiches were distributed to meet caloric demands. For example, if basal metabolic rate was 2100 kcal and a sandwich provided 210 kcal then 10 sandwiches were provided and instructed to be eaten over breakfast, lunch, and dinner beginning 24 h prior to the exercise trial. Each sandwich consumed provided on average 210 kcal, 9 g of total fat, 28 g of carbohydrates and 2 g of fiber, and 6 g of protein (Peanut Butter and Grape or Peanut Butter and Strawberry). Thus, 10 sandwiches would provide 2100 kcal, 90 g of total fat (39% of total kcal), 280 g of carbohydrates (50% of total kcal), and 60 g of protein (11% of total kcal).

Exercise Trial

Participants reported for the exercise trial between 0500-0800, 8 h fasted, not having consumed alcohol in 48 h, exercised in 72 h, or ingested caffeine within 8 h. Testing began with assessment of hydration status measured through urine testing by an ATAGO 2392 handheld refractometer (Bellvue, WA, USA). Participants moved forward with testing only if urine specific gravity level was less than 1.030 g/ml.

Following hydration assessment, muscle thickness was determined by ultrasound using a 3-12 MHz multi-frequency linear phase array transducer (Logiq S7 R2 Expert; General Electric, Fairfield, CT, USA). Briefly, the ultrasound probe was positioned horizontally on the vastus lateralis, halfway between the iliac crest and patella, where an image was captured. Another image was captured directly over the rectus femoris to determine midthigh thickness which was measured from the femur the inner-border of the skin. More detailed methods have been previously published by our laboratory [105].

Bioelectrical impedance spectroscopy was utilized to determine total body lean mass and total body fat mass (Imp SFB7, Impedimed, Pinkemba Queensland, Australia). Briefly, electrodes were set up according to manufacturer instructions whereby electrodes were placed on the right hand and right foot. Two electrodes were placed ~5 cm apart on the hand and on the foot. The ulnar styloid process was used as a middle point and electrodes were place 2.5 cm above and below this bony landmark. Similarly, the lateral malleolus was used as a starting point for one electrode while the accompanying electrode was placed 5 cm distal. Analysis was conducted twice and averaged. Further details regarding validity have been previously published by our laboratory [106].

Participants were instructed to lie supine on a treatment table in preparation for venipuncture and a skeletal muscle biopsy. Following 5 min of supine rest, venous blood samples were obtained from an antecubital vein. Blood samples were collected into a serum separating tube and a CPT tube with sodium citrate (BD Vacutainer; Franklin Lakes, NJ, USA). Serum and CPT tubes were spun at 1800 g for 30 min. Following venipuncture, the vastus lateralis was prepared for a skeletal muscle biopsy. Approximately halfway between the iliac crest and the patella, a 3x3 inch square was shaved, cleaned with alcohol, and cleansed with betadine. 1.0 mL of Lidocaine was injected to provide local anesthesia and given 5 min to enact effects. Using aseptic technique, a 1 cm incision was made into the skin and muscle fascia. Using a 5 gauge Bergstrom biopsy needle with suction, tissue was extracted from the vastus lateralis. Tissue was blotted of blood and any connective tissue was immediately removed. Tissue distribution for future analyses and analysis procedure is later described.

Following the skeletal muscle biopsy, participants were fitted with a heart rate monitor (Polar; Lake Success, NY, USA), mounted an electronically-braked cycle ergometer (Velotron, RacerMate, Inc., Seattle, WA, USA), and performed a self-selected pace 5 min warm up. Wattage was then manipulated to achieve 70% VO_2Reserve which was measured by corresponding HRReserve determined by VO_2max testing, and cycled for 60 min. Heart rate was continuously monitored throughout the cycling bout to ensure appropriate exercise intensity. Water was administered *ad libitum* throughout the trial.

Immediately following the 60 min cycling trial, venipuncture was performed as described prior. Given participants had not consumed calories since the day prior, a post-exercise recovery beverage (~30 g of carbohydrate and ~30 g of protein) was provided. Participants were monitored in the laboratory until 2 h following the cycling trial when a 2nd muscle biopsy and 3rd venipuncture

was performed. Participants were then instructed to consume standardized meals within 1 h which consisted of 25% of their basal metabolic rate in the form of commercially-prepared sandwiches (Uncrustables™, Smucker's, Orrville, OH, USA), and could leave the facility. Participants were instructed to consume no other calories aside from standardized meals. 8 h following the cycling trial, a 3rd muscle biopsy and 4th venipuncture were performed.

Skeletal Muscle Processing

Muscle tissue was rapidly removed from the needles, teased away from blood, connective tissue, and fat, wrapped in pre-labelled foils, and flash-frozen in liquid nitrogen. At the end of the day, tissue was stored at -80°C until further processing. For protein analysis, ~20 mg of frozen tissue was placed in 1.7 mL microcentrifuge tubes containing 500 µL of ice-cold cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton; Cell Signaling, Danvers, MA, USA] pre-stocked with protease and Tyr/Ser/Thr phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin). Samples were then homogenized by hand via micropestle manipulation, insoluble proteins were removed with centrifugation at 500 *g* for 5 min, and obtained sample lysates were stored at -80ºC prior to Western blotting.

~30 mg of frozen tissue was used for each total DNA and total RNA isolation. RNA-free DNA was prepared using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's instructions (Qiagen, Germantown, MD, USA). DNA-free RNA was prepared using the Quigen RNeasy Fibrous Tissue Kit and following manufacturer's instructions (Qiagen, Germantown, MD, USA). Sample concentrations were then quantified using a spectrophotometer (Nanodrop Lite; Thermo Scientific, Waltham, MA, USA) and subsequently frozen at -80ºC until later analysis.

Polymerase Chain Reaction

L1 DNA and mRNA expression were measured via real-time polymerase chain reaction (PCR). Following DNA quantification, samples were concentrated to one microgram of RNA-free DNA and subsequent PCR occurred. Following RNA quantification, DNA-free RNA was concentrated to 100 nanograms and was reverse transcribed to cDNA using a commercially available kit (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's recommendations. For DNA and mRNA expression analysis, real-time PCR was performed using gene-specific primers and SYBR green chemistry (Quanta Biosciences). Primer sequences used were as follows: L1 (ORF1p coding region) forward primer 5-TAAGGGCAGCCAGAGAGAAA-3, reverse primer 5-GCCTGGTGGTGACAAAATCT-3; B2M forward primer 5=-ATGAGTATGCCTGCCGTGTGA-3=, reverse primer 5=-GGCATCTTTCAAACCTCCATG-3=; Cyclophilin forward primer 5- CGATGTCTCAGAGCACGAAA-3, reverse primer 5-CCCACCTGTTTCTTCGACAT-3; PGC-1α forward primer 5-CAAGCCAAACCAACAACTTTATCTCT-3, reverse primer 5-CACACTTAAGGTGCGTTCAATAGTC-3; and GAPDH forward primer 5-AACCTGCCAAATATGATGAC-3, reverse primer 5- TCATACCAGGAAATGAGCTT-3.
To calculate the ΔCT value, the geometric mean CT of B2M, GAPDH, and Cyclophillin minus gene of interest CT was calculated. $2^{\Delta CT}$ values were then calculated. $2^{\Delta CT}$ values were normalized to the group mean of young PRE.

L1 Promoter Methylation

L1 promoter methylation analyses were performed from isolated DNA (described above) using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (Abcam, Cambridge, MA, USA) and L1 5'-UTR primers which spanned a CpG-rich region (positions 29 – 485 at the 5'-end). Primer sequences were as follows: forward primer, 5-

GAACAGCTCCGGTCTACAGC-3, reverse primer, 5- CCGGCTGCTTTGTTTACCTA-3. Prior to MeDIP being performed, 1.5 g of DNA was digested using MseI (New England BioLabs, Ipswich, MA). Thereafter, total methylated DNA from a total of 1 g input was immunoprecipitated using an anti-5-methylcytosine antibody provided within the kit. Additionally, 500 ng of residual input DNA from each sample was used as a control to normalize real-time PCR results. Promoter methylation expression values ($2^{\Delta CT}$ values) were calculated whereby ΔCT = input DNA CT – methylated DNA CT. $2^{\Delta CT}$ values were normalized to the group mean of young PRE.

Western Blotting

Whole-tissue sample lysates obtained through cell lysis buffer processing were batch process-assayed for total protein content using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Lysates were then prepared for Western blotting using 4x Laemmli buffer at 1 µg/µL. Following sample preparation, 25 µL samples were loaded onto 4-15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis (150 V for 75 min) using pre-made 1x SDS-PAGE running buffer (Ameresco, Solon, OH, USA). Proteins were then transferred (200 mA for 2 hours) to polyvinylidene difluoride membranes (Bio-Rad), Ponceau stained and imaged to ensure equal protein loading between lanes. Membranes were then blocked for 1 h at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST; Ameresco). Mouse anti-ORF1p (1:1,000; Abcam, catalog no. ab76726), was incubated with membranes overnight at 4°C in TBST with 5% bovine serum albumin (BSA). The following day, membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2,000, Cell Signaling, catalog no. 7074) in TBST with 5% BSA at room temperature for 1 h. Membrane development was performed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA, USA), and band densitometry was

performed using a gel documentation system and associated densitometry software (UVP, Upland, CA, USA). All densitometry values for proteins of interest were normalized to the ponceau densitometry values. Each participant's PRE, 2 h and 8 h samples were normalized to the group mean of young PRE for each protein target.

Accelerometry

Actigraph GT3X triaxial accelerometers (Actigraph) were worn on the right hip and attached with an elastic strap. Participants were asked to wear the device as often as possible with the exception of sleeping and water-based activities. To this end, participants filled out an on-off log, and were instructed to record an off time if the accelerometer was taken off for more than 5 min. Accelerometer data leading into the exercise trial was ignored due to physical activity restrictions. Scoring was conducted using the following algorithms: Energy Expenditure – Freedson Adult (1998), Metabolic equivalents (METs) – Freedson Adult (1998), and Cut Points and Moderate to vigorous physical activity (MVPA) – Freedson Adult (1998) [107].

Statistics

Shapiro-Wilk testing was used to determine normality for all dependent variables. When appropriate, Levene's test for equality of variances was determined. Baseline differences between age groups were determined using independent samples t-tests in select variables. Changes in dependent variables over time were tested using a repeated-measures analysis of variance (ANOVA) where within-subject factors included PRE, 2h, and 8h, and the between-subject factor was age. If majority of the groups within the repeated-measures ANOVA were normally distributed, then the test was carried out due to the robustness of an ANOVA. Lastly, Pearson correlations were performed between select variables.

Chapter 4: Completed Manuscript

Skeletal muscle LINE1 mRNA expression is higher in older humans but decreases with endurance exercise and physical activity

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Short title: Human skeletal muscle L1, aging, and endurance exercise

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INTRODUCTION: LINE1 (L1) is a retrotransposon which has the ability to copy and paste itself into the genome. L1 has been implicated with disease and aging. We sought to determine if skeletal muscle L1 markers in humans are affected by aging and cycling exercise. **METHODS**: 15 younger (23±3 y/o) and 15 older participants (58±8 y/o) donated a skeletal muscle biopsy (PRE) directly prior to one hour of cycling exercise at ~70% of heart rate reserve. A second (2 h) and third muscle biopsy (8 h) were donated 2 h and 8 h post-exercise, respectively. L1 DNA expression, mRNA expression, and other L1 markers were determined, and data are presented as relative expression units. **RESULTS**: PRE L1 DNA was not different between groups (younger – 1.00±0.05, older –

1.00±0.05; p>0.050). L1 mRNA demonstrated a time effect (p=0.034) and group effect (p=0.014). Older participants expressed more L1 mRNA than younger participants (younger – 0.90±0.39, older – 1.19±0.48; p=0.014). Cycling lowered L1 mRNA expression 2 h post-exercise (PRE – 1.15±0.53, 2 h – 0.89±0.25; p=0.027). L1 promoter methylation was higher in young compared to older participants (younger – 0.90±0.11, older – 0.30±0.04; p=0.006). When all participants were pooled, higher levels of moderate-to-vigorous physical activity per day, assessed via accelerometry, was associated with lower PRE L1 mRNA expression (r=-0.398, p=0.032). **CONCLUSIONS**: Skeletal muscle L1 mRNA expression is higher in older humans which may be related to lower L1 promoter methylation. L1 mRNA expression is reduced with endurance exercise and is negatively associated with higher levels of physical activity.

Keywords: LINE1, Endurance, Aging, Cycling, Methylation, Transposable Elements

INTRODUCTION

Transposable elements are repetitive DNA sequences that exist within mammalian genomes and possess the ability to mobilize and change positions. These sequences are generally classified as retrotransposons and DNA transposons. DNA transposons are suggested to play a role in human evolution but have not been active in the human genome for over 50 million years [1]. Retrotransposons, however, are still active within the human genome and the Long Interspersed Nuclear Element-1 (L1) is highly abundant [2]. The human genome is estimated to contain over 500,000 L1 copies, and these repeats comprise ~17% of the human genome [3]. L1 is ~6.0 kilobases (kb) in length, contains a 5' untranslated region (UTR) with an internal promoter, two open reading frames that encode for an ORF1 protein and an OFR2 protein, and a 3' UTR

containing a polyadenylation (polyA) signal and tail [4]. The ORF1 protein is ~40 kDa, and acts to form trimeric complexes with other ORF1 proteins to exhibit chaperone activity [5, 6] The ORF2 protein contains endonuclease and reverse transcriptase activity [7]. The retrotransposition process is termed target-site primed reverse transcription (TPRT) [8]. Following L1 transcription via RNA polymerase II in the nucleus, the biscistronic L1 mRNA is exported to the cytoplasm where it associates with ribosomes. Translated ORF1 and ORF2 proteins show strong cis-preference to bind to their original mRNA intermediate [9]. The resultant molecule, or ribonucleoprotein particle (RNP), enters the nucleus, the endonuclease activity of the ORF2 protein cleaves DNA [10], and the reverse transcriptase activity of the ORF2 protein catalyzes the integration and insertion of a new L1 copy into the genome [11]. Integration of new L1 sequences appears to be random, and insertion of these sequences within protein-coding sequences can alter protein structure and function [12].

Kazazian et al. reported over 120 different retrotransposition events are implicated with disease [13, 14]. Epigenetic alterations, specifically L1 promoter methylation, reduces L1 transcription [15]. In this regard, Baccarelli et al. determined L1 hypomethylation of blood DNA was associated with heart disease and stroke [16]. Furthermore, cardiac ischemia in rats upregulates L1 transcription, and when L1 transcripts were silenced by antisense oligonucleotides, infarct size was significantly lower compared to controls receiving a scrambled oligonucleotide [17]. L1 hypomethylation appears to be related to aging [18, 19] as well as various cancers [20]. L1 mRNA has also been shown to be upregulated in synovial fluid samples obtained from patients suffering from rheumatoid arthritis [21]. These lines of evidence suggest markers of increased L1 activity are detrimental to various health outcomes.

Several studies have attempted to gain perspective on environmental factors that might affect L1 DNA methylation status and mRNA levels. For instance, women who possessed lower white blood cell L1 methylation were more likely to have unfavorable body composition [22]. Marques-Rocha et al. observed similar findings in that favorable body composition metrics were associated with higher white blood cell L1 methylation [23]. To this end, obesity may alter L1 DNA methylation as well as L1 transcript levels across numerous tissues.

While no investigations have examined if skeletal muscle L1 markers are differentially expressed between younger and older humans, De Cecco et al. [18] demonstrated skeletal muscle L1 mRNA expression was 4-fold greater in 36-month old mice versus 5-month old mice. Likewise, skeletal muscle L1 DNA content was ~80% greater in 36-month old mice versus 5-month old mice. These data suggest L1 DNA hypomethylation, as well as increased L1 mRNA transcription, in skeletal muscle may be a consequence of aging or physical inactivity.

Physical activity and exercise improve body composition and, importantly, overall health outcomes [24-26]. An acute bout of endurance exercise has been shown to alter global methylation status in skeletal muscle [27], and chronic exercise activity has been shown to be associated with higher L1 methylation percentage in white blood cells [23]. Resistance exercise training has been shown to reverse the transcriptomic profile associated with aging in skeletal muscle [28]. Furthermore, our laboratory has shown that acute bouts of resistance exercise, and chronic resistance exercise, increase L1 methylation with an accompanied reduction in L1 mRNA expression in human skeletal muscle [29]. Given the metabolic demand differences and cellular signaling disparities between endurance versus resistance exercise [30], it is possible endurance exercise may also alter L1 methylation and mRNA expression.

The collective evidence above suggests skeletal muscle L1 activity increases with aging, and an acute bout of endurance exercise may increase L1 methylation and decrease L1 mRNA expression regardless of age. Moreover, a chronic engagement in higher levels of physical activity, regardless of age, may accomplish a similar molecular phenotype. Therefore, the purpose of this study is to determine baseline differences in skeletal muscle L1 DNA, L1 mRNA, and L1 DNA methylation status between younger and older humans. Further, we sought to determine if an acute bout of endurance exercise alters these markers, and whether daily physical activity levels were associated with these markers.

METHODS

Participants

This study was approved by the Auburn University Institutional Review Board (IRB) and conformed to the standards set by the latest revision of the Declaration of Helsinki (IRB protocol #: 18-226 AR 1806). Participants read and signed an informed consent form prior to participating in the study. Inclusion criteria were as follows: a) participants could be a male or female between the ages of 18-30 and 50-80 years old, b) participants had to be able to cycle for at least 60 min, and c) participants had to be apparently healthy and free of any known disease determined by a medical history questionnaire. A physical activity questionnaire and medical history questionnaire were filled out prior to participation to establish physical activity participation, health, and to identify potential risk factors that could be aggravated by training.

Experimental Design

Participants were initially screened using medical history and physical activity questionnaires. Thereafter participants were split into two groups based on age ("younger" (18-30

years old), and "older" (50-80 years old)), and were given an Actigraph GT3X accelerometer (Actigraph, Pensacola, FL, USA) to monitor physical activity throughout participation in the study. Figure 1 presents a graphical illustration of the experimental design. Following one to seven days, maximum leg extensor torque was assessed on the right leg to determine muscle function. During the same session, VO_2max testing occurred on a cycle ergometer to determine each participant's maximal aerobic power and heart rate. Following at least seven days, but no more than 14 days, the exercise trial began which consisted of body composition testing, ultrasonography for vastus lateralis thickness, a resting biopsy and blood draw (PRE), cycling for one hour at 70% VO_2max, an immediate post-exercise blood draw (POST), a two hours post-exercise muscle biopsy and blood draw (2 h), and an eight hours post-exercise muscle biopsy and blood draw (8 h). Notably, blood draws were performed for other analyses not included in this manuscript but are included in the methods to disclose all procedures being performed on participants. Each test is described in greater detail in the following paragraphs.

[***INSERT FIGURE 1***]

Muscle Function and VO_2max Testing

Participants first performed maximal isokinetic right leg extensions on an isokinetic dynamometer (System 4 Pro, BioDex Medical Systems, Shirley, NY, USA) at 30° and 120° $\cdot$ sec^{-1}. Participants warmed up prior to exercise by completing one set of 4 repetitions at 30° $\cdot$ sec^{-1} at 80% maximal effort. Participants then performed two sets of four repetitions for each velocity. The first two repetitions served as a warm-up to maximal effort while the following two repetitions

were maximal effort. Sets were separated by 60 sec of rest. The highest value for each velocity was determined as maximal torque.

Following muscle function testing, maximal aerobic power (VO_2max) and peak power (Wmax) testing were performed on an electronically-braked cycle ergometer (Velotron; RacerMate Inc., Seattle, WA, USA). Subjects performed a self-selected warm-up for five minutes and began the test at an intensity corresponding to what the subject perceived as comfortable for a 60 min cycling session. Intensity was increased by 30 W every two min until voluntary termination or until cadence dropped below 50 revolutions · min^{-1}. Participants then rested 10 min and performed a validation stage thereafter. The validation stage consisted of participants cycling to exhaustion at the wattage at which they ceased the continuous test. Using indirect calorimetry (Parvomedics Inc., Sandy, UT, USA), expired air was continuously analyzed for oxygen uptake (VO_2) and respiratory exchange ratio (RER). Values were averaged in 30-sec intervals. Heart rate (HR) was measured using a heart rate monitor (Polar; Lake Success, NY, USA). Subjects indicated rating of perceived exertion (RPE) by pointing to a Borg RPE scale (6-20) at the end of each stage. A test was accepted if heart rate was within 10% of age-predicted max HR, RPE $\geq$ 17, RER $\geq$ 1.10, and a plateau in VO_2 occurred (<250 ml increase in VO_2 from the end of the original test, and over the validation stage).

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As mentioned previously, the exercise trial occurred 7-14 days following muscle function and VO_2max testing. To control for differences in dietary intakes, participants consumed standardized meals 24 h prior to their exercise trial. Standardized meals were based on basal metabolic rate determined by the Harris-Benedict Equation. Each participant's basal metabolic rate was determined and then multiplied by 1.5 to ensure participants were adequately nourished

given long-term caloric restriction has been shown to alter L1 expression in mouse skeletal muscle [18].

Standardized meals were provided in the form of commercially-prepared sandwiches (Uncrustables™, Smucker's, Orrville, OH, USA). Based on basal metabolic rate, sandwiches were distributed to meet caloric demands. Given that each sandwich provided 210 kcal, if a participant's estimated basal metabolic rate was 2100 kcal per day then 10 sandwiches were provided, and the participant was instructed to consume them over breakfast, lunch, and dinner 24 h prior to the exercise trial. Each sandwich provided on average 210 kcal, 9 g of total fat, 28 g of carbohydrates, 2 g of fiber, and 6 g of protein. Thus, 10 sandwiches provided 2100 kcal, 90 g of total fat (39% of total kcal), 280 g of carbohydrates (50% of total kcal), and 60 g of protein (11% of total kcal).

Exercise Trial

Participants reported for the exercise trial between 0500-0800, 8 h fasted, not having consumed alcohol in 48 h, exercised in 72 h, or ingested caffeine within 8 h. Testing began with assessment of hydration status measured through urine testing by an ATAGO 2392 handheld refractometer (Bellevue, WA, USA). Participants moved forward with testing only if urine specific gravity level was less than 1.030 $g \cdot mL^{-1}$; however, all participants presented values that were below this cutoff. Following hydration assessment, muscle thickness was determined by ultrasound using a 3-12 MHz multi-frequency linear phase array transducer (Logiq S7 R2 Expert; General Electric, Fairfield, CT, USA). Briefly, the ultrasound probe was positioned horizontally on the vastus lateralis, halfway between the iliac crest and patella, where an image was captured. Another image was captured directly over the rectus femoris to determine midthigh thickness which was measured from the femur the inner-border of the skin. Further details regarding validity have been previously published by our laboratory [31].

Bioelectrical impedance spectroscopy was utilized to determine whole-body lean soft tissue mass and fat mass (Imp SFB7, Impedimed, Pinkemba Queensland, Australia). Briefly, electrodes were set up according to manufacturer instructions whereby electrodes were placed on the right hand and right foot. Two electrodes were placed ~5 cm apart on participants' hands and feet. For hand placement, the ulnar styloid process was used as a middle point and electrodes were place 2.5 cm above and below this bony landmark. For foot placement, the lateral malleolus was used as a starting point for one electrode while the accompanying electrode was placed 5 cm distal. Analysis was conducted twice and averaged. Further details regarding validity have been previously published by our laboratory [32].

Following body composition testing, participants were instructed to lie supine on a treatment table in preparation for venipuncture and a skeletal muscle biopsy. Following 5 min of supine rest, venous blood samples were obtained from an antecubital vein. Blood samples were collected into a serum separating tube and a CPT mononuclear cell preparation tube with sodium citrate (BD Vacutainer; Franklin Lakes, NJ, USA). Serum and CPT tubes were spun at 1,800 *g* for 30 min. Following venipuncture, the vastus lateralis of the right leg was prepared for a skeletal muscle biopsy. Approximately halfway between the iliac crest and the patella, a 3x3 inch square was shaved, cleaned with alcohol, and cleansed with betadine. Lidocaine (1.0 mL) was injected to provide local anesthesia and given five min to enact effects. Using aseptic technique, a 1 cm incision was made into the skin and muscle fascia. Using a 5-gauge Bergstrom biopsy needle with suction, tissue was extracted from the vastus lateralis. Tissue was blotted of blood, and any connective tissue was immediately removed. Tissue distribution for future analyses and analysis procedure is later described.

Following the skeletal muscle biopsy, participants were fitted with a heart rate monitor (Polar), mounted an electronically-braked cycle ergometer (Velotron, RacerMate, Inc., Seattle, WA, USA), and performed a self-selected pace 5 min warm-up. Wattage was then adjusted to achieve 70% VO_2Reserve which was measured by corresponding HRReserve determined by VO_2max testing, and participants cycled for 60 min. Heart rate was continuously monitored throughout the cycling bout to ensure appropriate exercise intensity. Drinking water was administered *ad libitum* throughout the trial.

Immediately following the 60 min cycling exercise trial, venipuncture was performed as described prior. Given participants had not consumed calories since the day prior, a post-exercise recovery beverage (~30 g of carbohydrate and ~30 g of protein) was provided. Participants were monitored in the laboratory until two hours following the cycling trial when a second muscle biopsy and third venipuncture was performed. Participants were then instructed to consume standardized meals within one hour which consisted of 25% of their basal metabolic rate in the form of commercially-prepared sandwiches (Uncrustables™) and could leave the facility. Participants were instructed to consume no other calories aside from standardized meals. Eight hours following the cycling trial, a third muscle biopsy and fourth venipuncture were performed.

Skeletal Muscle Processing

As stated prior, muscle tissue was rapidly removed from the needles, teased away from blood, connective tissue, and fat, wrapped in pre-labelled foils, and flash-frozen in liquid nitrogen. At the end of the day, tissue was stored at -80°C until further processing. For protein analysis, ~20 mg of frozen tissue was placed in 1.7 mL microcentrifuge tubes containing 500 µL of ice-cold cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton; Cell Signaling, Danvers, MA, USA] pre-stocked with protease and Tyr/Ser/Thr

phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin). Samples were then homogenized by hand via micropestle manipulation, insoluble proteins were removed with centrifugation at 500 *g* for 5 min, and obtained sample lysates were stored at -80ºC prior to Western blotting.

Frozen tissue was used for total DNA and total RNA isolation (~30 mg for each assay). RNA-free DNA was prepared using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's instructions (Qiagen, Germantown, MD, USA). DNA-free RNA was prepared using the Quigen RNeasy Fibrous Tissue Kit and following manufacturer's instructions (Qiagen). Sample concentrations were then quantified using a spectrophotometer (Nanodrop Lite; Thermo Scientific, Waltham, MA, USA) and subsequently frozen at -80ºC until later analysis.

Polymerase Chain Reaction

L1 DNA and mRNA expression were measured via real-time polymerase chain reaction (RT-PCR). Following DNA quantification, samples were concentrated to one microgram of RNA-free DNA for PCR analysis. Following RNA quantification, DNA-free RNA was concentrated to 100 nanograms and was reverse transcribed to cDNA using a commercially available kit (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's recommendations prior to PCR analysis. For DNA and mRNA expression analysis, real-time PCR was performed using gene-specific primers and SYBR green chemistry (Quanta Biosciences). Primer sequences used were as follows: L1 (ORF1 coding region) forward primer 5-TAAGGGCAGCCAGAGAGAAA-3, reverse primer 5-GCCTGGTGGTGACAAAATCT-3; B2M forward primer 5=-ATGAGTATGCCTGCCGTGTGA-3=, reverse primer 5=-GGCATCTTTCAAACCTCCATG-3=; Cyclophilin forward primer 5- CGATGTCTCAGAGCACGAAA-3, reverse primer 5-CCCACCTGTTTCTTCGACAT-3; PGC-1α forward primer 5-

CAAGCCAAACCAACAACTTTATCTCT-3, reverse primer 5-CACACTTAAGGTGCGTTCAATAGTC-3; and GAPDH forward primer 5-AACCTGCCAAATATGATGAC-3, reverse primer 5- TCATACCAGGAAATGAGCTT-3.

To calculate the ΔCq value, the geometric mean Cq of B2M, GAPDH, and Cyclophilin minus gene of interest Cq was calculated. $2^{\Delta Cq}$ values were then calculated. $2^{\Delta Cq}$ values were normalized to the group mean of young PRE.

L1 Promoter Methylation

L1 promoter methylation analyses were performed from isolated DNA (described above) using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (Abcam, Cambridge, MA, USA) and L1 5'-UTR primers which spanned a CpG-rich region (positions 29 – 485 at the 5'-end). Primer sequences were as follows: forward primer, 5-GAACAGCTCCGGTCTACAGC-3, reverse primer, 5- CCGGCTGCTTTGTTTACCTA-3. Prior to MeDIP being performed, 1.5 g of DNA was digested using MseI (New England BioLabs, Ipswich, MA). Thereafter, total methylated DNA from a total of one gram of input was immunoprecipitated using an anti-5-methylcytosine antibody provided within the kit. Additionally, 500 ng of residual input DNA from each sample was used as a control to normalize real-time PCR results. Promoter methylation expression values ($2^{\Delta Cq}$ values) were calculated whereby ΔCq = input DNA Cq – methylated DNA Cq. $2^{\Delta Cq}$ values were normalized to the group mean of young PRE.

Western Blotting

Whole-tissue sample lysates obtained through cell lysis buffer processing were batch process-assayed for total protein content using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Lysates were then prepared for Western blotting using 4x Laemmli buffer

at 1 μg/μL. Following sample preparation, 25 μL samples were loaded onto 4-15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis (150 V for 75 min) using pre-made 1x SDS-PAGE running buffer (Ameresco, Solon, OH, USA). Proteins were then transferred (200 mA for 2 hours) to polyvinylidene difluoride membranes (Bio-Rad), Ponceau stained and imaged to ensure equal protein loading between lanes. Membranes were then blocked for 1 h at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST; Ameresco). Mouse anti-ORF1 (1:1,000; EMD Millipore, Billerica, MA, USA; catalog no. MABC1152) was incubated with membranes overnight at 4°C in TBST with 5% bovine serum albumin (BSA). The following day, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:2,000, Cell Signaling, catalog no. 7076) or anti-rabbit IgG (1:2,000, Cell Signaling, catalog no. 7074) in TBST with 5% BSA at room temperature for 1 h. Membrane development was performed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore), and band densitometry was performed using a gel documentation system and associated densitometry software (UVP, Upland, CA, USA). All densitometry values for proteins of interest were normalized to the Ponceau densitometry values. Each participant's PRE, 2 h and 8 h samples were normalized to the group mean of young PRE for each protein target.

Accelerometry

Actigraph GT3X triaxial accelerometers (Actigraph) were worn on the right hip and attached with an elastic strap. Participants were asked to wear the device as often as possible with the exception of sleeping and water-based activities. To this end, participants filled out an on-off log, and were instructed to record an off time if the accelerometer was taken off for more than 5 min. Accelerometer data leading into the exercise trial was ignored due to physical activity

restrictions. Scoring was conducted using the following algorithms: Energy Expenditure – Freedson Adult (1998), Metabolic equivalents (METs) – Freedson Adult (1998), and Cut Points and Moderate to vigorous physical activity (MVPA) – Freedson Adult (1998) [33].

Statistics

Shapiro-Wilk testing was used to determine normality for all dependent variables. When appropriate, Levene's test for equality of variances was determined. Baseline differences in dependent variables between age groups were determined using independent samples t-tests. Changes in dependent variables over time were tested using a repeated-measures analysis of variance (ANOVA) where within-subject factors included PRE, 2h, and 8h, and the between-subject factor was age. If majority of the groups within the repeated-measures ANOVA were normally distributed, then the test was carried out due to the robustness of an ANOVA. In the event that a significant effect or interaction was observed, pairwise comparisons were utilized. Lastly, Pearson correlations were performed between select variables. Fold change was determined by dividing each participant's value over the young group's mean, and then averaging each group's values at each time point. All data are presented as mean ± standard deviation values, and statistical significance was established as $p<0.05$.

RESULTS

Participant Characteristics

Participant characteristics are summarized in Table 1. Notably, older participants (58 ± 8 y/o) were older than younger participants (23 ± 3 y/o; $p<0.001$). In conjunction, resting heart rate (younger: 69 ± 13 bpm; older: 58 ± 8 bpm) and maximal heart rate (younger: 190 ± 9 bpm; older: 165 ± 17 bpm) were lower in older participants ($p<0.001$). VO_2max, relatively (younger: 38.0 ±

7.7 ml/kg/min; older: 35.6 ± 7.3 ml/kg/min) and absolutely (younger: 2.70 ± 0.63 l/min; older: 2.56 ± 0.66 l/min), were not different between older and younger participants (p>0.050). Maximal cycling power (Wmax) was not different between groups (younger: 209 ± 46 W; older: 204 ± 52 W; p=0.768). Whole-body lean soft tissue mass (younger: 58.27 ± 12.48 kg; older: 57.72 ± 7.47 kg) and fat mass (younger: 13.25 ± 6.99 kg; older: 13.30 ± 4.51 kg) were not different either (p>0.050).

During the exercise trial, heart rate was higher in younger (156 ± 12 bpm) compared to older participants (135 ± 13 bpm; p<0.001); however, percent of heart rate reserve was similar between groups (younger: 71.4 ± 4.4%; older: 71.5 ± 1.8%; p=0.944). Average power output during exercise was not different between groups (younger: 112 ± 30 W; older: 107 ± 24 W; p=0.639).

[***INSERT TABLE 1***]

Skeletal Muscle L1 DNA and RNA Expression

Skeletal muscle L1 DNA expression is presented in Figure 2, and data are represented as fold change values from the younger group. L1 DNA expression was not different between younger (1.00 ± 0.05) and older participants (1.00 ± 0.05; p=0.867).

[***INSERT FIGURE 2***]

L1 mRNA expression is presented in Figure 3, and data are represented as fold change values from the younger group. mRNA expression analysis was conducted in 15 young

participants and 13 old participants due to low RNA yield for select participants. L1 mRNA expression demonstrated a time effect ($p=0.034$) and group effect ($p=0.014$), but not a group by time interaction ($p=0.526$). Older participants (1.19 ± 0.48) expressed more L1 mRNA than younger participants (0.90 ± 0.39) irrespective of exercise ($p=0.014$). Cycling lowered L1 mRNA expression 2 h post-exercise regardless of group (PRE: 1.15 ± 0.53, 2 h: 0.89 ± 0.25; $p=0.027$). L1 mRNA levels significantly increased from 2 h post-exercise to 8 h post-exercise (2 h: 0.89 ± 0.25, 8 h: 1.08 ± 0.52; $p=0.052$); however, 8 h post-exercise was not different compared to baseline (PRE: 1.15 ± 0.53, 8 h: 1.08 ± 0.52; $p=0.543$).

PGC-1α mRNA expression was also assessed as a positive control given this gene is responsive to acute endurance exercise. PGC-1α mRNA demonstrated a group by time interaction ($p=0.010$) as well as a time effect ($p<0.001$). Both groups experienced increases in PGC-1α mRNA expression from PRE to 2 h (younger – PRE: 1.00 ± 0.19, 2 h: 4.44 ± 1.21, $p<0.001$; older – PRE: 1.07 ± 0.18, 2 h: 5.71 ± 2.32, $p<0.001$) and decreases in PGC-1α mRNA expression from 2 h to 8 h (younger – 2 h: 4.44 ± 1.21, 8 h: 3.11 ± 0.99, $p<0.001$; older – 2 h: 5.71 ± 2.32, 8 h: 2.52 ± 0.95, $p<0.001$); however, PGC-1α mRNA expression was still elevated at 8 h compared to PRE for both groups ($p<0.001$). While a group by time interaction was found, expression was not different between groups at each time point following pairwise comparisons ($p>0.050$).

[***INSERT FIGURE 3***]

Skeletal Muscle ORF1 Protein Content

ORF1 protein content is presented in Figure 4, and data are represented as fold change values from the younger group. Protein content determination was conducted in 15 younger

participants and 14 older participants due to limited tissue availability in one older participant. ORF1 protein content violated the assumption of sphericity; thus, a Huynh-Feldt correction was utilized. ORF1 protein content did not reveal a time effect (p=0.805), group effect (p=0.242), or group by time interaction (p=0.789).

[***INSERT FIGURE 4***]

Skeletal Muscle L1 Methylation Status

L1 methylation status is presented in Figure 5, and data are represented as fold change values from the younger group. Methylation status was conducted in 15 young participants and 14 old participants due limited tissue availability in one older participant. L1 promoter methylation did not reveal a time effect (p=0.226) or group by time interaction (p=0.502). However, L1 promoter methylation demonstrated a group effect (p=0.006). Younger participants possessed higher L1 promoter methylation than older participants (younger – 0.90 ± 0.11; older – 0.30 ± 0.04).

[***INSERT FIGURE 5***]

Associations

Associations are shown in Table 2. Both groups wore the accelerometer 13 ± 4 days. Moderate-to-vigorous physical activity (MVPA) · day^{-1} was 40 ± 15 min in the younger group and 35 ± 16 in the older group (p=0.431). Notably, age correlated with L1 methylation (r=-0.371, p=0.048). Also, MVPA · day^{-1} correlated with PRE L1 RNA (r=-0.398, p=0.032).

[***INSERT TABLE 2***]

DISCUSSION

The participants utilized in this study were on average approximately 20 years old in the young group and 60 years old in the old group. Despite this age gap, an interesting observation was that both groups were similarly fitness-matched given there were no significant differences in metrics related to body composition or aerobic capacity. To be expected, resting HR, max HR, and, as a result of max HR, time trial HR were significantly lower in older compared to younger participants. Lowering of max HR with aging is thought to be related to reductions in intrinsic heart rate and beta-adrenergic responsiveness [34, 35]. Strength also declines with aging [36, 37] which is corroborated in this study by the significant difference between groups in maximal knee extension torque at $30^{o}{\cdot}sec^{-1}$. While this functional difference exists herein, maximal knee extension torque does not seem to translate to functional metrics of endurance exercise performance [38] or resistance exercise performance [31]. Based on these findings and the exercise prescribed, it could be suggested differences found herein would relate mostly to aging and not training status.

While skeletal muscle L1 DNA copy number has been found to be elevated in older mouse skeletal muscle [18], the current study found L1 DNA expression patterns to be nearly identical in younger and older human skeletal muscle. De Cecco et al. [18] reported L1 DNA copy number is higher in 36-month old mice compared to 5-month old mice, although they did not find a difference in L1 DNA copy number between 5- and 24-month old mice. Likewise, other researchers have noted that no differences in cardiac muscle L1 mRNA existed between 4- and 24-month old mice;

however, L1 transcript levels were elevated in brain and liver between the aged groups [39]. Given the older participants population age was approximately 60 years old, it is possible this population was not old enough to observe increases in skeletal muscle L1 DNA. It has been suggested 24-month old mice have a human age equivalent of approximately 70-years old [40, 41], and 36-month old mice have a human age equivalent of approximately 95-years old. Assuming these animal data translate, it would suggest humans would need to be very old for L1 DNA copy number differences to be found. It should also be noted that cellular turnover differences among various tissues appears to play a role in L1 DNA copy number wherein cell types that undergo more rapid proliferation are susceptible to L1 insertions. Billingsley et al. [42] demonstrated differences in L1 enrichment and total repetitive elements between skin cells and white blood cells. Since skeletal muscle fibers are post-mitotic, it is possible skeletal muscle is naturally protected from *de novo* L1 insertions due to lack of cellular turnover.

Even though skeletal muscle L1 DNA expression was not different between younger and older participants, this is the first study to demonstrate skeletal muscle L1 mRNA expression is higher in older versus younger participants. Furthermore, regardless of age, moderate-intensity endurance exercise transiently decreased skeletal muscle L1 mRNA expression and increased daily physical activity levels correlated with lower L1 mRNA expression. These findings are similar to Romero et al. [29] who found L1 mRNA expression is lower following acute resistance exercise and chronic resistance exercise in college-aged males. Other groups have shown similar findings in that blood cell L1 hypermethylation is associated with increased physical activity [23, 43]. It is interesting both exercise modalities lower skeletal muscle L1 mRNA expression given the vastly different molecular signaling associated with each exercise [30, 44, 45], which suggests a common exercise-induced molecule may be mediating L1 RNA expression. A possible mechanism for

regulation of L1 may be through the Sirtuin (SIRT) family which are histone deacetylase proteins. In this regard, SIRT6 knockout mice exhibit greater L1 mRNA expression and DNA integration in isolated mouse embryonic fibroblasts, while SIRT6 overexpression repressed these events [39]. However, within the context of exercise, these data are difficult to reconcile as 3- and 26-month old endurance-trained rodents possess lower skeletal muscle SIRT6 protein content compared to sedentary controls [46]. Furthermore, these authors found that with exercise training, skeletal muscle SIRT1 protein content decreases in young and old rodents, but SIRT1 activity increases in both groups [46]. To corroborate these findings, Ryan et al. [47] found SIRT1 mRNA expression decreases with an acute bout of resistance exercise. Thus, L1 mRNA suppression may rely on global SIRT activity as opposed to individual SIRT activity. Notably, we recently observed that rats bred to participate in high amounts of wheel running and have access to running wheels for five months [48, 49] exhibit lower skeletal muscle L1 mRNA expression increased and skeletal muscle SIRT activity compared to littermates who never engaged in wheel running (*unpublished findings*).

Higher skeletal muscle L1 mRNA expression in older participants is supported by the concomitant finding that L1 promoter methylation is lower in these individuals. Global methylation has been found to be lower with aging in human white blood cells [50, 51]. Due to the abundance of L1 in the genome, L1 methylation has been used as a surrogate marker for global methylation. For this reason, it could be concluded not only is the L1 promoter hypomethylated with aging but so is the genome as a whole. Interestingly, Barres et al. [27] demonstrated acute maximal aerobic exercise lowers global methylation in human skeletal muscle. The present study did not find that an acute bout of moderate-intensity aerobic exercise affected L1 promoter methylation. Moreover, we have previously reported that three consecutive days of resistance

exercise did not promote increased L1 promoter methylation, whereas 12 weeks of resistance training increased L1 promoter methylation [29]. As a result, it could be posited chronic endurance exercise could promote increases in skeletal muscle L1 promoter methylation.

Certain limitations exist within the current study. While the present study did not find increased skeletal muscle L1 DNA expression with aging, our older participants may not have been old enough to demonstrate this effect. Furthermore, the older individuals utilized were relatively healthy for their age suggesting disease may play a prominent role for L1 reintegration to occur. Lastly, we did not determine a mechanism to how exercise affects L1 mRNA expression.

CONCLUSIONS

This study is the first to put forth findings regarding how aging and endurance exercise influence L1 markers in human skeletal muscle. These findings build upon past literature showing skeletal muscle L1 markers are reduced with exercise and increase with aging. To this end, it appears exercise and engaging in regular physical activity appears to be beneficial in silencing skeletal muscle L1 activity. Future research is needed in determining how exercise affects mechanisms altering L1 markers in skeletal muscle and, more importantly, how aberrant L1 activity affects skeletal muscle health.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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TABLES AND FIGURE LEGENDS

Table 1. Baseline characteristics between age groups

	Young	**Old**	**p-value**
Sample Size	15	15	
Male:Female	7:8	10:5	
Age (years)	23 ± 3	58 ± 8	**<0.001**
Body Composition			
Height (cm)	172 ± 9	172 ± 8	0.861
Mass (kg)	71.9 ± 14.9	71.4 ± 8.7	0.913
WB LSTM (kg)	58.27 ± 12.48	57.72 ± 7.47	0.886
WB Fat mass (kg)	13.25 ± 6.99	13.30 ± 4.51	0.979
Midthigh Thickness (cm)	5.10 ± 0.82	4.78 ± 0.76	0.283
VL Thickness (cm)	2.45 ± 0.52	2.22 ± 0.44	0.207
Aerobic Measures			
VO_2peak (ml/kg/min)	38.0 ± 7.7	35.6 ± 7.3	0.394
VO_2peak (l/min)	2.70 ± 0.63	2.56 ± 0.66	0.552
RestingHR (bpm)	69 ± 13	58 ± 8	**0.005**
HRmax (bpm)	190 ± 9	165 ± 17	**<0.001**
ET HR (bpm)	156 ± 12	135 ± 13	**<0.001**
ET HRR (%)	71.4 ± 4.4	71.5 ± 1.8	0.944
Max Power (W)	209 ± 46	204 ± 52	0.768
ET Power (W)	112 ± 30	107 ± 24	0.639
Strength Measures			
120º Extension (N·m)	148.1 ± 42.2	124.0 ± 22.6	0.064
120º Flexion (N·m)	70.2 ± 15.9	67.6 ± 12.7	0.622
30º Extension (N·m)	196.1 ± 50.6	158.0 ± 31.7	**0.020**
30º Flexion (N·m)	92.0 ± 24.6	85.3 ± 14.6	0.374

Abbreviations: WB LSTM, whole-body lean soft tissue mass; VL thickness, vastus lateralis thickness; HR, Heart rate; ET, Exercise trial; HRR, Heart rate reserve; VO2, Volume of oxygen.

Table 2. Select correlations between skeletal muscle L1 markers and participant characteristics

Variable 1	**Variable 2**	**r**	**p-value**
Age	L1 DNA	-0.116	0.542
Age	L1 PRE RNA	0.088	0.645
Age	ORF1 Protein	0.161	0.405
Age	L1 Methylation	-0.371	**0.048**
L1 DNA	L1 Methylation	-0.532	**0.003**
L1 PRE RNA	MVPA/day	-0.398	**0.032**

Legend: These data are Pearson correlation coefficients between select dependent variables. Bold-faced p-values are significant ($p<0.050$).

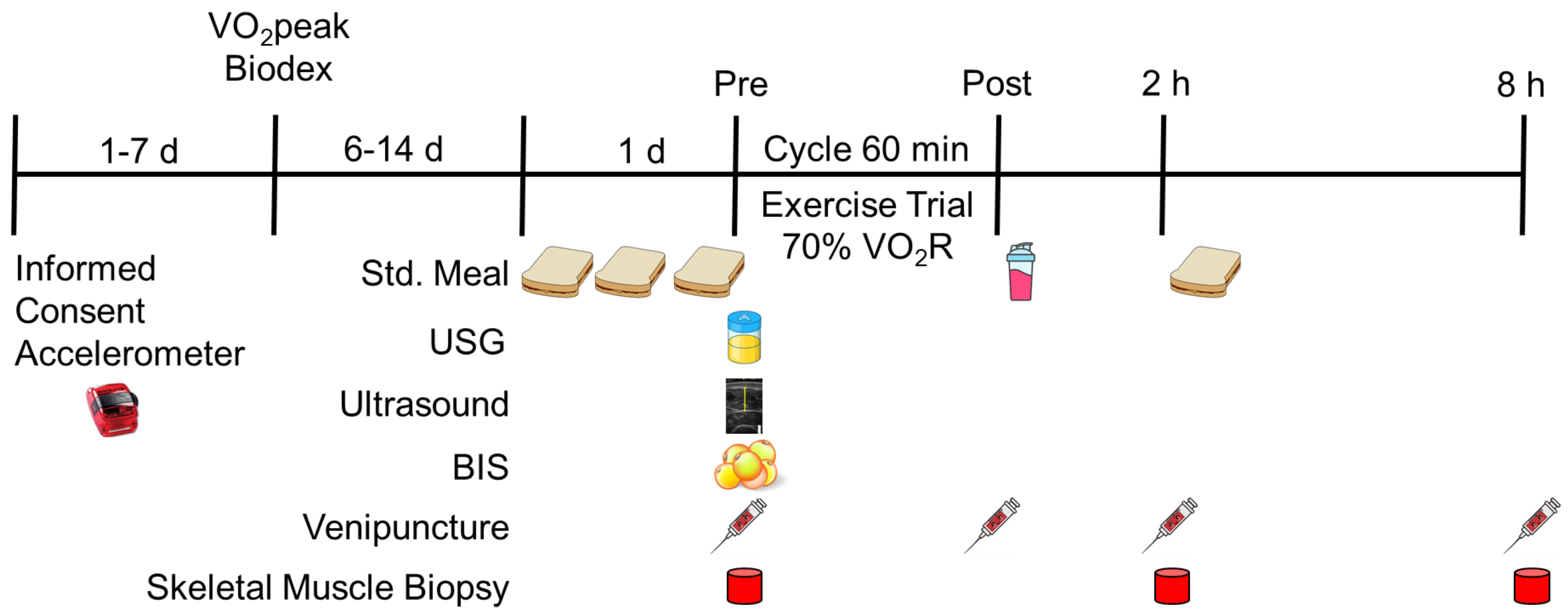

Figure 1. Graphical illustration of experimental design

Legend: Detailed methods are described in-text. Abbreviations: USG, urine specific gravity; BIS, bioelectrical impedance spectroscopy for body composition assessment; VO_2R, VO_2 Reserve.

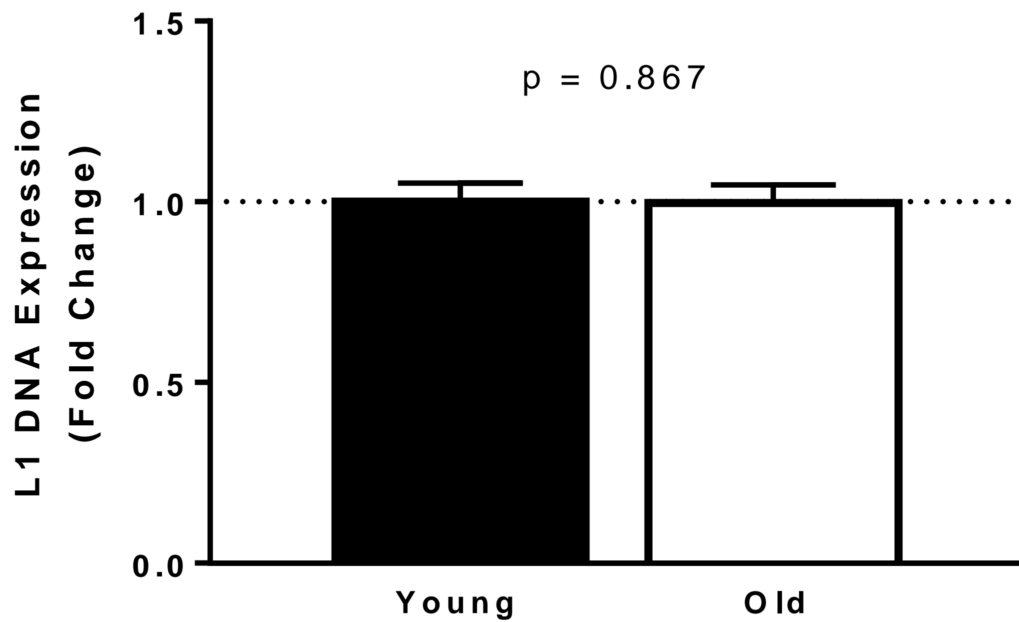

Figure 2. Skeletal muscle L1 DNA expression at PRE between age groups

Legend: These data are L1 DNA expression between younger and older participants only at the PRE time point. Data are presented as fold change from the younger groups PRE mean average (± standard deviation values).

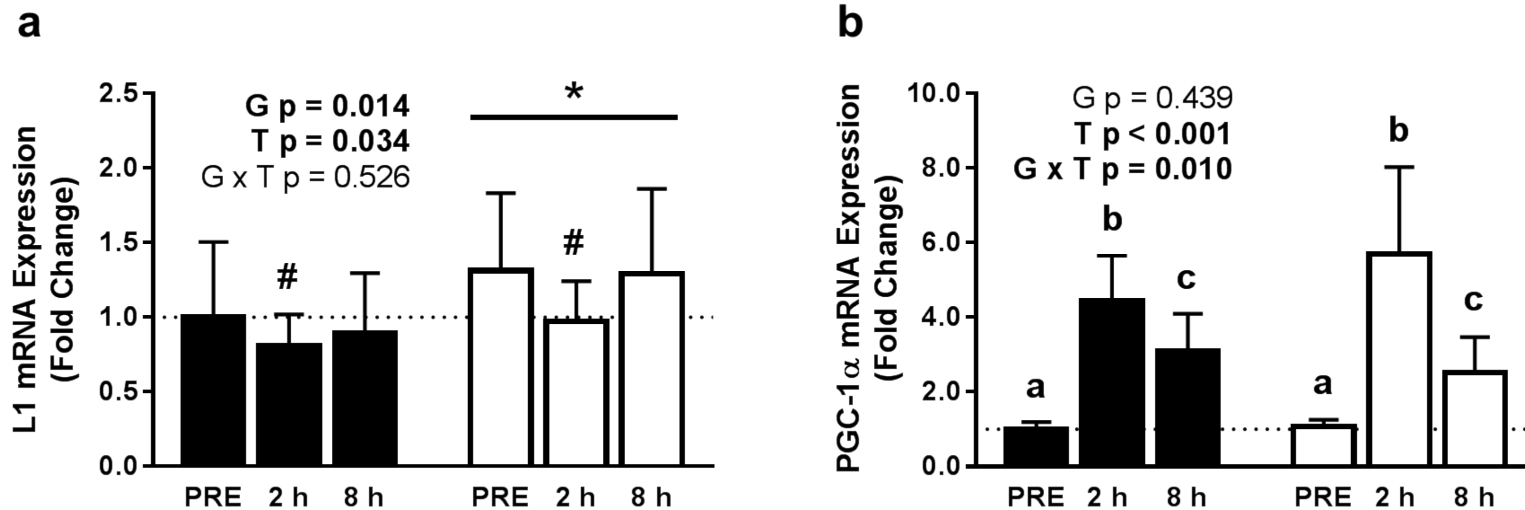

Figure 3. Skeletal muscle mRNA expression between age groups

Legend: a) The expression of L1 mRNA and b) PGC-1α mRNA between younger and older participants. Notably, PCG-1α was assessed given the robust response the gene has to acute endurance exercise. Data are presented as fold change from the younger groups PRE mean average (± standard deviation values). The young group is represented by the black bars while the older group is represented by the white bars. "G" = Group p-value, "T" = Time p-value, "G x T" = Group by Time Interaction p-value. "*" represents group differences whereby older participants were significantly higher than younger participants. "#" represents time differences whereby 2 h was significantly lower than PRE and 8 h. Differing letters denote significant differences.

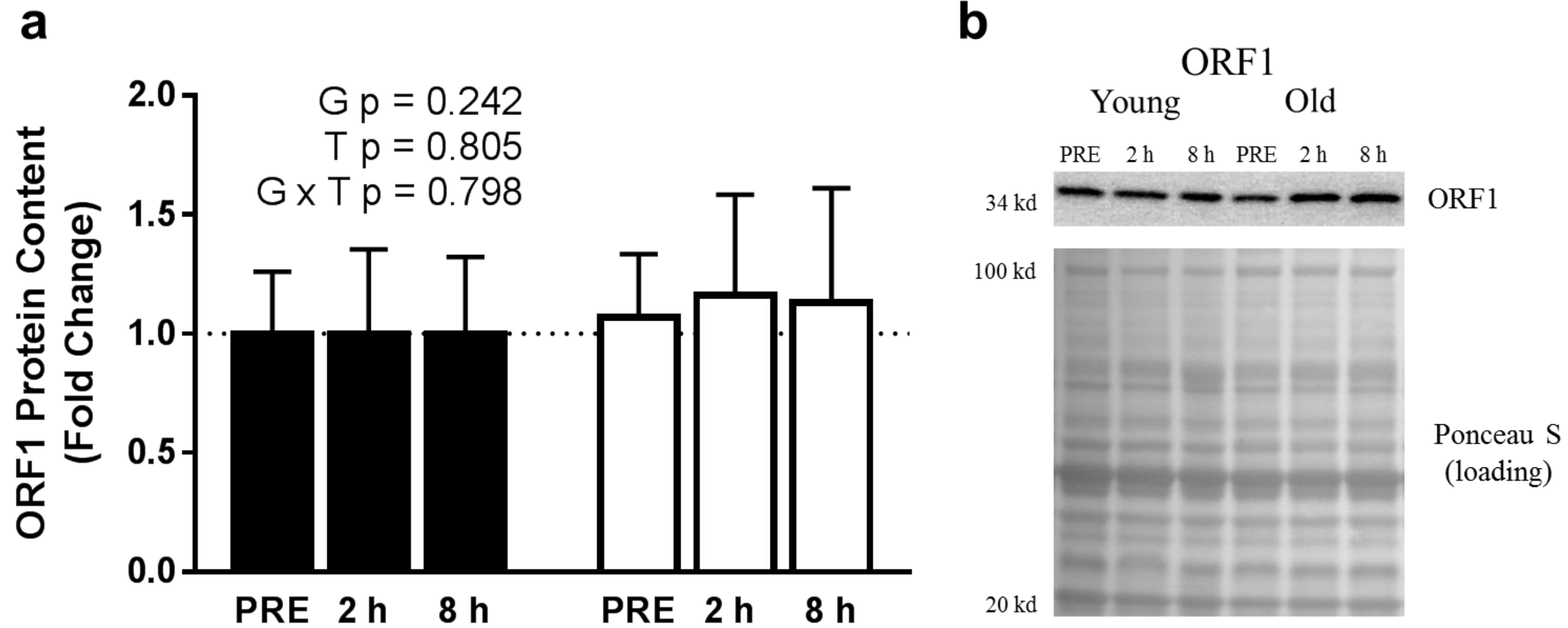

Figure 4. Skeletal muscle protein content between age groups

Legend: a) L1 protein content. b) western blot representative image. Data are presented as fold change from the younger groups PRE mean average (± standard deviation values). The younger group is represented by the black bars while the older group is represented by the white bars. “G” = Group p-value, “T” = Time p-value, “G x T” = Group by Time Interaction p-value.

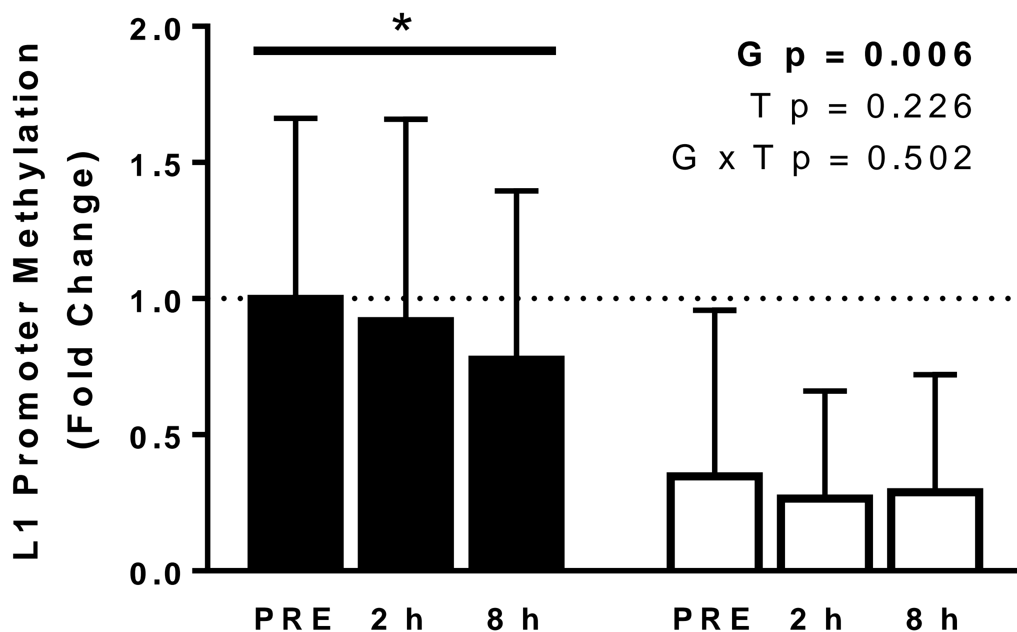

Figure 5. Skeletal muscle L1 promoter methylation between age groups

Legend: Data are presented as fold change from the younger groups PRE mean average (± standard deviation values). The younger group is represented by the black bars while the older group is represented by the white bars. "G" = Group p-value, "T" = Time p-value, "G x T" = Group by Time Interaction p-value. "*" represents group differences whereby younger participants were significantly higher than older participants.

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