Experimental Gerontology 110 (2018) 23–34

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

# Sestrins are differentially expressed with age in the skeletal muscle of men: A cross-sectional analysis

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ARTICLE INFO

Section Editor: Christiaan Leeuwenburgh

*Keywords:*
Aging
Sarcopenia
Sestrins
mTORC1
Autophagy
Antioxidant

ABSTRACT

A gradual loss of skeletal muscle mass is a common feature of aging, leading to impaired insulin sensitivity and mobility. Sestrin1, 2, 3 are multifunctional proteins that regulate the mammalian target of rapamycin complex (mTORC1), autophagy and redox homeostasis. It is unclear how aging affects Sestrins and their downstream targets in human, therefore this study examined the basal expression of Sestrins in three age groups, young, middle-aged and older men and explored the mTORC1 pathway, autophagy markers and antioxidant regulation. Older men had less Sestrin1 and 3 protein and a different pattern of Sestrin2 electrophoretic mobility. The mRNA expression of *SESN1* was upregulated in older men, but the discrepancy was not by microRNA expression. Although protein expressions of Sestrins were downregulated with aging, phosphorylation of AMP-dependent protein kinase (AMPK$\alpha^{Thr172}$) and read-outs of mTORC1 activation, ribosomal protein S6 kinase 1 (p70S6K1$^{Thr421/Ser424}$) and 4E-binding protein 1 (4E-BP1) mobility shift were unaltered. However, total p70S6K1 and 4E-BP1 were reduced in middle-aged and older men. The mRNA expressions of autophagic markers including microtubule-associated protein 1 light chain 3 (*LC3*) and BCL2 interacting protein 3 (*BNIP3*) were upregulated in middle-aged and older men. Although nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) was upregulated in older men, the protein and mRNA expressions of its downstream antioxidants were either increased, decreased or unaltered. No clear relationship was observed between Sestrins and their downstream targets, yet it can be concluded that Sestrins proteins are clearly downregulated with aging.

## 1. Introduction

Skeletal muscle mass and function are key determinants of metabolic health and physical performance (Wolfe, 2006). After reaching its peak in early adulthood, skeletal muscle mass and function gradually decline from the fourth decade of life, a condition known as sarcopenia (Janssen et al., 2002). This is associated with impaired whole-body insulin sensitivity (Srikanthan and Karlamangla, 2011) and mobility (Visser et al., 2005). Sestrins have been recently identified as a family of stress-responsive proteins composed of 3 members: Sestrin1, 2 and 3 (Budanov et al., 2002). They play important roles in aging by attenuating oxidative stress (Bae et al., 2013), stimulating autophagy (Maiuri et al., 2009), and regulating the AMP-dependent protein kinase (AMPK) and mammalian target of rapamycin complex (mTORC1) axis (Lee et al., 2013). Genetic ablation of Sestrin in *Drosophila* (*dSesn*) and *Caenorhabditis elegans* (*cSesn*), lead to the development of several age-related pathologies, including triglyceride accumulation, excess reactive oxygen species (ROS) production and skeletal muscle degeneration (Yang et al., 2013; Lee et al., 2010). The relationship between Sestrins and aging in human skeletal muscle is yet to be elucidated.

mTORC1 is a critical regulator of cell growth, metabolism and autophagy (Laplante and Sabatini, 2012). Sestrins can suppress mTORC1 by phosphorylating AMPK$\alpha^{Thr172}$ (Budanov and Karin, 2008) and have been proposed to regulate autophagy, likely via AMPK and mTORC1 (Lee et al., 2010; Budanov, 2011). Accumulation of oxidized protein and protein aggregates are common features in aged tissue (Grune et al., 2004). These could be causative factors in some age-associated diseases (Vilchez et al., 2014); therefore, some degree of autophagy is important for healthy aging (Rubinsztein et al., 2011). How aging affects Sestrins and its association with the mTORC1 pathway and autophagy have not been explored in human skeletal muscle previously.

Apart from promoting autophagy via mTORC1, Sestrins can suppress ROS formation through nuclear factor (erythroid-derived 2)-like 2 (Nrf2) upregulation. This occurs by promoting the degradation of Nrf2 inhibitor, Kelch-like ECH-associated protein (Keap1). Once released, Nrf2 acts as a key factor to facilitate gene transcription of multiple

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https://doi.org/10.1016/j.exger.2018.05.006
Received 9 January 2018; Received in revised form 21 March 2018; Accepted 7 May 2018
Available online 08 May 2018
0531-5565/ 

antioxidant genes (Ichimura et al., 2013). Increased oxidative stress is a common feature of aging and Nrf2 activation is often impaired (Shih and Yen, 2007). However, controversy remains around the direction of change in the basal expression of antioxidants (Zhang et al., 2015). The association between Sestrins and antioxidant response has also not previously been explored in human skeletal muscle.

Dysregulation of mTORC1 signaling, accumulated protein aggregates and increased oxidative stress alters whole body metabolism and contributes to the etiology of age-related diseases (Vilchez et al., 2014; Shih and Yen, 2007; Cornu et al., 2013). Given that Sestrins regulate mTORC1, autophagy and suppress ROS accumulation, it is hypothesized that Sestrins may attenuate stress-dependent acceleration of aging (Lee et al., 2010). Mammals have three Sestrins paralogs, how each might be affected by aging is unclear. As Sestrins may play a role in muscle maintenance (Yang et al., 2013; Lee et al., 2010), the objective of this study is to explore Sestrin-related signaling pathways and characterize the expression of all three Sestrins in the skeletal muscle of a healthy cohort including from young, middle-aged and older men.

## 2. Materials and method

### 2.1. Participants

This study reports cross-sectional observations collected from the baseline period of multiple clinical trials conducted at the University of Auckland Nutrition and Mobility Clinic and Deakin University by the same lab group led by the same principal investigator. A total of seven clinical trials were included (D'Souza et al., 2014; Mitchell et al., 2015, 2016, 2017a, b, c), with one not yet published (ACTRN12617000393358). Participants were assessed for baseline physical and biochemical characteristics. In total, 182 healthy men from three different age groups, 31 young (18–30 years), 78 middle-aged (40–55 years) (Mitchell et al., 2015; Mitchell et al., 2016, 2017b, c) and 73 older men (65–80 years) (D'Souza et al., 2014; Mitchell et al., 2017a) were included in the analyses.

Participants were all within the BMI range of 18 to 35 $kg/m^2$. All were largely sedentary while healthy and did not engage in regular resistance training. However, those involved in aerobic exercises were not excluded. Participants lived in New Zealand and Australia. They did not consume any dietary supplements in the one month preceding the trial and were able to perform activities of daily living independently without mobility aids. All were non-smokers, free of cancer, diabetes, mental illness, digestive disorders, thyroid diseases, cardiovascular diseases, metabolic diseases and conditions affecting neuromuscular functions. Young and middle-aged participants taking medications such as statins, steroids or nonsteroidal anti-inflammatory drugs which may affect muscle biology were excluded. However, older men that were taking anti-coagulation or anti-hypertensive medications were not excluded.

Thigh muscle cross-sectional area (CSA) and isometric knee extension torque were unavailable for 40 middle-aged, while grip strength was unavailable for 60 middle-aged men. 43 older men were recruited at Deakin University (D'Souza et al., 2014), which only measured weight and BMI, therefore baseline physical and biochemical characteristics were only available from the 30 older men recruited at the University of Auckland (Mitchell et al., 2017a). All participants were informed of the requirements and potential risks of the studies prior providing their written informed consent. The experimental procedures adhered to the standards set by the latest version of the Declaration of Helsinki and were approved by the Health and Disability Ethics Committee (New Zealand) and Deakin University Human Research Ethics Committee (Australia).

### 2.2. Imaging and physical function

Participant's body weight was measured without shoes in light clothing and height was measured on a stadiometer without shoes. Full body dual-energy X-ray absorptiometry (DXA) scans (Lunar Prodigy, GE, Waltham, MA, USA) was used to quantify total lean and fat mass. The whole body scan was automatically segmented by the software. Thigh muscle CSA at 50% femur length of the dominant leg was assessed by using a Stratec XCT 3000 peripheral quantitative computed tomography (pQCT) with software version 6.20C (Stratec Medizintechnik, Pforzheim, Germany). A detailed description has been previously reported (Mitchell et al., 2017c). Briefly, participants were positioned supine with the dominant leg centered within the machine's gantry and anchored by a footrest with straps to limit movement during each scan.

Isometric muscle strength of knee extensors was tested by using a Biodex System 4 dynamometer (Shirley, New York, United States) with the knee angle set to 90° of flexion. Lower limb strength testing occurred at least 72 h prior to collections of muscle biopsies. Isometric grip strength was measured by using a Jamar dynamometer (Patterson Medical) with the grip set to position 2 (Mitchell et al., 2017a).

### 2.3. Muscle biopsy sampling

Participants were asked to refrain from intense physical activity prior to the experimental trial. On the evening before the trial, participants were instructed to consume their normal diet before 10 pm and were instructed to consume nothing except water for the rest of the evening. Participants arrived fasted to the lab at 7 am, a cannula (20-gauge) was inserted into an antecubital vein and a baseline blood sample was obtained. Plasma was collected in 4 mL EDTA vacutainers and centrifuged immediately upon collection at 4 °C at 1,900*g* for 15 min. The supernatant was collected in 1.6 mL sterile tubes as 1 mL aliquots and stored at −80 °C until further analysis. A muscle biopsy (~100 mg) was collected at rest from the *vastus lateralis* muscle under local anesthesia (1% Xylocaine) using a Bergstrom needle modified for manual suction. Biopsies were quickly frozen in liquid nitrogen and stored at −80 °C until further analyses.

### 2.4. Plasma analysis

Plasma biochemistry analysis was performed using a Roche C311 autoanalyzer (Roche, Mannheim, Germany) by enzymatic colorimetric assay. Plasma insulin concentrations were analyzed using electrochemiluminescence immunoassay on a Cobas e11 (Roche, Mannheim, Germany). The homeostatic model assessment (HOMA) was used to estimate insulin resistance (HOMA2-IR) and beta cell function (HOMA2-%B) by using the nonlinear computer model obtained from HOMA Calculator v2.2.2 (The Oxford Centre for Diabetes, Endocrinology and Metabolism, UK) (Wallace et al., 2004).

### 2.5. Immunoblotting

~20 mg of muscle biopsies were homogenized for 40 s at 20 Hz using a TissueLyser (Qiagen, Venlo, Netherlands) in lysis buffer containing 10 μL/mg 25 mM Tris 0.5% v/v Triton X-100 supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). The supernatant was collected after centrifugation to determine protein concentration using the BCA protein assay kit (Thermo Scientific, Waltham, MA). Laemmli's buffer was added to protein homogenates and boiled for 5 min at 95 °C. Denatured proteins were separated by SDS-PAGE and proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA) using TransBlot semidry transfer apparatus (Bio-Rad, Hongkong, China). Membranes were blocked in 5% skim milk in TBST for 1 h at room temperature, and probed using specific antibodies for 4E-BP1, p-p70S6K$^{Thr421/Ser424}$, total p70S6K, p-AMPKα$^{Thr172}$ and total AMPKα (Cell Signaling, 9644, 9204, 2708, 2535 and 2532 respectively), Sestrin2 (ProteinTech, 10795-1-AP), NADPH oxidase 4, glutathione reductase, SOD1, SOD2, SQSTM1/p62, Sestrin1 and 3 (Abcam, ab133303, ab16801, ab13498, ab13533, ab56416, ab103121 and ab97792 respectively). Primary antibodies were incubated overnight at 4 °C (1:1,000 dilution, except Sestrin1 which is 1:100) with