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RESEARCH ARTICLE

Muscle Wasting: Cellular and Molecular Mechanisms

## The effect of young and old ex vivo human serum on cellular protein synthesis and growth in an in vitro model of aging

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### Abstract

In vitro models of muscle aging are useful for understanding mechanisms of age-related muscle loss and aiding the development of targeted therapies. To investigate mechanisms of age-related muscle loss in vitro utilizing ex vivo human serum, fasted blood samples were obtained from four old (72 ± 1 yr) and four young (26 ± 3 yr) men. Older individuals had elevated levels of plasma CRP, IL-6, HOMA-IR, and lower concentric peak torque and work-per-repetition compared with young participants ( $P < 0.05$ ). C2C12 myotubes were serum and amino acid starved for 1 h and conditioned with human serum (10%) for 4 h or 24 h. After 4 h, C2C12 cells were treated with 5 mM leucine for 30 min. Muscle protein synthesis (MPS) was determined through the surface sensing of translation (SUnSET) technique and regulatory signaling pathways were measured via Western blot. Myotube diameter was significantly reduced in myotubes treated with serum from old, in comparison to young donors (84%,  $P < 0.001$ ). MPS was reduced in myotubes treated with old donor serum, compared with young serum before leucine treatment (32%,  $P < 0.01$ ). MPS and the phosphorylation of Akt, p70S6K, and eEF2 were increased in myotubes treated with young serum in response to leucine treatment, with a blunted response identified in cells treated with old serum ( $P < 0.05$ ). Muscle protein breakdown signaling pathways did not differ between groups. In summary, we show that myotubes conditioned with serum from older individuals had decreased myotube diameter and MPS compared with younger individuals, potentially driven by low-grade systemic inflammation.

*anabolic resistance; leucine; muscle protein synthesis; serum; skeletal muscle cells*

### INTRODUCTION

Sarcopenia (1), defined as the age-related loss of skeletal muscle mass, strength, and function, is associated with an increased risk of mortality, physical disability, and metabolic disease which can reduce the quality of life and independence (2–6). This phenomenon is predicted to affect ~32 million older individuals (>65 yr) across Europe by 2045 (7), placing a significant strain on healthcare resources (8, 9). A number of factors contribute to the etiology of sarcopenia, including alterations in lifestyle, hormone concentrations, and peripheral nervous system changes (10). In particular, aging is often associated with a state of chronic low-grade inflammation, termed inflammaging, consisting of raised

levels of c-reactive protein (CRP) and pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which have been associated with reductions in muscle mass, strength (11–16), and diminished muscle protein synthesis (MPS) (17, 18). Previous acute metabolic investigations show that the postprandial MPS response to protein/amino acid ingestion is blunted in older individuals, particularly at moderate-to-low protein doses (<20 g) (19), which may contribute to the progression of sarcopenia (20, 21). Due to the significant costs, complexities, and often invasive nature of in vivo human studies of muscle protein turnover, there is a need for in vitro models to identify mechanisms of sarcopenia and expedite the development of targeted therapeutic interventions.



To date, in vitro models to probe mechanisms of sarcopenia have been conducted using pharmacological treatments to induce myotube atrophy, such as TNF- $\alpha$  (22, 23) and dexamethasone (DEX) (24–27). Although these models provide an insight into the potential mechanisms of muscle wasting, the supraphysiological dosages used hinder the translation of this work to humans. Alternatively, C2C12 skeletal muscle cells subjected to replicative aging, a process in which a population of cells undergoes multiple passages, provide a potential in vitro model of musculoskeletal aging. Using this approach, Sharples et al. (28) demonstrated reductions in IGF-1, myogenic regulatory factors, and a diminished capacity for myoblast differentiation that was associated with impaired Akt signaling. However, undergoing multiple passages within cell lines such as C2C12 skeletal muscle cells does not induce senescence (28). In fact, immortalized cell lines have been shown to maintain their telomerase activity (29, 30). Thus, as senescence is considered to be a key hallmark of aging (31), replicative aging may not closely represent inherent aging processes.

To overcome the potential drawbacks of in vitro experimental models outlined in the previous paragraph, studies have used ex vivo human plasma (32, 33) and serum (34–37) to condition cell culture media. These studies have investigated a range of factors including changes in myotube diameter (32), MPS (34, 37), anabolic and catabolic regulatory signaling (33, 36) in response to divergent nutrient status, states of disease and injury (i.e., septic shock and burns), and aging. Recently, Kalampouka et al. (32) identified an increase in myotube diameter in C2C12 cells in response to 24-h and 48-h treatments with 5% plasma from young versus older participants. In addition, Carson et al. (34) identified an increase in MPS in response to an acute 4-h treatment with ex vivo human serum in comparison to serum-free control cells. Furthermore, the authors detected differences in anabolic signaling with the addition of serum obtained after the ingestion of a bolus of whey protein compared with the ingestion of nonessential amino acids (37) or fasted serum (34). Collectively, this evidence suggests that ex vivo human serum or plasma can be used to condition cell culture media to investigate changes in MPS and muscle cell morphology.

Although there is growing evidence demonstrating the use of ex vivo human serum to condition cell culture media in vitro, no studies have used serum to create an in vitro model of aging in response to an acute stimulus. In addition, no studies have conditioned media with human serum before in vitro nutritional treatment, which would enhance our understanding of mechanisms of age-related muscle anabolic resistance. Therefore, the primary purpose of this study was to assess acute changes in MPS and regulatory signaling in response to leucine treatment, after conditioning media with ex vivo human serum from young and old participants to investigate mechanisms of age-related muscle wasting. Second, we aimed to investigate age-related alterations in myotube growth in response to a chronic 24-h conditioning period with ex vivo human serum, to identify whether acute signaling responses correlated with chronic morphological changes. We hypothesized that myotubes conditioned with serum from older men would display impairments in MPS and lower myotube diameter in comparison to those treated with serum from young men.

## MATERIALS AND METHODS

### Subject Characteristics and Ethical Approval

Four young ( $26 \pm 3$  yr) and four old ( $72 \pm 1$  yr) healthy male participants provided written informed consent to participate in this study. All participants were screened with a general health questionnaire and deemed healthy for the completion of this study. Participants were expected to be normotensive ( $<140/90$  mmHg), free from inflammatory and metabolic disease conditions (i.e., rheumatoid arthritis, type II diabetes, hyperlipidemia), not prescribed any medications such as anticoagulants or nonsteroidal anti-inflammatory drugs (NSAIDs), and be habitually and/or recreationally active, that is, participate in regular physical activity such as walking. Ethical approval for this study was obtained through the local ethics committee at the University of Birmingham (ERN\_19-0831) and conformed to the standards set by the Declaration of Helsinki.

### Study Design

Participants reported to the laboratory after an overnight fast and were asked to refrain from the consumption of caffeine on the morning of the trial. Participants were additionally asked to refrain from involvement in strenuous forms of exercise 24 h before their visit. A fasted blood sample was obtained from the antecubital vein and collected into EDTA and serum separator vacutainers (BD, Oxford, UK). After collection, the serum separator tubes were allowed to sit for 30 min at room temperature to enable the blood to clot. Both types of the tube were then centrifuged at 3,000 rpm for 10 min at 4°C to obtain serum and plasma. Aliquots were frozen at  $-80^{\circ}\text{C}$  for further analysis. Finally, participants underwent body composition analysis and completed basic functional assessment in the form of isokinetic functional assessment and handgrip strength (HGS).

### Experimental Procedures

#### Body composition.

Body mass was measured using digital scales by weighing each participant in loose, light clothing without shoes to the closest 0.1 kg. Height was measured using a stadiometer and made to the nearest 0.1 cm. After assessment of body mass and height, body composition was measured using 8-electrode bioelectrical impedance analysis (BIA), allowing for the noninvasive measurement of fat mass (FM), fat-free mass (FFM), and skeletal muscle mass (SMM) (mBCA 525, SECA, Hamburg, Germany). BIA has previously been observed to be a valid and clinically reliable measure of body composition in younger and older adults (38).

#### Handgrip strength.

Handgrip strength (HGS) assessment was used as a clinically relevant measure of muscle strength (39). Participants were asked to hold a handheld dynamometer (Jamar Hydraulic Handheld Dynamometer, Patterson Medical, Warrenville, IL) with their arm straight and palm facing medially in a semipronated position. Participants completed three trials on each hand with a 30-s rest period between each test. Participants were asked to exert their maximal grip strength throughout the test with standardized verbal encouragement.

The highest recording across all six measurements was used for analysis (39).

### Leg strength.

Eccentric and concentric muscle strength of the knee flexors and extensors was assessed using an isokinetic dynamometer (Cybex, division of Lumex Inc., Ronkonkoma, NY) for the evaluation of isokinetic muscle strength of both limbs. Participants were seated with their hips and knees flexed at a 90° angle, with the tested limb, hips, and chest secured. The rotational axis of the lever arm was aligned with the lateral epicondyle of the femur. The knee range of motion was set between 5° and 90° of knee flexion. Participants were asked to start the test at full flexion, extend their leg, and return to flexion with as much force as possible, completing a total of five repetitions at an angular velocity of 60 deg/s. After the procedure was explained in full, participants were asked to complete a short warm-up set of five repetitions to ensure familiarity with the test, without reaching the point of fatigue. Standardized verbal encouragement was given by the operator throughout the test.

### Blood analyses.

Fasting plasma glucose concentrations were measured using a Glucose-Glo Assay (J6021, Promega Corporation, Madison, WI) following the manufacturer's instructions. Plasma insulin (DINS00), IL-6 (D6050), and c-reactive protein (CRP) (DCRP00) concentrations were determined through the use of commercially available enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN).

### Cell culture.

Murine C2C12 skeletal muscle cells (passage numbers 10–12) were cultured in Dulbecco's modified Eagle's medium (DMEM) (11966025, Gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (F9665, Sigma-Aldrich, St. Louis, MO), 1% (vol/vol) penicillin-streptomycin (P/S) (15070-063, Gibco), 5 mM glucose (G7021, Sigma-Aldrich), 1 mM sodium pyruvate, and 1 mM GlutaMAX (35050-038, Gibco) (growth media) in standard conditions (5% CO<sub>2</sub>, 100% humidity, 37°C). Media were changed every 48 h until a confluency of ~70%–80% was reached. Passage numbers between 10 and 12 were selected to ensure C2C12s before treatment showed normal levels of MPS and growth were present, in contrast to using cells considered as an aging model, investigated in previous work (28). Cells were then seeded onto 6-well plates or 24-well plates at a density of  $1.0 \times 10^5$  and  $2.5 \times 10^3$  cells·mL<sup>-1</sup>, respectively. At a confluency of ~90%, myoblasts were differentiated in DMEM supplemented with 2% (vol/vol) horse serum (16050, Gibco), 1% (vol/vol) P/S, 5 mM glucose, 1 mM sodium pyruvate, and 1 mM GlutaMAX (differentiation media) over the course of 5 days. During the differentiation period, media were changed every 24 h. All in vitro experiments were performed with  $n = 4$  in each group and were repeated in triplicate.

### Myotube diameter.

C2C12 cells were seeded in 24-well plates and differentiated once a confluency of ~90% was reached. On *day 6* of differentiation, myotubes were incubated in differentiation media supplemented with 10% ex vivo human serum from either

fasted young or old male donors for 24 h. A subset of myotubes was maintained in differentiation media throughout the treatment period, providing a comparison to normal growth conditions. Similar to previous work by Kalampouka et al. (32), we chose to add serum to differentiation media, as we aimed to observe growth following normal conditions, rather than after the stress of a serum and amino acid starvation period. After a 24-h incubation period, media were removed and myotubes were washed with phosphate-buffered saline (PBS). Myotubes were incubated with 2% paraformaldehyde for 30 min. Following a PBS wash, cells were incubated for 10 min with 100% methanol. Cells were washed three times in PBS before blocking in 5% (vol/vol) goat serum in PBS. After three PBS washes, cells were then incubated with the primary antibody Desmin (1:100, D8281, Sigma-Aldrich) for 1 h. After incubation, cells were washed twice with PBS and incubated with the secondary antibody (1:200, Alexa Fluor Plus 488 goat anti-rabbit IgG H + L, A32731, Thermo Fisher Scientific, Waltham, MA) for 1 h in darkness. Cells were washed once with PBS and incubated in the dark with DAPI (1:5,000, 4083, Cell Signaling Technology, Leiden, Netherlands) for 5 min. Finally, cells were washed with PBS before the addition of mountant (P36930, Thermo Fisher Scientific) to each well. A 13-mm coverslip was applied and image acquisition was performed after an overnight incubation using a fluorescent microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Hessen, Germany). For analysis, ~10 fields per well were randomly selected and at least 100 myotubes were included in the analysis per well (40). The mean of five measurements along each myotube was used to calculate the average myotube diameter. Nuclear fusion index (NFI) was defined as the number of nuclei within myotubes, expressed as a percentage of the total number of nuclei within a field of view (41). Images were analyzed using ImageJ software (US National Institutes of Health, Bethesda, MD).

### Muscle protein synthesis.

C2C12 myoblasts were seeded in six-well plates and maintained in DMEM supplemented with FBS. Once a confluency of ~90% was reached, growth media were replaced with differentiation media and changed every 24 h. On *day 7* of differentiation, myotubes were changed to an amino acid and serum-free media (D9800-13, US Biological, Salem, MA) (pH 7.3) and underwent a 1-h serum and amino acid starvation period before treatment. Myotubes were subsequently treated with media containing 10% human serum from fasted young or old participants for 4 h. This time period of "preconditioning" was chosen as previous research has shown that MPS induced by growth medium is reduced within 30–60 min of serum and amino acid starvation and is significantly downregulated alongside key mTOR pathway signaling proteins (34, 42). A range of plasma and serum doses have previously been used in previous investigations with similar outcomes, ranging from 5% to 20% (32, 34, 37). Throughout our preliminary experimental trials, we identified similar viability across dilutions (data not shown). Therefore, given limited serum sample availability and the detection of protein expression during preliminary experiments, a dilution of 10% was deemed optimal for the current

experimental outcomes. In addition, conditioned media containing 10% human serum have been shown to exhibit high cell adherence and viability throughout a 4-h time-course treatment (34). In a subset of C2C12 myotubes, 5 mM leucine was added to wells for 30 min after the 4-h serum conditioning treatment with ex vivo human serum with either young or old donor serum to investigate the anabolic response to the addition of an essential amino acid. The in vitro leucine stimulation used provides an alternative model to investigate the effect of a nutritional supplement, providing us with an alternative “fed” state. This concentration was selected due to its ability to increase the phosphorylation of mTOR, 4EBP-1, and RPS-6 in C2C12 myotubes, without any prior treatment (43).

Acute measures of MPS were assessed using the surface sensing of translation (SUnSET) technique, as previously described (44). Briefly, this method uses the antibiotic puromycin, a tyrosyl-tRNA analog, and immunoblotting to assess the incorporation level of puromycin into newly synthesized peptide chains (44, 45). Myotubes were incubated with puromycin (P8833, Sigma-Aldrich) (1  $\mu$ mol/L) for the final 30 min of treatment. Subsequently, cellular protein lysates were obtained by aspirating the culture media and washing the cells with cold PBS (Gibco, 10010015) three times over ice. The PBS was aspirated and RIPA lysis buffer (Merck Millipore, Watford, UK) (150  $\mu$ L) was added to each well. After 20 min on ice, cell lysates were scraped and collected in Eppendorfs. Lysates were centrifuged at 13,000 rpm for 15 min at 4°C.

### Immunoblotting.

Protein lysate concentrations were determined through the use of a DC assay (Bio-Rad, Hercules, CA) using an FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK). Concentrations were read at an absorbance of 750 nm and equal amounts of protein from each sample were added to 1 $\times$  Laemmli sample buffer. Protein (15–30  $\mu$ g) was separated on 4%–20% linear graded precast gels (5671095, Bio-Rad) by SDS-PAGE for 1 h. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) at 100 V for 1 h. Membranes were stained with Ponceau S solution as a loading control (46) and blocked with 5% BSA or 5% milk diluted in Tris-buffered saline and 0.1% Tween-20 (TBST) for 1 h. Membranes were incubated overnight at 4°C with the following antibodies: mouse IgG2a monoclonal antipuromycin (clone 12D10, 1:5,000, Merck Millipore), Phospho-mTOR (Ser2448) (#2971, CST), total mTOR (#2972, CST), Phospho-protein kinase B (Akt) Ser473 (#3787, CST), total Akt (#9272, CST), phospho-ribosomal protein S6 kinase  $\beta$ -1 (p70S6K1) Thr389 (#9205, CST), total p70S6K1 (#9202, CST), phospho-eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP-1) Thr37/46 (#9459, CST), total 4EBP1 (#9452, CST), phospho-ribosomal protein S6 (RPS-6) Ser240/244 (#5364, CST), total RPS-6 (#2217, CST), phospho-eukaryotic elongation factor 2 (eEF2) Thr56 (#2331, CST), total eEF2 (#2332, CST), LC3A/B (#12741, CST), Caspase-3 (D3R6Y) (#14220, CST), muscle RING finger 1 (MuRF-1) (#sc-398608, Santa Cruz Biotechnology, TX), and muscle atrophy F-box (MAFbx) (AM3141, ECM Biosciences, KY). After 15 min of washing with TBST, membranes were incubated for 1 h in an anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (#7074, CST) with the

exception of MAFbx (anti-rat IgG, HRP-linked antibody, #7077, CST), puromycin and MuRF-1 (anti-mouse IgG, HRP-linked antibody, #7076, CST). Following 15 min of washing in TBST, protein content was quantified using Immobilon Western chemiluminescent HRP substrate (Merck Millipore) and imaged on a G:BOX Chemi XT4 imager using GeneSys capture software (Syngene, Cambridge, UK). Once the image was captured, band quantification was conducted through the use of ImageJ software. For puromycin blots, the whole lane was quantified in order to account for the range of proteins which incorporate puromycin (44). Relative arbitrary units were normalized to a protein band stained with ponceau S solution (46).

### Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 26 (IBM SPSS Inc., Chicago, IL). Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene’s test. Independent samples *t* tests were used to assess differences in anthropometric measures and body composition components (fat-free mass, skeletal muscle mass, fat mass, and body fat %). In addition, measures of muscle strength, both HGS and isokinetic dynamometry measures, were also assessed using an independent samples *t* test. Independent samples *t* tests were also used to analyze differences in plasma insulin, glucose, and IL-6. A one-way ANOVA was used for the analysis of myotube diameter, NFI, puromycin incorporation, and markers anabolic and catabolic signaling. Whereas the results of the one-way ANOVA revealed a positive interaction effect, post hoc analysis *t* tests were completed using Tukey’s honestly significant difference test (HSD). The effect size was calculated using Cohen’s *d* for *t* test and post hoc comparisons, while partial eta squared ( $\eta^2$ ) was used for omnibus tests. Statistical significance was set at  $P < 0.05$ . The data presented include the mean of each participant, over the three passage numbers to provide one data point per participant  $\pm$  SD, unless stated otherwise. Figures were generated in GraphPad Prism v8.4.3.

## RESULTS

### Body Composition and Strength

Body composition and strength measures are shown in Table 1. No significant difference was observed between young and old participants for whole body FM, body fat percentage, FFM, or SMM. Concentric peak torque ( $P < 0.05$ ,  $d = 2.70$ ) and concentric WPR ( $P < 0.05$ ,  $d = 2.03$ ) were significantly greater in the young compared to old participants. No significant difference was identified in eccentric peak torque, eccentric WPR, or HGS between groups.

### Inflammatory Markers and Blood Analysis

Plasma IL-6 and CRP were significantly greater in old, compared with young participants ( $P < 0.001$ ,  $d = 4.92$ ,  $P < 0.001$ ,  $d = 4.3$ ), respectively (Table 1). No significant difference was identified in fasting plasma insulin ( $P = 0.08$ ,  $d = 1.52$ ) and plasma glucose ( $P = 0.11$ ,  $d = 1.71$ ) between young and old participants. Homeostatic model assessment for insulin resistance (HOMA-IR) was significantly greater in old, compared with young participants ( $P = 0.03$ ,  $d = 2.2$ ).

**Table 1.** Participant characteristics for anthropometric, body composition, and strength data in young and old males

	Young (n=4)	Old (n=4)	P Value
<b>Anthropometrics</b>			
Age, yr	26.5 ± 3.1	72.7 ± 0.8	<0.001
Body mass, kg	80.6 ± 19.2	77.1 ± 12.0	0.77
Height, cm	174.9 ± 11.8	179.3 ± 4.4	0.50
BMI, kg m <sup>-2</sup>	26.1 ± 3.1	24.0 ± 3.8	0.43
<b>Body composition</b>			
FFM, kg	62.4 ± 11.8	59.4 ± 6.3	0.67
WBFM, kg	18.2 ± 8.8	17.7 ± 9.7	0.95
BF, %	21.6 ± 8.0	22.2 ± 8.9	0.92
SMM, kg	31.7 ± 6.3	27.0 ± 3.7	0.25
<b>Strength</b>			
HGS, kg	54.75 ± 12.1	45.8 ± 10.4	0.30
Con PT, nm	209.0 ± 69.1	91.0 ± 18.3	0.016*
Ecc PT, nm	93.8 ± 46.1	63.0 ± 19.2	0.27
Con WPR, nm	217.5 ± 74.8	90.3 ± 51.1	0.03*
Ecc WPR, nm	97.3 ± 23.7	69.5 ± 28.1	0.18
<b>Blood analyses</b>			
Fasting plasma insulin, μU/mL	6.4 ± 0.8	8.22 ± 1.6	0.08
Fasting plasma glucose, mmol/L	4.5 ± 0.4	4.9 ± 0.04	0.11
HOMA-IR	1.3 ± 0.13	1.8 ± 0.4	0.03*
Plasma IL-6, pg/mL	0.41 ± 0.07	0.78 ± 0.08	<0.001‡
Plasma CRP, ng/mL	0.40 ± 0.05	0.53 ± 0.01	<0.001‡

BF, body fat %; BMI, body mass index; con PT, concentric peak torque; con WPR, concentric work per repetition; CRP, c-reactive protein; ecc PT, eccentric peak torque; ecc WPR, eccentric work per repetition; FFM, fat-free mass; HGS, handgrip strength; HOMA-IR, homeostatic model assessment for insulin resistance; IL-6, interleukin-6; SMM, skeletal muscle mass; WBFM, whole body fat mass. \*Significantly different from young ( $P < 0.05$ ). †Significantly different from young ( $P < 0.001$ ).

### Myotube Diameter and Nuclear Fusion Index

Differentiated myotubes were cultured in media conditioned with ex vivo human serum from young and old participants over a 24-h time period. A one-way ANOVA revealed a significant main effect between groups ( $P < 0.001$ ,  $\eta^2 = 0.98$ ,  $F = 196.1$ ) (Fig. 1). Myotube diameter was significantly greater (84%) in response to conditioning with ex vivo human serum from young participants, in comparison to serum from old participants ( $P < 0.001$ ,  $d = 14.11$ ). In addition, the myotube diameter of those treated with serum from young participants was significantly greater (48%) than untreated control myotubes, receiving normal differentiation media over the 24-h treatment period ( $P < 0.001$ ,  $d = 9.82$ ). In contrast, cells treated with serum from older participants showed a significant decrease (20%) in myotube diameter in comparison to the untreated control myotubes ( $P < 0.001$ ,  $d = 4.05$ ).

A one-way ANOVA revealed a significant main effect between groups for the analysis of NFI ( $P < 0.5$ ,  $\eta^2 = 0.62$ ,  $F = 7.3$ ) (Fig. 1). NFI was significantly greater (20%) in myotubes conditioned with young serum, in comparison to the untreated control myotubes ( $P < 0.5$ ,  $d = 2.71$ ). The NFI tended to be greater in myotubes conditioned with young serum, in comparison to old serum, though significance was not reached ( $P = 0.06$ ,  $d = 2.02$ ). No difference was identified in NFI between myotubes conditioned with old serum and untreated control myotubes.

### Muscle Protein Synthesis

There was a significant main effect of MPS, measured by the incorporation of puromycin through the SUNSET technique ( $P < 0.001$ ,  $\eta^2 = 0.83$ ,  $F = 20.11$ ) (Fig. 2). After conditioning with serum from young participants, MPS was significantly increased (32%) in comparison to serum from old participants ( $P < 0.01$ ,  $d = 3.7$ ). In addition, leucine treatment significantly elevated MPS in myotubes conditioned with young serum compared with myotubes treated with young serum only (25%,  $P < 0.05$ ,  $d = 1.7$ ) and myotubes treated with old serum and leucine (14%,  $P < 0.001$ ,  $d = 3.8$ ). The addition of leucine did not significantly increase MPS in myotubes conditioned with old serum.

### Anabolic Signaling

In response to conditioning media with human serum, with and without the addition of leucine treatment, significant main effects were identified in the phosphorylation of Akt ( $P < 0.001$ ,  $\eta^2 = 0.77$ ,  $F = 13.1$ ), p70S6K ( $P = 0.03$ ,  $\eta^2 = 0.51$ ,  $F = 4.2$ ), and eEF2 ( $P = 0.026$ ,  $\eta^2 = 0.51$ ,  $F = 4.3$ ) (Fig. 2). No significant difference was identified in the phosphorylation of Akt, eEF2, and p70S6K in response to conditioning with fasted ex vivo serum, from young and old participants. However, the activation of Akt (68%,  $P < 0.001$ ,  $d = 4.97$ ), eEF2 (120%,  $P = 0.032$ ,  $d = 2.2$ ), and p70S6K (45%,  $P < 0.5$ ,  $d = 2.35$ ) significantly increased in response to leucine treatment in myotubes conditioned with ex vivo serum from young participants. In contrast, there was no significant increase in the activation of Akt and p70S6K in response to the addition of leucine in myotubes conditioned with serum from older individuals. In addition, no significant difference in eEF2 phosphorylation was identified between myotubes treated with ex vivo human serum from older participants and with the addition of leucine. Akt phosphorylation was significantly elevated in myotubes conditioned with young serum and leucine treatment in comparison to those conditioned with old serum before leucine treatment (73%,  $P < 0.05$ ,  $d = 3.29$ ).

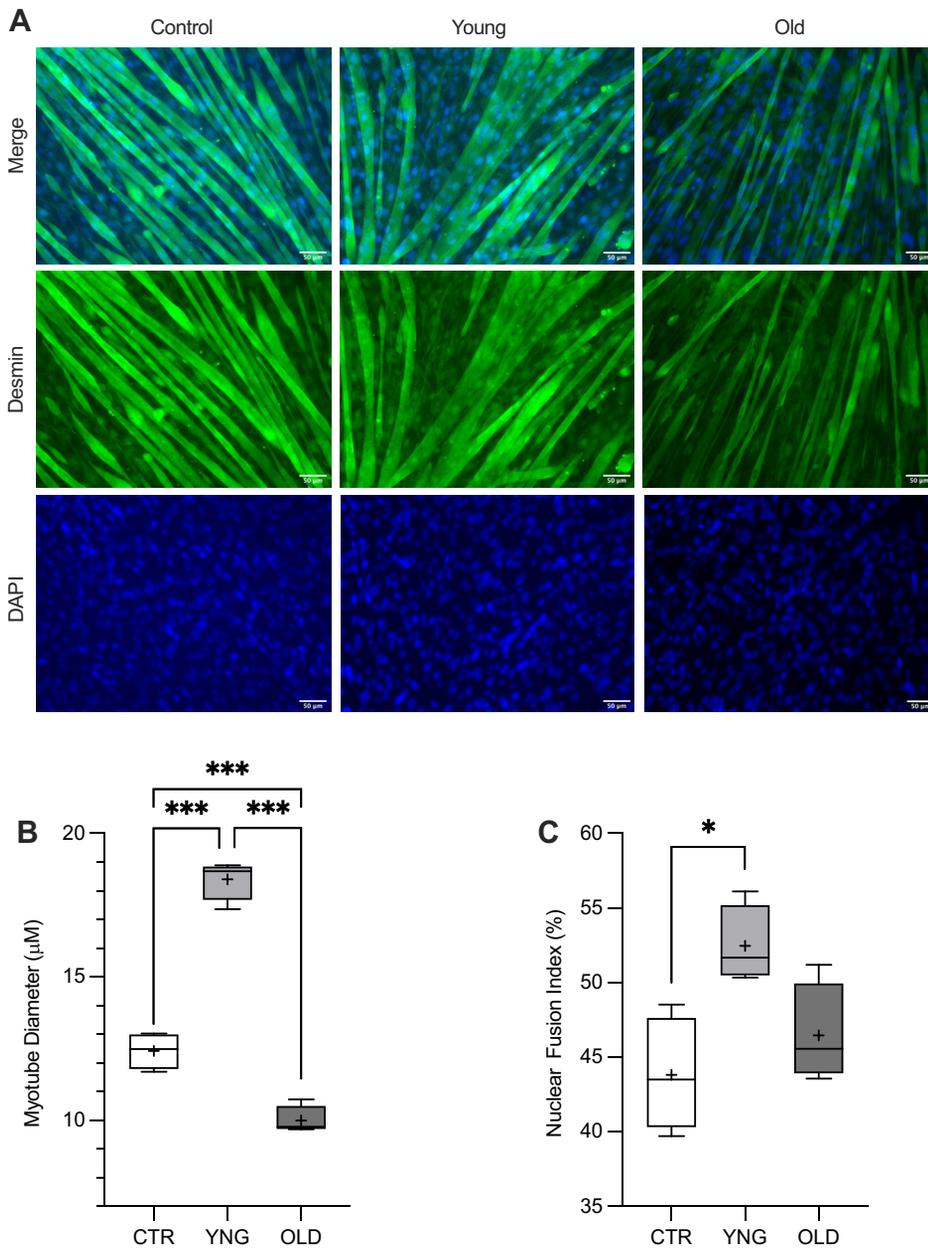
There were no significant differences identified in the phosphorylation of mTOR ( $P = 0.77$ ,  $\eta^2 = 0.38$ ,  $F = 2.46$ ), RPS6 ( $P = 0.82$ ,  $\eta^2 = 0.07$ ,  $F = 0.30$ ), and 4EBP-1 ( $P = 0.95$ ,  $\eta^2 = 0.03$ ,  $F = 0.12$ ) between myotubes treated with ex vivo human serum from young and old serum alone, and with additional leucine treatment.

### Catabolic Signaling

No significant differences were identified between myotubes conditioned with young and old serum, with or without leucine treatment, in MuRF-1 ( $P = 0.74$ ,  $\eta^2 = 0.1$ ,  $F = 0.42$ ), MAFbx ( $P = 0.81$ ,  $\eta^2 = 0.1$ ,  $F = 0.31$ ), Caspase 3 ( $P = 0.9$ ,  $\eta^2 = 0.1$ ,  $F = 0.25$ ), and LC3A/B I/LC3A/B II ( $P = 0.056$ ,  $\eta^2 = 0.5$ ,  $F = 3.33$ ) protein content (Fig. 3).

## DISCUSSION

The development of an in vitro model of age-related muscle wasting is useful for the investigation of potential mechanisms and interventions, with potential application to myriad disease conditions. Recent developments have involved the use of replicative aging (28), TNF- $\alpha$  (22, 23),

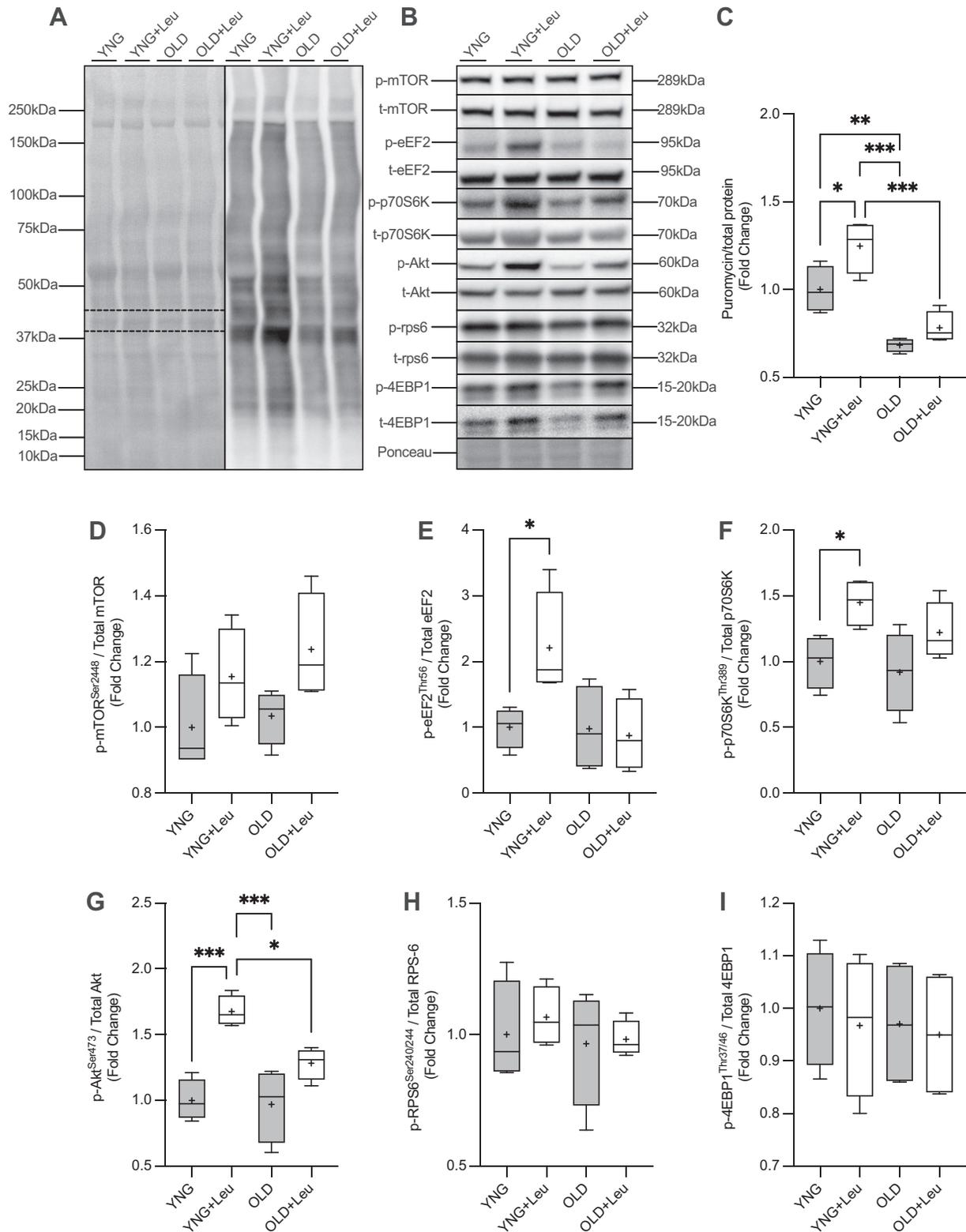


**Figure 1.** Myotube diameter and nuclear fusion index (NFI). The effect of ex vivo human serum-conditioned media on myotube diameter and NFI in vitro. **A:** representative images illustrating atrophy in myotubes conditioned with serum from older males in comparison to untreated control C2C12 myotubes and myotubes treated with serum from young males. **B:** mean myotube diameter. **C:** mean NFI. Data are expressed as the means (cross), median (central horizontal line), 25th and 75th percentiles (box), and the minimum and maximum values (vertical lines), with  $n=4$  per group, corresponding to treatment of each participant's serum, repeated in triplicate. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

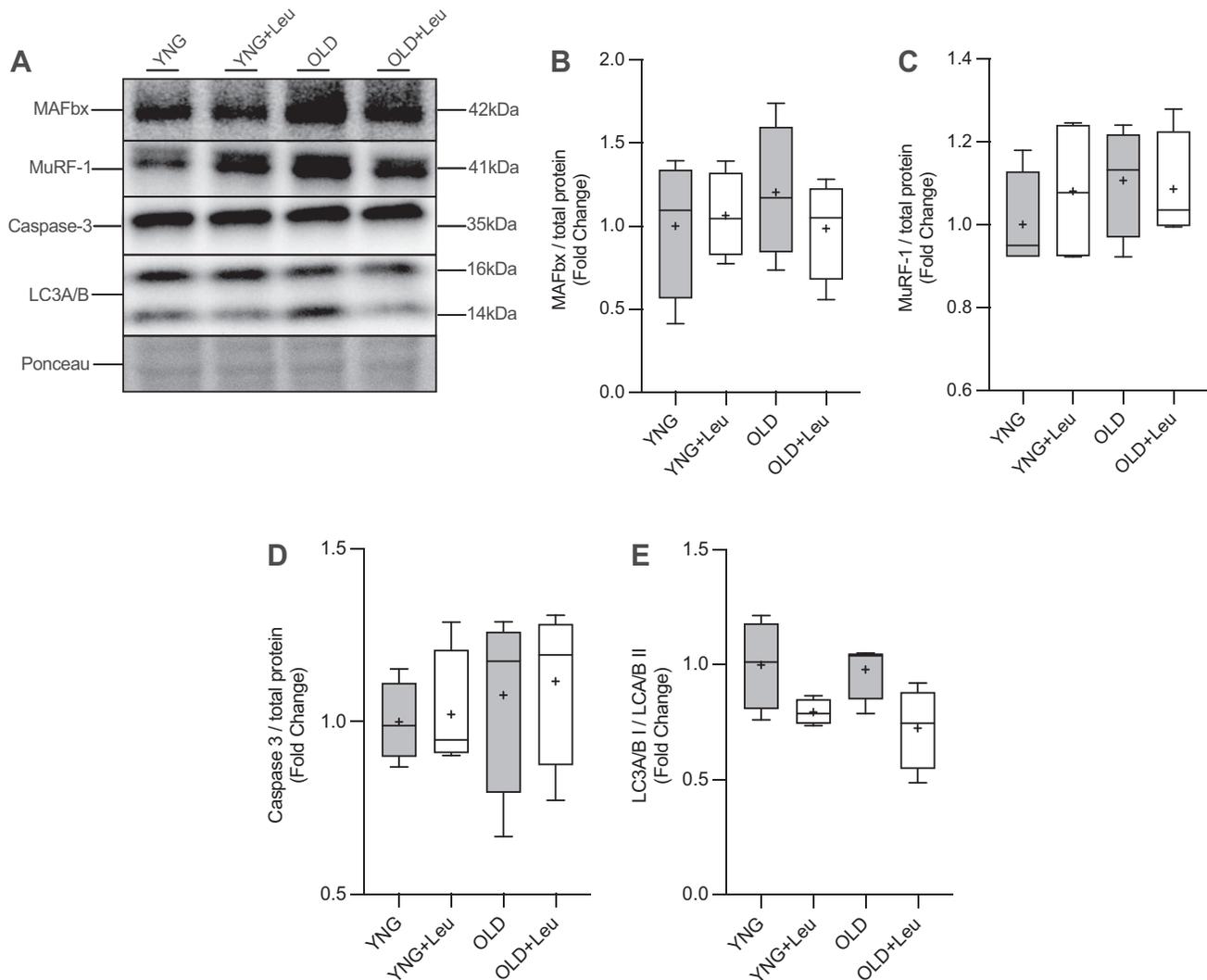
and DEX (24–27) treatment, but these approaches may not closely represent inherent physiological aging processes. Therefore, we used ex vivo human serum from young and old men to condition C2C12 skeletal muscle myotubes, with and without subsequent in vitro anabolic treatment with leucine, to develop a more physiologically relevant model to study regulatory mechanisms of muscle aging. In line with our hypotheses, we demonstrated that cells conditioned with serum from older men led to a decrease in myotube diameter compared with serum from young men. Mechanistically, the smaller myotube diameter appeared to be underpinned by a reduced MPS in older versus younger serum treated cells. In addition, myotubes treated with old versus young serum showed a blunted MPS and anabolic signaling response to in vitro leucine treatment (i.e., ana-

bolic resistance). Collectively, these data highlight that conditioning cell culture media with human serum can be effectively used to study mechanisms of age-related muscle loss, and that systemic inflammatory factors may contribute to age-related muscle loss through impaired anabolism, though blockade of cytokine signaling would be required to confirm this.

Following a 24-h treatment with ex vivo human serum, we found that serum from young men led to a significant increase in myotube diameter in comparison to old serum and untreated control myotubes, whereas old serum decreased myotube diameter compared with untreated control myotubes. This observation is consistent with work by Kalampouka et al. (32), who identified a reduction in myotube diameter after 24 and 48 h of treatment with ex vivo human plasma



**Figure 2.** Measures of muscle protein synthesis (MPS) and anabolic signaling in response to the addition of ex vivo human serum with and without leucine treatment. Data are represented as fold change in relation to the myotubes treated with ex vivo serum from young males. **A:** representative Western blot images for puromycin incorporation and total protein. **B:** representative Western blot images for anabolic signaling markers. **C:** puromycin incorporation. **D:** phospho-mTOR<sup>Ser2448</sup>/total-mTOR. **E:** phospho-eEF2<sup>Thr56</sup>/total-eEF2. **F:** phospho-p70S6K<sup>Thr389</sup>/total-p70S6K. **G:** phospho-Akt<sup>Ser473</sup>/total-Akt. **H:** phospho-RPS6<sup>Ser240/244</sup>/total-RPS6. **I:** phospho-4EBP1<sup>Thr37/46</sup>/total-4EBP-1. Data are expressed as the means (cross), median (central horizontal line), 25th and 75th percentiles (box), and the minimum and maximum values (vertical lines), with  $n=4$  per group, corresponding to treatment of each participant's serum, repeated in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 3.** Changes in markers of catabolic signaling in response to the addition of ex vivo human serum with and without leucine treatment. Data are represented as fold change in relation to the myotubes treated with ex vivo serum from young males. *A*: representative Western blot images. *B*: MAFbx. *C*: MuRF-1. *D*: Caspase-3. *E*: LC3A/B. Data are expressed as the means (cross), median (central horizontal line), 25th and 75th percentiles (box), and the minimum and maximum values (vertical lines), with  $n = 4$  per group, corresponding to treatment of each participant's serum, repeated in triplicate.

from old versus young humans. In contrast, previous work investigating myotube diameter within human primary skeletal muscle cells failed to identify a difference between young and old donor cells, when cultured under standard conditions (47). It is possible that the absence of any age-related atrophic effect in this study may result from primary skeletal muscle cells not retaining their phenotype. Similar to the effects of young and old serum on myotube growth, conditioning media with ex vivo human serum from young participants resulted in a significant increase in NFI compared with untreated control myotubes, whereas NFI was numerically lower in myotubes treated with old compared with young serum (large effect size). Previous work investigating the myogenic fusion index in primary myoblasts identified a decrease in myoblast fusion in cultures from older compared with younger donors (48). In contrast, no difference in myogenic fusion index was observed in myoblasts treated with serum from young and old participants, suggesting that

myoblasts sustain the capacity to fuse, independent of the donor age (49). However, the absence of an age-related difference in NFI herein may be due to the lower percentage (2%) serum-enriched media used throughout the experiment. Notwithstanding, the present data reinforce the concept that serum from human participants can modulate myotube growth and NFI and be applied to probe mechanisms and countermeasures to age- and disease-related muscle atrophy.

Analogous to myotube growth data, we report that acute MPS stimulation and anabolic signaling were dysregulated in myotubes conditioned with serum from old donors. This observation is in line with in vivo human trials highlighting a reduction in basal MPS in older men (50–52). However, it is important to note that others have suggested that basal MPS rates are not influenced by the aging process (53). Despite the age-related difference in MPS, no difference in the phosphorylation of mTOR, p70S6K, Akt, 4EBP-1, and RPS-6 was found between young and old serum-treated myotubes.

Although this observation is generally consistent with data from in vivo human trials showing no age-related impairment in basal anabolic signaling (20, 54, 55), it should be noted that the phosphorylation of mTOR, p70S6K, and Akt increased only in myotubes conditioned with young serum. Similarly, in response to the addition of leucine, a blunted MPS response and mTOR, p70S6K, and Akt phosphorylation was observed in myotubes conditioned with older versus younger donor serum. This suggests that old serum-treated myotubes experience a diminished anabolic response to leucine treatment, which aligns with the concept of age-related muscle anabolic resistance to protein provision demonstrated in in vivo human and animal experimental studies (20, 21, 56, 57). In addition, we identified no difference in markers of MPB, including MuRF-1 and MAFbx, in response to the addition of young versus old serum alone, which is consistent with previous in vivo work in rats that identified no difference in MuRF-1 protein content between middle-aged and older mice (58). However, despite the fact that MuRF-1 and MAFbx have been shown to be upregulated in models of atrophy such as immobilization and limb unloading, the role of the ubiquitin-proteasome pathway in sarcopenia progression is unclear (59, 60). Overall, this suggests that the addition of serum from older individuals contributes to a dysregulation in MPS, that may underpin changes in myotube atrophy identified.

The differences in myotube growth and MPS between cells treated with ex vivo serum from young and old individuals may partially be underpinned by the low-grade inflammatory status that is a hallmark of aging (31). Aging is often associated with chronic elevations in concentrations of pro-inflammatory cytokines including CRP, IL-6, and TNF- $\alpha$  (61) and may increase sarcopenia risk (15) through association with MPS (17). Indeed, the present data show elevated concentrations of pro-inflammatory cytokines in old versus young individuals. In rodents, it has been suggested that low-grade inflammation (LGI) influences the age-related anabolic resistance to food intake, whereas rodents without LGI appear to restore robust postprandial MPS responses (62, 63). Further in vivo human studies are required to resolve the role of LGI on postprandial MPS in older humans, which is currently unclear (64, 65). Other systemic factors, such as glucose (66) and insulin (67), have been suggested to influence the growth of C2C12s through myogenesis, although these factors did not differ between young and old donors in the present study. We acknowledge that alterations in factors not measured in this study including myostatin (68), irisin (69), testosterone (70), insulin growth factor-1, and growth hormone (71) may also influence age-related muscle atrophy. Finally, the relative role of inherent aging processes [e.g., senescence (72), endocrine changes and mitochondrial dysfunction (73)] and artifacts of biological aging [e.g., inactivity (74–76) and raised BMI (77)], that induce LGI, in age-related muscle loss and MPS dysregulation requires further attention. It is likely that all of these factors play some role in sarcopenia progression, with the degree varying between individuals.

Although this in vitro model provides a novel, valuable means of investigating mechanisms of age-related muscle loss, it is worth noting that there are limitations to this approach. In the present study, myotubes were conditioned with only 10% human serum in order to maintain cell

viability. Although this induced some changes in anabolic signaling markers, this low concentration may not maximally stimulate changes in MPS, anabolic or catabolic markers. In addition, we did not condition primary human skeletal muscle cells with young and old human serum, instead using C2C12 murine cells, which may cause issues related to cross-species differences. Although this approach is beneficial for use when human primary skeletal muscle cells are unavailable, we are unable to discount the potential role of intrinsic muscle properties and cellular environmental factors on the regulation of growth with serum treatment. Future research should, therefore, aim to replicate this work using human primary skeletal muscle cells from young and old donors, matched with serum from these participants. Finally, the dose of leucine applied herein could be deemed “supraphysiological,” and repeat experiments using fed versus fasted serum would be a useful means of confirming and expanding on the current findings.

In conclusion, conditioning cell culture media with ex vivo human serum enabled us to establish a novel model to study the effects of the young and old systemic milieu on regulatory mechanisms of myotube growth. We report that ex vivo serum from young participants increased MPS and induced myotube hypertrophy, whereas serum from old participants induced myotube atrophy in comparison to untreated control myotubes and young treated myotubes, allied to lower rates of MPS. Furthermore, we showed that myotubes conditioned with old serum display a diminished anabolic response to the addition of leucine treatment, in comparison to those treated with young serum. Collectively, this study indicates that ex vivo human serum can be successfully used to create an in vitro model to study potential mechanisms of and countermeasures to age-related muscle loss, providing a more physiologically relevant model than current pharmacological treatments.

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## DISCLAIMERS

The views expressed are those of the authors and not necessarily those of the NIHR or the Department of Health and Social Care.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

S.L.A., G.G.L., and L.B. conceived and designed research; S.L.A. and R.N.M. performed experiments; S.L.A. and S.J.E. analyzed data; S.L.A., R.N.M., S.J.E., J.M.L., G.G.L., and L.B. interpreted results of experiments; S.L.A. prepared figures; S.L.A., G.G.L., and

L.B. drafted manuscript; S.L.A., R.N.M., S.J.E., J.M.L., G.G.L., and L.B. edited and revised manuscript; S.L.A., R.N.M., S.J.E., J.M.L., G.G.L., and L.B. approved final version of manuscript.

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