The influence of systemic inflammation on skeletal muscle in physically active elderly women

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Abstract The biological mechanisms responsible for the decline in skeletal muscle mass during aging remain unknown. It is hypothesized that elevations in the level of the acute phase C-reactive protein (CRP) negatively affect skeletal muscle mass in elderly. We examined the relationship between serum CRP and muscle mass in a population of active elderly women (65–70 years; n =23). Though all subjects were physically active, serum CRP levels were negatively associated to the amount of time spent in moderate-to-vigorous physical activity (R^2=0.20, P=0.032) and to skeletal muscle mass (R^2= 0.28, P=0.009). We further aimed to determine the potential mechanisms behind the action of systemic inflammation on skeletal muscle by exposing myoblasts isolated from vastus lateralis to the different sera from each elderly woman. The doubling time (DT) of myoblasts increased when cells were exposed to sera with high CRP levels (R^2=0.27, P=0.011), indicating that CRP contributes to the impairment of the proliferative rate of myoblasts in elderly. In order to further confirm our findings, we incubated human myoblasts in exogenous CRP. Exposition to exogenous CRP induced an increase in myoblast DT by 1.21-fold (P=0.007) and a reduction in the expression of the proliferation marker ki-67 confirming the negative influence of CRP on myoblast proliferative rate. Collectively, these findings highlight the contribution of the systemic inflammatory status in the age-related decline in skeletal muscle function.

Keywords Aging · Skeletal muscle · Inflammation · CRP · Myoblast · Physical activity

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Introduction

The functional status in elderly is greatly influenced by the gradual loss of skeletal muscle mass (Metter et al. 2002; Short et al. 2004). Muscle weakness, especially in lower extremities, is an independent risk factor for falls in elderly (Moreland et al. 2004), and chronic muscle loss is estimated to affect 5–13 % of adults aged between 60 and 70 years and may affect more than 50 % of those older than 80 years (Baumgartner et al. 1998; Cruz-Jentoft et al. 2010; Morley 2008).

The exact underlying biological mechanisms responsible for the gradual decline in skeletal muscle mass during aging are not clearly understood. A hypothesis that has gained a large consideration in the scientific community during the past decade is the association between chronic systemic inflammation and many age-related deleterious changes in different tissues (Eklund 2009; Franceschi et al. 2000; Giunta 2008; Pearson et al. 2003; Rieu et al. 2009). Serum level of the circulating

acute phase C-reactive protein (CRP) is an established marker of systemic inflammation and is recognized as an important factor underlying several age-related deleterious changes (Ansar and Ghosh 2013; Blake and Ridker 2002; Eklund 2009; Ferrucci et al. 2005). Although some reports failed to show any evidence of increases in inflammatory biomarkers in healthy elderly (Ahluwalia et al. 2001; Beharka et al. 2001), associations between chronic systemic inflammation and muscle mass and strength have been supported by several investigations (Cesari et al. 2005; Hamer and Molloy 2009; Schaap et al. 2009; Visser et al. 2002). However, less is known about the relationship between chronic systemic inflammation and muscle mass in healthy, physically active elderly knowing that the level of physical activity (PA) has been shown to be inversely associated to chronic systemic inflammation (Geffken et al. 2001; Shanely et al. 2013).

The regenerative rate of skeletal muscle is supported by muscle satellite cells, which under the influence of increased workload or injury are activated and start to proliferate to generate myoblasts that differentiate and fuse together to form new muscle tissue (Hawke and Garry 2001). An impairment in the proliferative rate of satellite cells in elderly would result in a slower ability to respond to hypertrophic stimuli, a delayed muscle regeneration and a longer recovery time from intensive workload, which ultimately negatively affects muscle mass (Hawke and Garry 2001; Kadi and Ponsot 2010). The influence of the systemic environment on skeletal muscle has been supported by the seminal work conducted by Conboy and collaborators who used a parabiotic model in mice to show an improvement of the regenerative capacity of injured old skeletal muscle exposed to a youthful systemic milieu (Conboy et al. 2005). It has been shown that human myoblasts obtained from young and old donors have similar myogenic behavior (Alsharidah et al. 2013; George et al. 2010). However, the exposure of myogenic cells from old mice to serum from young mice induces an enhancement of the proliferative capacity (Conboy et al. 2005). Similar findings were reported in two other studies using human myogenic cells (Barberi et al. 2013; Carlson et al. 2009), indicating that the aged systemic environment can be the cause of the age-related decline in the proliferative rate of myoblasts. However, the chronological age of serum donors per se might not explain the deleterious effects of the systemic environment on myogenic cells as it has been shown that the ability of human myoblasts to proliferate is not impaired by culture in an aged milieu (George et al. 2010). Therefore, we hypothesized that the level of systemic inflammation in elderly is an important factor able to influence skeletal muscle mass and the proliferative rate of myoblasts. For that purpose, we first evaluated the relationship between the systemic inflammatory marker CRP and skeletal muscle mass as well as physical activity level in healthy and recreationally active elderly women. Next, we examined the influence of the systemic inflammatory marker CRP on the proliferative rate of myogenic cells by exposing myoblasts isolated from vastus lateralis of elderly to the different sera from all elderly women. In order to further ascertain the influence of CRP on myogenic cells, we examined the effect of exogenous CRP on the proliferative rate of myoblasts obtained from each elderly woman.

Methods

Subjects

Twenty-three healthy and physically active elderly women (mean age 67; SD 1.5 years; mean body weight 70.6; SD 13 kg; mean height 164; SD 5 cm) were included in the study. All subjects were examined by a physician and had no history of cardiac and pulmonary diseases, uncontrolled hypertension (seven out of 23 subjects used β-blockers or ACE-inhibitors) or diabetes and had normal levels of fasting blood glucose, cholesterol (including HDL and LDL), and triglycerides. To be included, the subjects had to be recreationally active. All subjects included in the study participated in various recreational physical activities such as walking, Nordic walking, jogging, cycling, golf, swimming, skiing, and aerobics. A 6-day objective measurement of physical activity was performed on all subjects included in the study. After verbal and written information, all participants signed an informed written consent and the ethical approval was obtained from the regional Ethical Review Board in Uppsala.

Assessment of physical activity

PA was assessed by accelerometry (ActiGraph model GT3x, Pensacola, FL, USA). The monitor was worn with an elastic belt on the right hip for six consecutive days. Participants were informed to wear the monitor all

waking hours except for bathing or swimming. The monitors were initialized and downloaded using the manufacturer software ActiLife 6. None-wear time was defined as periods of at least 60 consecutive minutes of zero values. The total number of counts per minute (cnts min^{-1}) and the number of minutes spent in moderate-to-vigorous physical activity (MVPA) were derived. MVPA was defined as all activities with intensity over 2000 cnts min^{-1}, a threshold frequently used and equivalent to an energy expenditure of ≥3 METs (Hansen et al. 2012; Nilsson et al. 2009).

Body composition

All assessments were performed between 7:00 and 9:00 am after an overnight fast. Body composition was measured using bioelectrical impedance analysis (BIA) (TANITA BC-420MA, Tanita Corporation, Japan). Skeletal muscle mass was calculated using the equation by Janssen et al. (2002): Skeletal muscle mass (kg)=[(height^2/BIA-resistance 0.401)+(gender 3.825)+(age −0.071)]+5.102, where, height is in cm; BIA-resistance is in ohms; gender=0 for women; and age is in years. A skeletal muscle mass index (SMI) in percentage is calculated as follows: skeletal muscle mass/body mass×100.

Laboratory measurements

Blood samples were obtained between 7:00 and 9:00 am, after an overnight fast. The participants were asked to avoid smoking and alcohol and not to engage in any strenuous physical activity 24 h before the blood sample. Blood was collected by venipuncture from an antecubital vein, centrifuged at 4000 rpm for 10 min, aliquoted, and stored in −80 °C. Fasting blood glucose was assessed using Reflotron Plus (Roche), and cholesterol (HDL and LDL) and triglycerides were assessed using the Vitros 5.1 Ortho Clinical Diagnostics. CRP level was measured using a high-sensitivity C-reactive protein (Hs-CRP) kit by a fully automated immunoturbidimetric assay (Advia 1800, Chemistry System, Siemens, Germany) and the lowest detection level was 0.16 mg/L.

Isolation and purification of human myoblasts

Muscle biopsies were performed by a trained medical doctor on the mid-portion of the vastus lateralis using the Weil-Blakesley conchotome and were minced using sterile scalpels. One-cubic millimeter explants were scissor-minced and were trapped inside a thin layer of 6 mg/mL Matrigel (BD Matrigel Matrix from BD Biosciences) in 35-mm collagen-coated Petri dishes with growth media (Dulbecco's modified Eagle medium (DMEM), Sigma) supplemented with 20 % fetal bovine serum (HyClone Perbio), 2 % Ultroser G (Pall, France), 10 mM Hepes (H0887, Sigma), and 50 μg/mL gentamicin (G1397, Sigma). After 6 to 8 days, cells migrated out of the explants. Migrating cells were enzymatically harvested using dispase (BD Biosciences) and subcultured in growth medium. Harvested cells were purified by immunomagnetic cell sorting using magnetic activated cell sorter (MACS) microbeads (Miltenyi Biotec) coupled to an antibody against CD56 (Barro et al. 2010). At cell isolation, all myoblasts were considered to be at 1 population doubling (PD). All experiments were performed at 4 PD.

Proliferation rate of human myoblast exposed to different sera

Myoblasts pooled from skeletal muscle samples of 10 elderly subjects were seeded at 3×10^4 cells/dish onto 35-mm collagen-coated dishes and were exposed to 15 % serum from each elderly woman. At 96 h, the cells were counted using a hemocytometer (Bürker). Myoblast doubling time (DT) was calculated as follows: DT=96/(ln (y/x)/ln2), where x is the number of cells counted at 0 h and y the number of cells counted at 96 h (Barro et al. 2010).

Immunohistochemistry

Cells were fixed in 4 % formaldehyde, treated with 0.5 % Triton and immunostained for 2 h at room temperature using the primary antibodies against desmin (mouse monoclonal Sigma D1033, 1:100) and Ki-67 (rabbit polyclonal Biocare Medical CP249, 1:100). Myoblasts expressing the muscle-specific marker desmin are labeled desmin$^+$myoblasts. Desmin$^+$myoblasts expressing the proliferation marker Ki-67 are labeled desmin$^+$ki-67$^+$myoblasts. Secondary antibodies Alexa Fluor 488 (goat anti-mouse) and Alexa Fluor 568 (goat anti-rabbit) were used. Nuclei are stained blue with DAPI. Myoblasts were viewed using a Zeiss Axiovert microscope, and at least seven randomly selected fields were used for the quantification of the number of

desmin$^+$myoblasts and desmin$^+$ki-67$^+$myoblasts (an average of 860 nuclei was counted for each well).

Effects of exogenous CRP

Isolated myoblast were seeded at 3×10^4 cells/dish onto 35-mm collagen-coated dishes and cultured in growth medium without (non-treated myoblasts) and with exogenous CRP (C1617, Sigma) at a concentration of 15 μg/mL (CRP-treated myoblasts). A total of five experiments in duplicate from five separate human primary myoblast cell cultures were performed.

Statistical analysis

Variables with a skewed distribution (DT and CRP) were logarithmically transformed. Linear regression was used to examine the relationship between serum CRP level (explanatory variable) and SMI as well as the proliferative rate of myoblasts. The association between MVPA (explanatory variable) and serum CRP level was assessed by linear regression. Unpaired *t* test was used to compare the proliferative rate between CRP-treated and non-treated myoblasts. Statistical significance was set at $P<0.05$. All analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL).

Results

The elderly women (65–70 years) included in the study were healthy and had normal cholesterol (mean 6.0; SD 1.0 mmol/L), HDL (mean 1.5; SD 0.3 mmol/L), LDL (mean 3.4; SD 0.9 mmol/L), fasting glucose (mean 5.5; SD 0.6 mmol/L), and triglycerides (mean 1.17; SD 0.46 mmol/L) levels. Physical activity was objectively measured during six consecutive days using accelerometry to determine the time (min/day) spent in moderate-to-vigorous intensity physical activity (MVPA). We found that the participants spent at least 38 min/day in MVPA, which is above the recommendations for physical activity in young as well as older adults (Haskell et al. 2007; Nelson et al. 2007). The measurement Hs-CRP in this population of recreationally active elderly women revealed large inter-individual variations as serum CRP levels ranged between 0.16 and 8.9 mg/L with an average of 2.5 mg/L. We found that serum CRP levels were inversely associated to MVPA ($R^2=0.20$, $P=0.032$), indicating that even in recreationally active elderly women, the amount of MVPA contributes to the control of systemic CRP levels. We also found an inverse relationship between serum CRP level and skeletal muscle mass index (SMI) ($R^2=0.28$, $P=0.009$) (Fig. 1) highlighting the important influence of the systemic marker CRP on skeletal muscle mass even in physically active elderly.

In order to determine the potential mechanisms behind the action of systemic inflammation on skeletal muscle, an in vitro model was used to evaluate the proliferative rate of myoblasts from vastus lateralis of elderly exposed to the different sera from all elderly women. The use of the immunomagnetic cell sorting system allowed obtaining highly purified myoblasts from vastus lateralis biopsies as evidenced by the staining with the muscle cell-specific marker desmin, which showed that more than 99 % of the cells were desmin$^+$myoblasts (Fig. 2). When myoblasts, pooled from skeletal muscle samples of 10 elderly subjects, were exposed to the different sera from all elderly subjects, we found that the doubling time (DT) of myoblasts increased when cells were exposed to sera with high CRP levels ($R^2=0.27$, $P=0.011$) (Fig. 3), indicating a decrease in the proliferative rate of myoblasts as serum CRP level increases. This implies that subtle differences in the concentration of CRP in human serum can affect the proliferative rate of myoblasts.

In order to confirm the finding that the concentration of CRP in human serum has an influence of the proliferative rate of myoblasts, we next exposed myoblasts from elderly woman to exogenous CRP. We found that myoblast DT significantly increased by 1.21-fold ($P=0.007$) after incubation with 15 μg/mL exogenous CRP, indicating a reduction of the proliferative rate and confirming the influence of the inflammatory marker

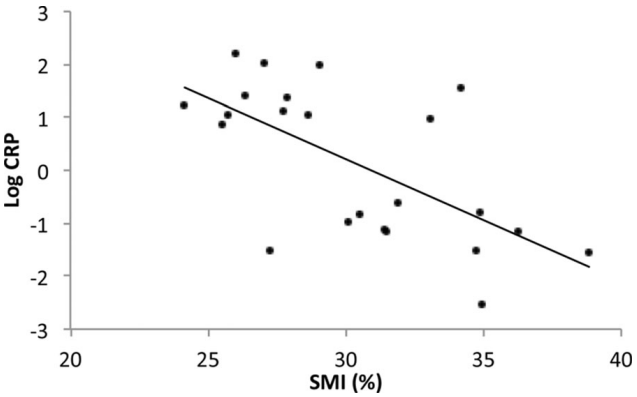

Fig. 1 The association between log CRP and skeletal muscle mass index (*SMI*%) in elderly women. $R^2=0.28$, $P=0.009$

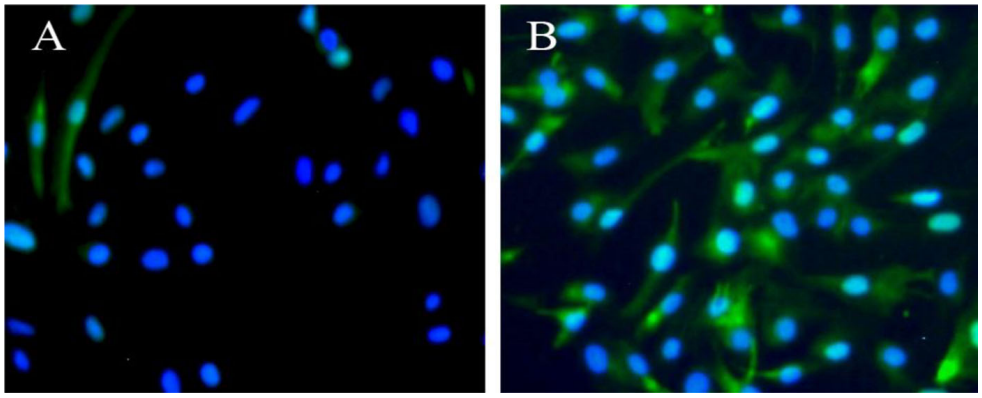

Fig. 2 Immunofluorescence staining for the visualization of desmin+myoblasts (*green*) before (**a**) and after (**b**) immunomagnetic cell sorting. Nuclei are stained blue with DAPI

CRP on the proliferative rate of myoblasts. In order to determine the mechanisms by which systemic inflammation might affect the proliferative rate of myoblasts, we next investigated the effect of exogenous CRP on the proliferation marker Ki-67 in myoblasts from elderly women. The proportion of desmin^{+}ki-67^{+}myoblasts expressed as a percentage of the total desmin^{+}myoblasts was evaluated after 12-, 48-, and 96-h incubation with 15 μg/mL exogenous CRP (Fig. 4). We found that the proportion of desmin^{+}ki-67^{+}myoblasts was lower in the CRP-treated myoblasts both at 48 h (P=0.005) and 96 h (P=0.01) compared to the non-treated myoblasts, which indicates that the effects of CRP on the proliferative rate of myoblasts might be mediated by a decrease in the expression of the proliferation marker ki-67.

Discussion

The gradual loss of skeletal muscle mass during aging has important clinical implications and contributes to the decline in quality of life in elderly. The present investigation is the first to combine the assessment of the relationship between skeletal muscle mass and its systemic environment with the study of the influence of the systemic inflammatory status in physically active elderly on the proliferative rate of myogenic cells using an in vitro approach. Notably, we demonstrated that (1) even in recreationally active elderly women, the level of the systemic inflammatory marker CRP is influenced by the time spent in moderate-to-vigorous physical activity, (2) serum CRP level in recreationally active elderly women is negatively associated with skeletal muscle mass, (3) elevations in serum CRP levels in elderly contribute to the reduction of the proliferative rate of myoblasts from elderly, (4) exogenous CRP reduces the proliferative rate of myoblasts from elderly, and (5) the effects of CRP on the proliferative rate of myoblasts might be mediated by a decrease in the expression of the proliferation marker ki-67.

Several previous investigations exploring the influence of factors contributing to the age-related decline in skeletal muscle mass included physically inactive or participants with unknown fitness level as well as frail subjects with chronic diseases. Here, we aimed to explore the influence of the systemic environment on muscle mass in a group of physically active elderly women. The time spent in moderate-to-vigorous physical activity was above the recommendations for physical activity in both young and older adults (Haskell et al. 2007; Nelson et al. 2007) and higher than what has been reported in previous studies assessing physical activity behaviors in elderly women (Hagstromer et al. 2010; Orsini et al. 2008). Earlier studies have reported an inverse relationship between physical activity and chronic systemic inflammation (Geffken et al. 2001;

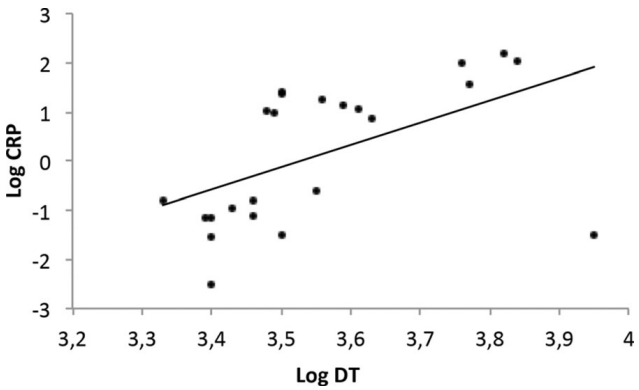

Fig. 3 The association between log CRP in serum and myoblast log doubling time, R^2=0.27, P=0.011

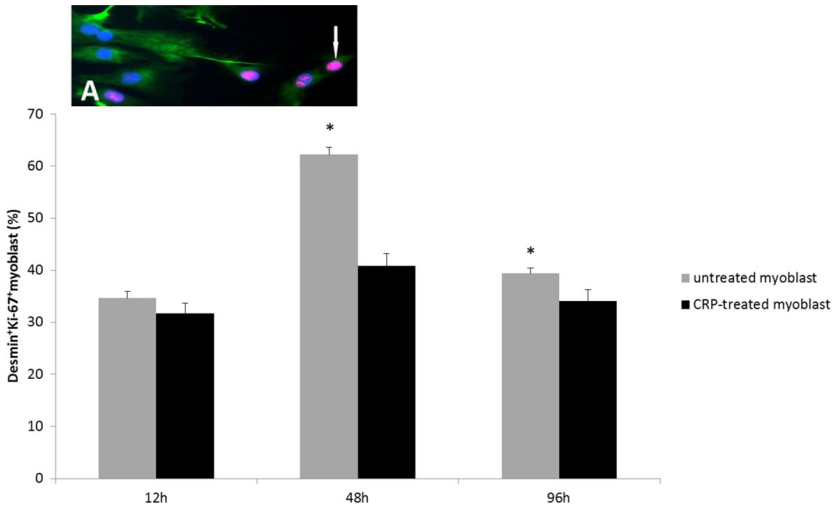

Fig. 4 The effect of exogenous CRP on the proportion of desmin+Ki-67+myoblasts at 12, 48, and 96 h of proliferation. *Insert* (*a*) shows desmin+Ki-67- myoblasts in *blue* and desmin+ Ki-67+myoblasts in *pink* (*arrow*). A total of five experiments in duplicate from five separate human primary myoblast cell cultures were performed. *Asterisks* indicate significant difference ($P<0.05$) between CRP-treated and untreated myoblasts. Values are mean±SEM

Prestes et al. 2009; Schaap et al. 2009; Shanely et al. 2013). Here, we show that even in physically active elderly women, the amount of moderate-to-vigorous physical activity is an important factor accounting for the variability in serum CRP levels, which highlights the importance of the intensity of physical activity in the control of the status of the chronic systemic inflammation. Importantly, our data demonstrate that elderly women with the highest serum CRP levels had the lowest skeletal muscle mass, which indicates that elevations in the inflammatory status can be detrimental for skeletal muscle mass even in physically active elderly women.

Despite the occurrence of associations between inflammatory serum markers and muscle mass in elderly, the underlying mechanisms remain unclear. Here, we show that when myoblasts are exposed to sera from elderly women with different CRP levels, the proliferative rate of myoblasts is reduced as serum CRP level increases. Serum CRP level significantly explained 27 % of the variability in myoblast proliferative rate, which is indicative of the influence of the systemic milieu on myogenic cells. To further elucidate the effect of CRP on myogenic cells, myoblasts from each elderly woman were incubated in the presence of exogenous CRP. Treatment with CRP significantly affected the proliferative rate of myoblasts from elderly, which further strengthens the hypothesis that the inflammatory status of the systemic environment in elderly can exert an important influence on muscle tissue.

In order to determine the mechanisms by which CRP influences the proliferative rate of myoblasts, we have examined the expression of the proliferation marker ki-67. This nuclear antigen has been traditionally used as a marker of proliferating cells and is expressed initially in mid-G1, increases during S and G2, and peaks in the M phase of the cell cycle (Gerdes et al. 1984). The CRP-induced downregulation of Ki-67 indicates that increases in systemic CRP can affect the proliferative rate of myoblasts. In support of the influence of CRP on the cell cycle, it has been shown that a p53-mediated cell cycle arrest occurs in CRP-treated cardiac myocytes via the ERK1/2 signaling pathway (Choi et al. 2011; Lee et al. 2014). It has also been shown that CRP induces G2/M phase cell cycle arrest and apoptotic cell death in human monocytes (Kim et al. 2014).

Our data suggest that the systemic inflammatory status in elderly has an influence of the behavior of myoblasts. This conclusion supports the hypothesis that age-related changes in the systemic environment can contribute to the decline in skeletal muscle function (Barberi et al. 2013; Carlson et al. 2009; Conboy et al. 2005). Previous studies showed that the proliferative potential of old cells can be rejuvenated if exposed to a young environment indicating that the proliferative potential can be modulated by changes in the systemic environment (Barberi et al. 2013; Carlson et al. 2009; Conboy et al. 2005; Conboy and Rando 2012). The rejuvenating action of young serum can be partly explained by the low systemic inflammatory status of young adults compared to older adults. It has previously been shown that myoblasts from young and old have similar myogenic behavior (Alsharidah et al. 2013; George et al. 2010) and that the age of the serum donor has no significant effect on the proliferative potential of myoblasts (Alsharidah et al. 2013; George et al. 2010), which together with our data suggests that the

inflammatory status is more important than the chronological age of serum donor. Noteworthy, our findings are based on an approach allowing studying the influence of the inflammatory status on the proliferative rate of myoblasts using a model that mimics the physiological environment of myogenic cells. Indeed, sera CRP levels in our study averaged 2.5 mg/L, which indicates that the final CRP concentration in the incubation medium is thus very low when compared to an experimental set up where exogenous CRP is added to the culture medium. This is suggestive of the strong influence that might exert subtle changes in the inflammatory status of the systemic environment on myogenic cells in elderly. Additionally, our data show that CRP level in serum reduces but does not inhibit the proliferative rate of myoblasts indicating that the inflammatory status only partly influences the proliferative rate of myogenic cells in elderly. It should be however acknowledged that the relationship between endogenous CRP concentrations and the proliferative rate of myoblasts does not necessarily imply that serum CRP level itself is a major factor influencing the proliferation of myoblasts as other factors reflected by CRP levels might act on myoblasts. Nevertheless, experiments using exogenous CRP are in favor of the impact of CRP on the proliferative rate of myoblasts.

In conclusion, this study revealed that even in physically active elderly, the systemic inflammatory status exerts a significant influence on skeletal muscle mass. A CRP-mediated decline in the proliferation rate of myoblasts together with a decrease in the expression of the proliferation marker ki-67 might ultimately lead to an age-related gradual decline in muscle mass. Finally, the influence of the systemic environment on skeletal muscle tissue opens new therapeutic avenues for improving muscle mass and function in elderly.

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