

## ***In vivo* and *in vitro* evidence that intrinsic upper- and lower-limb skeletal muscle function is unaffected by ageing and disuse in oldest-old humans**

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### **Abstract**

**Aim:** To parse out the impact of advanced ageing and disuse on skeletal muscle function, we utilized both *in vivo* and *in vitro* techniques to comprehensively assess upper- and lower-limb muscle contractile properties in 8 young (YG;  $25 \pm 6$  years) and 8 oldest-old mobile (OM;  $87 \pm 5$  years) and 8 immobile (OI;  $88 \pm 4$  years) women.

**Methods:** *In vivo*, maximal voluntary contraction (MVC), electrically evoked resting twitch force (RT), and physiological cross-sectional area (PCSA) of the quadriceps and elbow flexors were assessed. Muscle biopsies of the vastus lateralis and biceps brachii facilitated the *in vitro* assessment of single fibre-specific tension (Po).

**Results:** *In vivo*, compared to the young, both the OM and OI exhibited a more pronounced loss of MVC in the lower limb [OM (–60%) and OI (–75%)] than the upper limb (OM = –51%; OI = –47%). Taking into account the reduction in muscle PCSA (OM = –10%; OI = –18%), only evident in the lower limb, by calculating voluntary muscle-specific force, the lower limb of the OI (–40%) was more compromised than the OM (–13%). However, *in vivo*, RT in both upper and lower limbs (approx.  $9.8 \text{ N m cm}^{-2}$ ) and Po (approx.  $123 \text{ mN mm}^{-2}$ ), assessed *in vitro*, implies preserved intrinsic contractile function in all muscles of the oldest-old and were well correlated ( $r = 0.81$ ).

**Conclusion:** These findings suggest that in the oldest-old, neither advanced ageing nor disuse, *per se*, impacts intrinsic skeletal muscle function, as assessed *in vitro*. However, *in vivo*, muscle function is attenuated by age and exacerbated by disuse, implicating factors other than skeletal muscle, such as neuromuscular control, in this diminution of function.

**Keywords** *in vitro*, *in vivo*, oldest-old, sarcopenia.

The age-related loss of skeletal muscle mass and the concomitant decline in muscle strength often contribute to the disability and ultimately the mortality associated with the elderly (Janssen *et al.* 2002, Metter *et al.* 2002, Narici & Maffulli 2010, Sayer *et al.* 2013). The aetiology of this syndrome, sometimes termed sarcopenia, is multi-factorial and includes alterations in endocrine function, chronic disease, inflammation and nutritional disorders; however, ageing, *per se*, and muscle deconditioning caused by reduced physical activity appear to play a central role in this phenomenon (Doherty 2003, Fielding *et al.* 2011). Due to mobility limitations, primarily caused by a decline in both balance and ambulatory capacity, the oldest-old (>85 years) are more prone to periods of lower-limb disuse (Buford *et al.* 2012, Pojednic *et al.* 2012, Dufour *et al.* 2013), which can result in a rapid and accelerated loss of maximal force and skeletal muscle mass, most commonly in the legs (Berg *et al.* 1997, Gallagher *et al.* 1997, Janssen *et al.* 2000, Raue *et al.* 2009, Venturelli *et al.* 2012). Although there are some studies evaluating older subjects (>70 years) and the impact of disuse in skeletal muscle (D'Antona *et al.* 2003, Suetta *et al.* 2007, 2009, Hvid *et al.* 2011), very little is known about the specific and combined physiological consequences of advanced ageing and disuse in the oldest-old.

Ageing, itself, has been proposed to lead to a multitude of adaptations in the neuromuscular system that are similar to those associated with disuse (Vandervoort 2002); therefore, it is difficult to discern the exclusive effect of ageing rather than simply the effect of disuse (Suetta *et al.* 2004, 2009, Suetta & Kjaer 2010). However, it has been recognized that lower-limb disuse in old subjects appears to exacerbate the age-related decline in the contractile function of the quadriceps muscles (Suetta *et al.* 2007, 2009), and the same age–disuse interaction pattern appeared to hold true when single skeletal muscle fibre mechanics were assessed *in vitro* (D'Antona *et al.* 2003, Hvid *et al.* 2011). Yet, no one has parsed out the effect of ageing and disuse by adopting the novel approach of examining skeletal muscle function *in vivo* and *in vitro* in both the upper- and lower-limb muscles, with the former exhibiting less of a reduction in use with age as they are not relied upon for locomotion.

Several factors differentiate *in vitro* single muscle fibre function from *in vivo* whole muscle function (Bottinelli *et al.* 1999, Erskine *et al.* 2009, Canepari *et al.* 2010b). For instance, MVC, assessed *in vivo*, is influenced by neuromuscular factors downstream from central command, such as motor neurone excitability and muscle voluntary activation (Hakkinen *et al.* 1998, Izquierdo *et al.* 1999, Aagaard *et al.* 2010). Additionally, *in vivo*, the volume of skeletal

muscle mass recruited for a voluntary contraction and the muscle architecture (pennation angle) are the determining factors for *in vivo* force development (Campbell *et al.* 2013). However, the *in vitro* analysis of single muscle fibre function, such as the determination of maximal  $\text{Ca}^{2+}$ -activated isometric force ( $F_0$ ), focuses on the intrinsic contractile properties of muscle fibres. Thus, in combination, *in vivo* and *in vitro* assessments of skeletal muscle function should better parse out the contribution of the multitude of factors that are recognized to play a role in the age- and disuse-induced reduction in skeletal muscle force.

Utilizing both *in vivo* and *in vitro* approaches, this study sought to investigate the unique and combined effects of advanced ageing and long-term disuse on skeletal muscle function. Specifically, by studying the upper- and lower-limb muscles of OM (old mobile) and OI (old immobile, wheelchair bound) subjects, in comparison with the YG (healthy young), we tested the following hypotheses: (1) the lower-limb muscle of the OM and OI subjects, assessed both *in vivo* and *in vitro*, would exhibit attenuated muscle function, (2) if this *in vivo* and *in vitro* decrement in muscle function could be explained, perhaps at least partially, by the complex muscle-specific process of sarcopenia which may be accelerated by inactivity, the diminished function would therefore be more pronounced in the OI subjects, and (3) the upper-limb muscle of the OM and OI subjects, both *in vivo* and *in vitro*, would exhibit similarly attenuated muscle function, which could be attributed solely to ageing.

## Material and methods

### Subject characteristics

Twenty-four women, 8 YG (range 23–26 years), 8 OM (range 85–92 years), and 8 OI (range 86–90 years), volunteered and were approved to participate in this study based upon a physician's assessment of minimal cognitive, cardiovascular and musculoskeletal disease. This screening included a health history, a physical examination, an evaluation of lower-limb mobility (Tinetti 1986), an upper-limb activities of daily living performance test (Yozbatiran *et al.* 2008), a blood pressure assessment, a blood sample and a familiarization with the study exercise procedures. The local Ethics Committee approved the study, and all experimental procedures were performed in accordance with the Declaration of Helsinki. Written, informed consent was obtained from all participants before inclusion in the study. The young subjects were normally physically active college students. The OM subjects were all community dwelling, but able to

walk independently (Tinetti 1986). The OI subjects were selected from residents of the Mons. Mazzali Foundation, geriatric institute of Mantua, Italy. The medical staff recruited oldest-old women who at the time of the study were mobility limited in lower limbs (2 years of being wheelchair bound) and unable to stand up from a chair and walk independently (Tinetti 1986). The diagnosis of altered gait capacity, poor balance and an elevated risk falling was the cause of this lower-limb mobility restriction.

#### Upper- and lower-limb muscle volume

Magnetic resonance images (MRI) was performed using a clinical 1.5 Tesla MRI system (GE Signa hdx, Fairfield, CT, USA). T1-weighted images of the upper arm and thigh were acquired supine with the legs extended and the right arm adjacent to the trunk, elbow extended, supported on a foam pad. Images were obtained using a turbo spin echo sequence (slice thickness = 8.00 mm, gap thickness = 8–10 mm, turbo factor = 3, TE = 8–12 ms; TR = 550 ms; concatenation = 2, field of view =  $25 \times 25$  and  $20 \times 20$  cm; matrix size =  $384 \times 384$ ) with 12–18 axial slices from the top of the humerus to the elbow (arm) and 16–20 axial slices from the greater trochanter to the knee (thigh). The anatomic cross-sectional area of each scan was measured three times by a trained investigator, blinded to the subject's group, using the public domain image-processing software, IMAGE-J v1.46r (National Institute of Health, Bethesda, md, USA). On the basis of a signal-intensity threshold, muscle borders were selected to delineate and differentiate muscle, fat, and connective tissue, and the coefficient of variation between consecutive measurements was <5%. In the upper arm, the biceps brachii and brachialis muscles were identified in each slice and grouped together to constitute the elbow flexors (EF). Similarly, in the thigh area, the vastus lateralis (VL), vastus medialis, vastus intermedius and rectus femoris were traced in each image and in combination made up of the quadriceps (Q). EF and Q muscle volumes were then calculated by summing the areas of all the slices, taking into account the slice thickness and the interslice space (Deschenes *et al.* 2001). Muscle length was assessed as the distance between the most proximal and the most distal images in which the muscle was visible.

#### Muscle architecture

The physiological cross-sectional area (PCSA) (Petrella *et al.* 2008) was calculated in the arm by dividing EF muscle volume by the fascicle length (Fl). Fl was

calculated by multiplying muscle length (measured by MRI) by the fibre length-to-muscle length ratio, as previously reported in an autopsy-based study (Murray *et al.* 2000):

$$PCSA_{EF} = EF_{vol}/Fl$$

In the thigh, sagittal ultrasound images of the VL muscle were recorded with a Siemens ACUSON P50 ultrasound system (Siemens AG, Munich, Germany) equipped with an 8–12 MHz linear transducer. Images were obtained with a 90° flexion of hip and knee, at 50% of femur length corresponding to the mid-belly of the VL muscle. The pennation angle ( $\theta_p$ ) of the VL fascicles was measured as the angle between the VL muscle fascicles and the deep aponeurosis of the insertion. PCSA of the Q muscle was then calculated as the product of muscle volume times the cosine of  $\theta_p$  divided by the Fl (Powell *et al.* 1984):

$$PCSA_Q = Q_{vol} \cdot \cos \theta_p / Fl$$

#### In vivo experimental set-up

Maximal voluntary and electrically evoked muscle contractions of the EF and Q muscles were measured utilizing a custom-made set-up (Fig. 2, Panel a). Subjects were seated in an upright position with back support, and the right elbow was flexed to 90°, with the right hand supinated and the fingers extended. The hip and the knee were flexed at 90°, and both the right wrist and the right ankle were attached, via two straps and rigid steel bars, to the two force transducers (DBBSE-100 kg, A2829. Applied Measurements, Aldermaston Berkshire, UK). The output from the force transducers was amplified (INT2-L; London Electronics, Sandy Bedfordshire, UK) and recorded at a sampling rate of 5 KHz with a PowerLab-16/35 data acquisition system (ADInstruments, Bella Vista, NSW, Australia).

#### Voluntary- and RT-specific force

Q and EF voluntary muscle-specific force was calculated by dividing fascicle force, estimated from measurements of the EF and Q isometric maximal voluntary contraction (MVC), by the PCSA of the corresponding muscles (Maganaris *et al.* 2001). Similarly, RT-specific force was calculated by dividing fascicle force, estimated in this case from the measurements of EF and Q electrically evoked RT, by the PCSA of the corresponding muscles (Maganaris *et al.* 2001).

Voluntary- and RT-specific force = fascicle force/PCSA

### Electromyography

M-waves were recorded from the biceps brachii and triceps brachii in response to brachial plexus stimulation, while during femoral nerve stimulation, M-waves were recorded in the VL and biceps femoralis muscles (detailed in next section). Pairs of full-surface solid adhesive hydrogel electrodes (H59P; Tyco Healthcare Group, Mansfield, MA, USA) were positioned lengthwise over the respective muscle bellies, with an inter-electrode distance (centre to centre) of 20 mm. The ground electrodes were fixed over the ipsilateral elbow and patella. Light skin abrasion followed by skin cleansing kept electrical impedance below 10 k $\Omega$ . EMG signals were amplified with a pass-band of 10 Hz–1 kHz and digitized online at a sampling frequency of 5 kHz.

### EF and Q nerve stimulation

Each test procedure began with the determination of the maximal M-wave and RT responses in the resting EF and Q muscles. Briefly, current intensity was progressively increased from 0 mA to the value beyond which there was no further increase in M-wave amplitude. The stimulus utilized for the study was set at the 125% of the intensity required to produce a maximal M-wave response. For EF, electrical stimuli were delivered using circular (diameter 5.0 cm), self-adhesive electrodes (Dermatode; American Imex, Irvine, CA, USA) attached to the brachial plexus at Erb's point. The cathode was in the supraclavicular fossa, and the anode was over the acromion. The femoral nerve was stimulated with the cathode positioned in the femoral triangle, 3–5 cm below the inguinal ligament, and the anode placed over the iliac crest (Fig. 2, Panel a). The RT was evoked in the passive muscle using electrical stimulation consisting of single square-wave pulses of 0.1-ms duration, delivered by a Digitimer DS7 constant-current stimulator (Digitimer, Welwyn Garden City, UK). The RT was measured 5 s after a 5-s MVC of the EF, and this procedure was repeated six times. Hence, it should be noted that RT was assessed in the potentiated state. With a 5-min interval, the same procedure was repeated for the Q muscle. The interval between the MVCs was 30 s. Peak torque was assessed for each RT (Sandiford *et al.* 2005). Voluntary activation of the EF and Q muscles during the MVCs was assessed using a superimposed twitch technique (Merton 1954). Briefly, the force produced during a single twitch superimposed on the MVC was compared with the force produced by the electrically evoked RT produced, at rest, 5 s after the MVC.

### Muscle biopsies and in vitro single muscle fibre analysis

On a separate day, a sample of skeletal muscle was obtained with a 14-gauge tru-cut needle (Paoli *et al.* 2010) from both the *vastus lateralis* and *biceps brachii*. The muscle samples were immediately immersed in high potassium, high EGTA solution, often described as skinning solution (details below), mixed in equal parts with glycerol. Once immersed in this solution, the sample could be stored at  $-20^{\circ}\text{C}$  for up to 2 weeks. On the day of the *in vitro* experiment, the samples were washed with skinning solution containing ATP, and the single fibres were manually dissected under a stereomicroscope and permeabilized with 1% Triton X-100. Fibre segments of the average length of 0.7 mm were mounted, by means of light aluminium clips, between the force transducer (model AME-801; SensorOne, Sausalito, CA, USA) and the motor (SI, Heidelberg, Germany) equipped with a displacement transducer. The fibre segment was immersed in a drop of relaxing solution and, after measuring length, diameters and sarcomere length at  $400\times$  magnification, was stretched by 20%. Cross-sectional area (CSA) was calculated from the measurement of these three diameters (at  $400\times$  magnification) assuming the fibre to have a cylindrical shape. The fibre was then transferred into the pre-activating solution and finally maximally activated by immersion in the activating solution (pCa 4.6) at  $12^{\circ}\text{C}$ . Fo was measured in four subsequent maximal activations and the average value calculated. The fibre segment was then removed from the force assessment system and stored in Laemmli solution for electrophoretic determination of MyHC isoform composition.

Skinning, relaxing, pre-activating and activating solutions were prepared, as previously described (Toniolo *et al.* 2007). Their millimolar composition was as follows: 1) skinning solution contained 150 K-propionate, 5 Mg acetate, 5 ATP, 5 EGTA and 5  $\text{KH}_2\text{PO}_4$ ; 2) relaxing solution contained 100 KCl, 20 imidazole, 5  $\text{MgCl}_2$ , 5 ATP and 5 EGTA. Pre-activating solution was similar to relaxing solution except that EGTA concentration was reduced to 0.5 and 25 mM creatine phosphate and  $300\text{ U mL}^{-1}$  creatine kinase was added, whereas activating solution was similar to relaxing solution with the addition of 5 mM  $\text{CaCl}_2$ , 25 mM creatine phosphate and  $300\text{ U mL}^{-1}$  creatine kinase. The pH of all solutions was adjusted to 7.0 at the temperature at which the solutions were used ( $12^{\circ}\text{C}$ ). Protease inhibitors (10  $\mu\text{M}$  E-64 and 40  $\mu\text{M}$  leupeptin) were present in all solutions.

Each fibre was characterized by CSA, Fo and Po, determined by normalizing Fo by fibre CSA and then classified according to its MyHC isoform composition which was determined with gel electrophoresis, as described below. To ensure that the response of each subject was equally represented, the fibres from a given subject were pooled to calculate an individual weighted mean (Doria *et al.* 2011). Bundles of fibres remaining on the dissection plate at the end of the dissection were spread on a slide, images were taken at 400 $\times$  magnification, and CSA was determined from diameter measurement as performed for the fibres studied in the contraction experiments.

Proteins for gel electrophoresis were prepared from the remains of the sample after single fibre dissection and from a sample, specifically collected for this purpose. The tissue samples were solubilized in Laemmli solution (62.5 mM Tris, pH 6.8, 10% glycerol, 2.3% SDS, 5%  $\beta$ -mercaptoethanol, with 0.1% E-64 and 0.1% leupeptin as antiproteolytic factors). After heating for 5 min at 80 °C, appropriate amounts of the protein suspension were loaded onto polyacrylamide gels (about 1  $\mu$ g of total protein/lane). For single fibre identification, the whole fibre segment was solubilized in 10  $\mu$ L of Laemmli solution, and 2–3  $\mu$ L was loaded onto gels. The separation of MyHC isoforms was achieved on 8% polyacrylamide slab gels with a protocol derived from Talmadge and Roy (Talmadge & Roy 1993), with some minor modifications. Slabs – 18 cm wide, 16 cm high and 1 mm thick – were used. Electrophoresis was run at 4 °C for 24 h, at 70 V for 1 h and 230 V for the remaining time. Three bands were separated in the region of 200 kDa, corresponding (in order of migration from the fastest to the slowest) to MyHC-1, MyHC-2A and MyHC-2X. Gels were silver stained (Life Science Bio-Rad Laboratories S.r.l., Milan, Italy) for single fibre identification or stained with Coomassie Blue for the determination of the relative proportions of the three MyHC isoforms. The relative proportions of MyHC isoforms were determined with the measurement of the brightness-area product B.A.P. (i.e. the product of the area of the band by the average brightness, subtracted from local background after black–white inversion) with the accuracy of 600 dpi.

### Statistical analyses

Two-way ANOVA was used to establish differences between groups and limbs. Following these analyses, where indicated, a Tukey *post hoc* test was used to determine the group differences. Pearson correlation test was used to examine the relationship between variables. Significance was set at an  $\alpha$  level of 0.05, and the results are presented as mean  $\pm$  SE.

## Results

### Subject characteristics

The physical characteristics of the YG, OM and OI are documented in Table 1. Body weight of the participants was significantly reduced in the OM and OI. Additionally, the YG women were taller than the two oldest-old groups. By experimental design, the YG and OM were normally active individuals with typical and similar upper- and lower-limb mobility. The upper-limb function and activity in the OI was not different from the YG and OM, whereas, in contrast, the ambulatory capacity of the OI, and therefore the lower limb, use was significantly attenuated. Compared to the YG, both the OM and OI exhibited higher systolic blood pressure, blood glucose, low-density lipoprotein and a lower blood haemoglobin concentration.

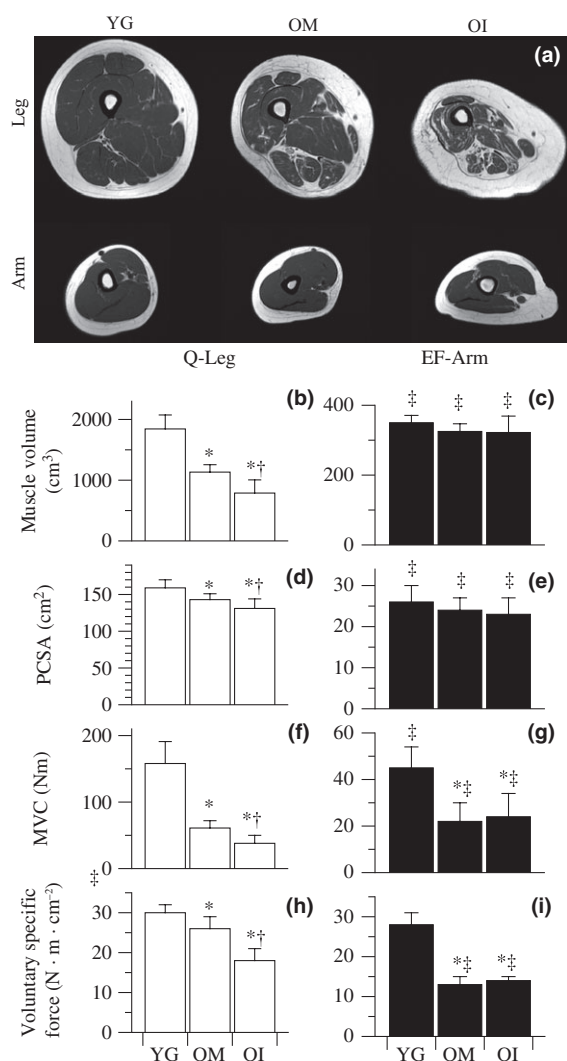
**Table 1** Subject's characteristics

|  | YG              | OM               | OI                      |
|--|-----------------|------------------|-------------------------|
| Age (years)  | 25 $\pm$ 2      | 87 $\pm$ 5*      | 88 $\pm$ 4*             |
| BMI  | 23 $\pm$ 4      | 21 $\pm$ 3       | 22 $\pm$ 4              |
| (kg m <sup>-2</sup> )                                      |                 |                  |                         |
| Body   | 69 $\pm$ 4      | 52 $\pm$ 5*      | 54 $\pm$ 3*             |
| weight (kg)  |                 |                  |                         |
| Stature (m)  | 1.75 $\pm$ 0.05 | 1.57 $\pm$ 0.03* | 1.58 $\pm$ 0.04*        |
| SBP  | 118 $\pm$ 4     | 135 $\pm$ 5*     | 133 $\pm$ 6*            |
| (mmHg)   |                 |                  |                         |
| DBP  | 82 $\pm$ 4      | 86 $\pm$ 6       | 87 $\pm$ 8              |
| (mmHg)   |                 |                  |                         |
| Glucose  | 86 $\pm$ 3      | 95 $\pm$ 8*      | 96 $\pm$ 7*             |
| (mg dL <sup>-1</sup> )                                     |                 |                  |                         |
| RBC  | 4.2 $\pm$ 0.1   | 4.0 $\pm$ 0.2    | 3.9 $\pm$ 0.3           |
| (10 <sup>6</sup> $\mu$ L <sup>-1</sup> )                   |                 |                  |                         |
| Hb   | 13.6 $\pm$ 0.4  | 11.6 $\pm$ 0.5*  | 11.2 $\pm$ 0.8*         |
| (g dL <sup>-1</sup> )                                      |                 |                  |                         |
| HDL  | 51 $\pm$ 8      | 52 $\pm$ 9       | 54 $\pm$ 6              |
| (mg dL <sup>-1</sup> )                                     |                 |                  |                         |
| LDL  | 96 $\pm$ 10     | 107 $\pm$ 11*    | 110 $\pm$ 14*           |
| (mg dL <sup>-1</sup> )                                     |                 |                  |                         |
| Lower-limb mobility capacity                               |                 |                  |                         |
| Balance  | 16 $\pm$ 0      | 16 $\pm$ 0       | 1 $\pm$ 1* <sup>†</sup> |
| test (0–16)  |                 |                  |                         |
| Gait   | 12 $\pm$ 0      | 12 $\pm$ 0       | 0 $\pm$ 0* <sup>†</sup> |
| test (0–12)  |                 |                  |                         |
| Upper-limb performance score of activities of daily living |                 |                  |                         |
| ARAT   | 57 $\pm$ 0      | 55 $\pm$ 3       | 55 $\pm$ 2              |
| test (0–57)  |                 |                  |                         |

Data are presented as means  $\pm$  SE. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RBC, red blood cells; Hb, haemoglobin; HDL high-density lipoprotein; LDL, low-density lipoprotein.

\*Significantly different from young subjects.

<sup>†</sup>Significantly different from old-mobile subjects.



**Figure 1** Muscle mass and maximal voluntary contraction characteristics. Panel (a) 3 mid-thigh and 3 mid-arm magnetic resonance images (MRI) of representative YG, OM and OI subjects. Panels (b–i) document maximal voluntary contraction (MVC), muscle volume of quadriceps (Q) and elbow flexors (EF) calculated from the MRI, physiological cross-sectional areas (PCSA) and maximal voluntary-specific force, respectively, of healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). Data in Panels (b–i) are presented as mean  $\pm$  SE; \*Significantly different from young subjects; †Significantly different from OM subjects; ‡Significant within-group difference between Q and EF.

### Muscle mass and maximal voluntary contraction

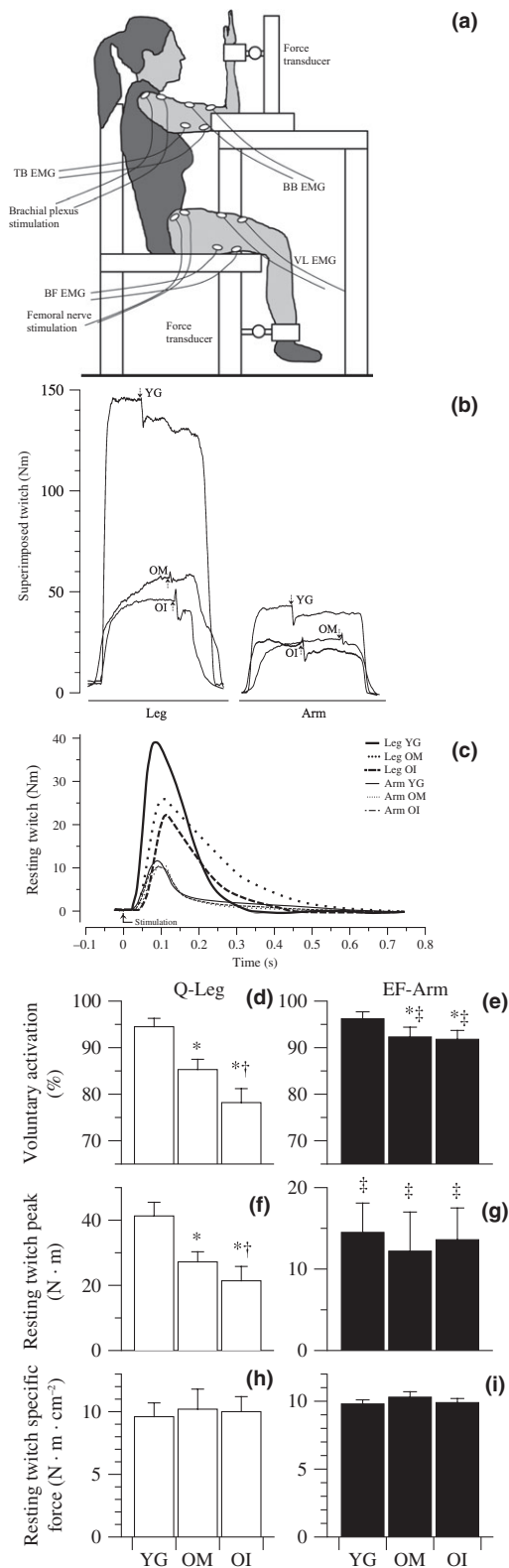
As illustrated in the representative T1-weighted images of the upper and lower limbs (Fig. 1, Panel a), there was a marked and progressive thigh muscle atrophy and a greater proportion of fat and connective tissue across the spectrum from YG to OM to OI subjects. This was in stark contrast to the upper arm in which muscle volume clearly remained preserved.

Quantitative analysis supported these visual impressions with a thigh muscle volume of  $1844 \pm 230$  cm<sup>3</sup> in the YG,  $1133 \pm 120$  cm<sup>3</sup> in OM and  $788 \pm 217$  cm<sup>3</sup> in OI (Fig. 1, Panel b). The upper arm muscle volume was similar between the three groups,  $350 \pm 21$  cm<sup>3</sup>,  $325 \pm 22$  cm<sup>3</sup> and  $322 \pm 47$  cm<sup>3</sup> for the YG, OM and OI respectively (Fig. 1, Panel c). Likewise, PCSA of the thigh also fell progressively across the three groups from the YG ( $159 \pm 11$  cm<sup>2</sup>) to the OM ( $143 \pm 8$  cm<sup>2</sup>) to the OI ( $131 \pm 13$  cm<sup>2</sup>) (Fig. 1, Panel d). While, again, there was no such changes in the PCSA of the upper arm muscle between groups (Fig. 1, Panel e).

Lower-limb MVC was progressively lower than the YG ( $158 \pm 33$  N m) in the OM ( $61 \pm 33$  N m) and the OI ( $38 \pm 12$  N m) (Fig. 1, Panel f). The MVC of the upper limb was similarly attenuated compared to that of YG ( $45 \pm 9$  N m) in the OM ( $22 \pm 8$  N m) and the OI ( $24 \pm 10$  N m) (Fig. 1, Panel g). Even after the normalization for the PCSA, the maximal force of the quadriceps muscle was progressively diminished across the three groups, with a quadriceps voluntary-specific force of  $30 \pm 2$  N m cm<sup>-2</sup> in the YG,  $26 \pm 3$  N m cm<sup>-2</sup> in the OM and  $18 \pm 3$  N m cm<sup>-2</sup> in OI (Fig. 1, Panel h). In contrast, in the upper limb, compared to the voluntary-specific force of the YG ( $28 \pm 3$  N m cm<sup>-2</sup>), there was an equal reduction in both the OM ( $13 \pm 2$  N m cm<sup>-2</sup>) and the OI ( $14 \pm 1$  N m cm<sup>-2</sup>) (Fig. 1, Panel i). Interestingly, only in the YG was the difference between upper- and lower-limb MVC ablated after the normalization for the PCSA (voluntary-specific force), while both the OM and the OI exhibited an attenuated voluntary-specific force in the upper limb compared to the lower limb (Fig. 1, Panel e).

### Neuromuscular activation and RT characteristics

Representative images of superimposed twitches evoked during the MVCs of the upper and lower limbs are displayed in Figure 2, Panel b. Representative RT tracings in the arm and leg muscle are displayed in Figure 2, Panel c. Voluntary activation of the quadriceps muscle was  $94.5 \pm 1.8\%$  in YG,  $85.3 \pm 2.2\%$  in OM and  $78.2 \pm 3.0\%$  in OI subjects (Fig. 2, Panel d), whereas voluntary activation of the upper arm muscle was more similar in the YG ( $96.2 \pm 1.5\%$ ), OM ( $92.3 \pm 2.1\%$ ) and OI ( $91.8 \pm 1.9\%$ ) (Fig. 2, Panel d). The peak quadriceps RT was significantly attenuated compared to the YG ( $41.3 \pm 4.2$  N m) in the OM ( $27.2 \pm 3.1$  N m) and even more so in the OI ( $21.4 \pm 4.4$  N m) (Fig. 2, Panel f). In contrast, peak RT of the upper limbs was not different in the YG, OM and OI ( $14.5 \pm 3.6$ ;  $12.2 \pm 4.8$ ;  $13.6 \pm 3.9$  N m respectively) (Fig. 2,

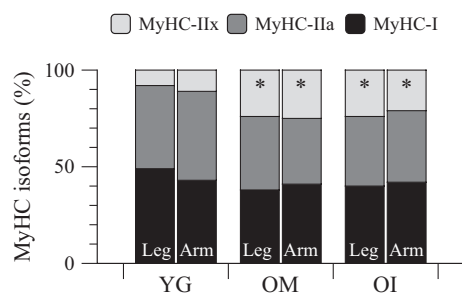


Panel g). Interestingly, by normalizing peak RT for the PCSA, all differences between groups in both upper and lower limbs were ablated (Fig. 2, Panels h,i).

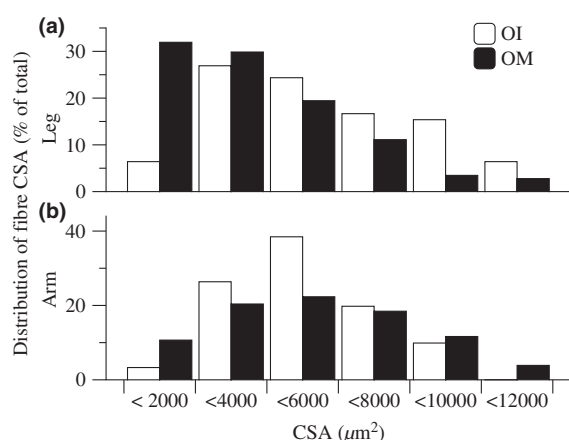
**Figure 2** Neuromuscular activation and resting twitch (RT) characteristics. Panel (a) illustrates the *in vivo* experimental set-up. Panel (b) presents example tracings of the superimposed twitch technique utilized to determine muscle voluntary activation in legs (quadriceps, Q) and arms (elbow flexors, EF). The superimposed twitches (arrows) were imposed at the highest volitional steady-state torque. Representative examples of RT torque–time curves from Q and EF are illustrated in Panel (c). Panels (d–i) document muscle activation, as determined by the superimposed twitch technique, RT peak torque and RT-specific force, respectively, in healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). Data in Panels (d–i) are presented as mean  $\pm$  SE; \*Significantly different from young subjects; †Significantly different from OM subjects; ‡Significant within-group difference between EF and Q.

### Fibre type distribution, CSA and single fibre mechanics

The distribution of the myosin isoforms, taken as molecular markers of fibre type, is illustrated in Figure 3. As documented, the proportion of both MyHC-1 and MyHC-2A isoforms were not statistically different in the legs or arms of YG, OM and OI subjects ( $P = 0.8$ ; Fig. 3); however, a tendency for a higher proportion of slow-type MyHC-1 was evident in the legs of YG but not in the legs of OM and OI. Conversely, the proportion of MyHC-2X isoform was significantly increased in the upper and lower limbs of both OM (approx. 24%) and OI (approx. 22%) in comparison with the YG (approx. 9%). To ascertain whether the muscle fibre sampled for mechanics were representative of the changes (atrophy) related to disuse, CSA was measured in the fibres belonging to the same sample used for *in vitro* force measurements, but not mounted on the force analysis system (Fig. 4). Interestingly, in OI lower-limb muscles, a significant population of thin fibres (below  $4000 \mu\text{m}^2$ , approx. 1/3 of the total, Fig. 4, Panel a) was detected. A total of 302 single fibres (127 in YG, 91 in OM and 84 in OI) were dissected and characterized for their contractile performance, and the results are reported in Figure 5. The CSA of fibres from the lower limb was similar across the three groups:  $4629 \pm 1239 \mu\text{m}^2$ ,  $4878 \pm 877 \mu\text{m}^2$ ,  $4758 \pm 1023 \mu\text{m}^2$  in YG, OM and OI respectively. Similarly, the CSA of fibres in the upper limb was not different between the YG ( $3499 \pm 634 \mu\text{m}^2$ ), OM ( $3941 \pm 801 \mu\text{m}^2$ ) and OI ( $3222 \pm 811 \mu\text{m}^2$ ) (Fig. 5a). However, all the groups exhibited a significantly higher CSA in the fibres of the lower limb compared to the corresponding upper limb. The maximally activated  $F_0$  assessed in the fibres dissected from leg muscles was  $0.46 \pm 0.09$  mN in YG subjects,  $0.42 \pm 0.03$  mN in OM and  $0.48 \pm 0.03$  mN in OI (Fig. 5, Panel b) with no



**Figure 3** Myosin isoform distribution in leg and arm muscle of healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). \*Significantly different from young subjects.

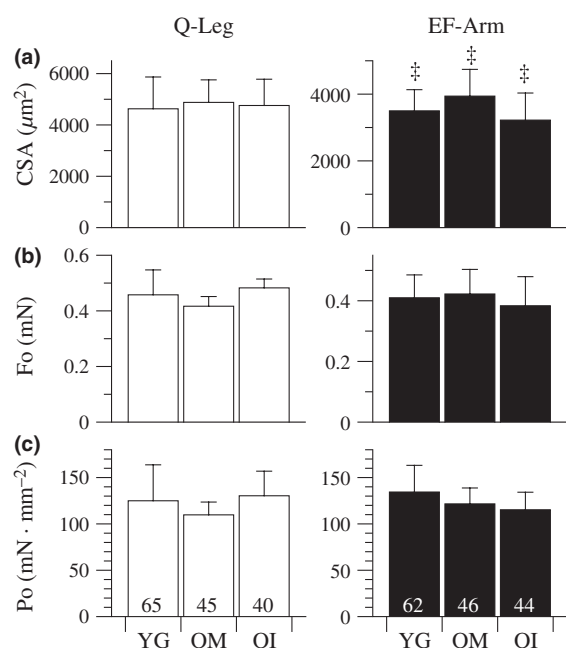


**Figure 4** Cross-sectional area (CSA) distribution in fibres from arm and leg muscle of mobile oldest-old (OM) and immobile oldest-old (OI).

difference between groups. In the arms,  $F_o$  was  $0.41 \pm 0.07$  mN,  $0.42 \pm 0.08$  mN and  $0.38 \pm 0.09$  mN for YG, OM and OI, respectively, again with no difference between groups (Fig. 5, Panel b). This lack of a difference in single fibre mechanics, this time between YG, OM and OI, as well as between muscle from the upper and lower limbs, also held true when the single fibre data were expressed as  $P_o$  (Fig. 5, Panel c). Importantly, even when fibres were grouped according to their composition in terms of myosin isoforms, no difference was detectable between YG, OM and OI (Table 2). When fibres were grouped according to their composition in terms of myosin isoforms, very few significant differences were detectable between YG, OM and OI (Table 2). Interestingly, fast 2A fibres were thicker and stronger in the biceps of OI.

## Discussion

This study sought to parse out the impact of advanced ageing and disuse on skeletal muscle function. Specifi-



**Figure 5** Cross-sectional area (CSA) and single fibre mechanics. Panels (a–c) illustrate CSA, maximal isometric force ( $F_o$ ) and specific tension ( $P_o = F_o/CSA$ ), respectively, of single fibres from the legs (quadriceps, Q) and arms (elbow flexors, EF) of healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). Data are presented as mean  $\pm$  SE; \*Significant within-group difference between EF and Q.

cally, both *in vivo* and *in vitro* techniques were utilized to comprehensively assess muscle contractile properties in combination with the innovative approach of assessing both upper- and lower-limb muscle, which experience differing degrees of disuse, in YG, OM and OI subjects. In partial agreement with our first and second hypotheses, the lower-limb muscle of the OM and OI subjects, assessed *in vivo* by volitional muscle contraction, exhibited attenuated muscle function compared to the YG, and this diminished lower-limb function was more pronounced in the OI subjects. Likewise, our third hypothesis, regarding *in vivo* function in the arms, was also partially correct, revealing an age-related reduction in volitional contraction assessed *in vivo*. However, in disagreement with all of our hypotheses, the *in vitro* assessment of YG, OM and OI skinned single skeletal muscle fibres implied preserved intrinsic contractile force in the oldest-old, regardless of age or inactivity. In support of this finding, the muscle twitch parameters, normalized for muscle mass, which reflects *in vivo* force without the effects of potentially limiting neuromuscular factors, also revealed preserved intrinsic contractile function in all muscles of the oldest-old. Moreover, muscle voluntary activation, assessed

**Table 2** *In vitro* skinned muscle fibre characteristics from the legs (quadriceps, Q) and arms (elbow flexors, EF) of healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). Data relative to the three major fibre types (slow, fast IIA and fast IIA-X) and covering approx. 90% of the fibre population are shown, whereas the minor groups of mixed I-IIA and pure IIX fibres are omitted

|    | MyHC                      | Q-Leg         |               |               | EF-Arm         |                |               |
|----|---------------------------|---------------|---------------|---------------|----------------|----------------|---------------|
|    |                           | I             | IIA           | IIA-X         | I              | IIA            | IIA-X         |
| YG | N                         | 27            | 20            | 10            | 20             | 20             | 20            |
|    | Fo (mN)                   | 0.399 ± 0.044 | 0.540 ± 0.055 | 0.461 ± 0.024 | 0.274 ± 0.031  | 0.559 ± 0.080  | 0.413 ± 0.020 |
|    | CSA ( $\mu\text{m}^2$ )   | 4259 ± 378    | 4770 ± 448    | 4156 ± 792    | 3423 ± 325     | 4046 ± 394     | 3153 ± 339    |
|    | Po (mN mm <sup>-2</sup> ) | 105 ± 12      | 136 ± 22      | 128 ± 14      | 88 ± 11        | 153 ± 27       | 144 ± 11      |
| OM | N                         | 22            | 11            | 10            | 23             | 7              | 10            |
|    | Fo (mN)                   | 0.435 ± 0.041 | 0.377 ± 0.064 | 0.561 ± 0.115 | 0.3756 ± 0.031 | 0.411 ± 0.052  | 0.535 ± 0.078 |
|    | CSA ( $\mu\text{m}^2$ )   | 5327 ± 598    | 3854 ± 820    | 4834 ± 545    | 4005 ± 333     | 3861 ± 330     | 3827 ± 419    |
|    | Po (mN mm <sup>-2</sup> ) | 93 ± 13       | 109 ± 12      | 112 ± 14      | 106 ± 13       | 112 ± 16       | 146 ± 23      |
| OI | N                         | 20            | 4             | 10            | 22             | 6              | 10            |
|    | Fo (mN)                   | 0.557 ± 0.042 | 0.378 ± 0.123 | 0.640 ± 0.075 | 0.305 ± 0.039  | 0.701 ± 0.028* | 0.431 ± 0.065 |
|    | CSA ( $\mu\text{m}^2$ )   | 5603 ± 464    | 2939 ± 521    | 3859 ± 403    | 3069 ± 377     | 6499 ± 706*    | 4092 ± 783    |
|    | Po (mN mm <sup>-2</sup> ) | 111 ± 12      | 131 ± 32      | 177 ± 7       | 108 ± 8        | 113 ± 14       | 119 ± 18      |

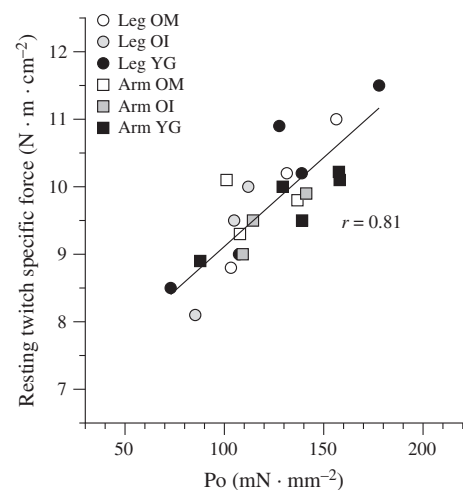
Mean ± SE. N, number of fibres analysed; CSA, cross-sectional area; Fo, maximal isometric force; Po, specific tension; MyHC, myosin heavy chain isoform.

\*Significantly different from corresponding values in YG and OM.

with a combination of RT and superimposed twitches, was attenuated with advanced age and more so with disuse. Therefore, it appears that in the oldest-old, neither advanced ageing nor disuse, *per se*, impacts intrinsic skeletal muscle function, as assessed *in vitro* and *in vivo*, but volitional muscle function is attenuated by age and exacerbated by disuse. These findings implicate factors other than skeletal muscle, such as neuromuscular control, in the diminution of skeletal muscle function with advanced ageing and disuse *in vivo* (Fig. 6).

#### Differential impact of sarcopenia on the skeletal muscle of the upper and lower limbs

Despite several investigations emphasizing the greater impact of sarcopenia on lower limbs in comparison with upper limbs, the reason for this difference has not been fully elucidated (Gallagher *et al.* 1997, Janssen *et al.* 2000, Narici & Maffulli 2010). However, it has been speculated that this disparity may be the result of a greater loss of motor units in the legs than in the arms (Galea 1996). Specifically, with advancing age, and especially in the oldest-old, humans are more prone to become less physically active with significant periods of lower-limb disuse (Buford *et al.* 2012, Pojednic *et al.* 2012, Dufour *et al.* 2013), which may accelerate the loss of skeletal muscle mass and subsequently lower-limb force generating capacity (Berg *et al.* 1997, Venturelli *et al.* 2012). Conversely, the muscle volume of the upper limbs is less affected by



**Figure 6** The relationship between intrinsic muscle function measured *in vitro*, single fibre-specific tension (Po) and *in vivo*, resting twitch (RT)-specific force. Each point represents the average *in vitro* and *in vivo* value obtained from either the leg or arm muscle of a subject ( $n = 5$  healthy young (YG),  $n = 3$  mobile oldest-old (OM) and  $n = 3$  immobile oldest-old (OI)).

the reduction in physical activity across the lifespan (Venturelli *et al.* 2014), likely because the arms are constantly utilized for the activities of daily living, even in those with mobility limitations (Onder *et al.* 2005).

Recently, utilizing the same human model of the oldest-old and varying levels of limb disuse, as in the

current study, our group demonstrated that the progressive fall in skeletal muscle use, as seen in the legs, likely plays a significant role in the exacerbation of cellular ageing and the loss of muscle mass (Venturelli *et al.* 2014). However, in terms of the impact of sarcopenia, the current findings expand our previous work into the functional consequences of advanced age-related changes in the upper and lower extremities. Specifically, this study reveals that, despite the maintenance of muscle mass in the arms in the oldest-old, there was still a significant decrement in voluntary force production in this limb of the OM and OI compared to the YG (Fig. 1, Panel g,i). Although complicated somewhat by clear evidence of sarcopenia in the leg, the equivalent findings of diminished force regardless of muscle mass, achieved by calculating muscle-specific force, are also apparent in the lower limbs, in this case, as a consequence of both advanced age and disuse (Fig. 1, Panel f,h). These first very simplistic observations, supported later by several more complex assessments, imply that the limiting factors for the reduction in force generation capacity with advanced ageing reside outside the contractile machinery of the skeletal muscle cells.

#### *Muscle atrophy and voluntary force production with advanced ageing and disuse*

The results obtained in this study reveal that the loss of muscle mass in the oldest-old was far greater in lower limb than in upper limb where this did not reach statistical significance. The loss of muscle mass is the consequence of a greater loss of muscle fibres, caused by denervation (Doherty *et al.* 1993), and a reduction in size of the remaining fibres. Interestingly, the assessment of the single fibres utilized for contractile measurements did not reveal this latter effect. This is, however, at least partially, likely due to the involuntary selection of fibres during the manual dissection procedure, which tends to result in the very thin fibres being discarded. Indeed, the assessment of larger muscle tissue samples revealed that in all the oldest-old, but particularly in the OI subjects, a large population of fibres (up to 30%) undergo pronounced atrophy.

The relevance of atrophy as explanation for the functional impairment associated with ageing is somewhat controversial. The literature based on cross-sectional studies has indicated that the reduction of *in vivo* muscle performance with ageing is a direct result of the muscle atrophy (Frontera *et al.* 1991). However, other longitudinal investigations have suggested a dissociation between the drop of skeletal muscle mass and the consequent decrease in voluntary force among older adults (Goodpaster *et al.* 2006, Delmonico *et al.* 2009). For instance, Hughes and

co-authors (Hughes *et al.* 2001) demonstrated that in physically active elderly, morphological variations in the skeletal muscle explained only 5% of the corresponding decrease in voluntary muscle force. Therefore, this dissociation between atrophy and the more pronounced reduction in skeletal muscle voluntary force with advancing age implies that additional physiological mechanisms are responsible for this phenomenon. Data from the current study are in agreement with these previous findings; in fact, even after the normalization for the PCSA, voluntary-specific force was significantly reduced in both the upper and lower limbs of OM and OI (Fig. 1, Panel e). In this scenario, it has been suggested that age- and disuse-related changes in neural control may play a substantial role in this recognized decline in muscle voluntary force (Delbono 2003, 2011, Payne & Delbono 2004, Aagaard *et al.* 2010, Reid *et al.* 2012).

#### *Single muscle fibre contractile function with advanced ageing and disuse*

Previously, it has been recognized that both ageing and skeletal muscle disuse are related to the progressive impairment in muscle mechanical function assessed *in vivo*, which may, at least in part, be a consequence of altered single muscle fibre mechanics (Yu *et al.* 2007, Frontera *et al.* 2008, Aagaard *et al.* 2010, Canepari *et al.* 2010a, Callahan *et al.* 2014a). However, the present study reveals no substantial differences between single fibre size and contractile performance from either the upper or lower limbs of the YG, OM or OI subjects. This unaltered single fibre mechanics (i.e. unchanged tension development) disagrees with some previous findings of an age- and disuse-related decline in single fibre contractile properties (Larsson *et al.* 1997, Frontera *et al.* 2000, D'Antona *et al.* 2003, Ochala *et al.* 2007, Yu *et al.* 2007, Callahan *et al.* 2014b), but is in accordance with other investigations that are more similar to the current work (Raue *et al.* 2009, Hvid *et al.* 2010, 2011, Reid *et al.* 2012). Indeed, the present findings, of maintained single fibre contractile function, in the oldest-old are in best agreement with previous studies that employed a similar *in vivo* and *in vitro* approach to compare young and oldest-old subjects (approx. 85 years) (Trappe *et al.* 2003, Raue *et al.* 2009). The apparent contrast between the overall decline of muscle mass and performance, assessed *in vivo*, and the preservation of single fibre function, as determined *in vitro*, can be viewed as the final result of a progressive selective loss of fibres. Likely in relation to motor neurone death (see below for further discussion), during which many fibres disappear and the surviving fibres undergo compensatory hypertrophy. Another

factor that may contribute to this phenomena is the age- and inactivity-related changes in skeletal muscle extracellular matrix (Kragstrup *et al.* 2011). Specifically, in an animal study, it has been demonstrated that exercise training did not prevent the increase of muscle collagen with advancing age (Zimmerman *et al.* 1993). These findings suggest that the age-related increase in skeletal muscle extracellular matrix is not just a result of decreasing physical activity with age.

It is well established that ageing and disuse are two stimuli that are critical in determining skeletal muscle phenotype. Specifically, ageing is typically associated with a fast-to-slow fibre type shift, while disuse predominantly results in a slow-to-fast fibre type change (Schiaffino & Reggiani 2011, Blaauw *et al.* 2013, Ciciliot *et al.* 2013). Interestingly, in previous studies, it has been indicated that ageing or disuse can alter the contractile properties of single muscle fibres without evident changes in the fibre type composition. This intriguing observation suggests that single fibre mechanics may change independently from the fibre phenotype (Bottinelli *et al.* 1999, Canepari *et al.* 2010a). The current data suggest that the preserved capacity to produce tension of the single skeletal muscle fibres from the OM and OI is accompanied by significant changes in the distribution of MyHC isoforms (Figs 3 and 5, Panel c). Indeed, both OM and OI showed similar transition towards expression of the fast 2X MyHC isoform (Table 2), implying some degree of limited motor activity, not only in the OI, but also potentially in the OM subjects (Purves-Smith *et al.* 2014). This is not so surprising given the advanced age of these individuals.

Some important caveat must accompany the discussion of the results obtained on single fibres. In the first place, the number of single fibres analysed represents in all cases a very small fraction of the whole fibre population composing the muscle. This is even more true for this study where a less invasive biopsy technique adopted in this study (14-gauge tru-cut needle) yielded limited, and in some cases insufficient, samples of skeletal muscle tissue for the *in vitro* measurements. In addition, whenever performing *in vitro* studies with single skeletal muscle fibres, it is important to take into account a possible unwanted selection of fibres with the thinnest fibres discarded and to underline that the force measurements were performed with maximal calcium activation ( $pCa = 4.6$ ) and at a temperature lower than the physiological body temperature. Available evidence suggests that such maximal activation is never attained by muscle fibres *in vivo*, and this may be particularly the case in old subjects. Specifically with regard to ageing, it has been documented that there is a significant age and

neural-dependent decrease in dihydropyridine receptor (DHPR) functional expression that is responsible for an uncoupling of the excitation–contraction process that results in an incomplete activation of the myofibrillar machinery (Delbono 2011). Interestingly, exercise and chronic nerve stimulation increase the expression of DHPR (Saborido *et al.* 1995, Pereon *et al.* 1997), and this may be one of the mechanisms responsible for the attenuated function *in vivo*, with both advanced ageing and disuse, which was not evident *in vitro*.

#### Neuromuscular factors and voluntary force production with advanced ageing and disuse

Maximal voluntary force represents the integration of neural, cellular and integrated muscle function (Payne & Delbono 2004, Aagaard *et al.* 2010). The current data provide evidence that neuromuscular activation may be a modulator of advanced ageing- and disuse-related decline in muscle voluntary force (Fig. 2, Panels h,i). Specifically, in line with this progressive reduction in voluntary force generation, the lower limbs of the OM and OI exhibited a similar fall in muscle voluntary activation (Fig. 2, Panel d). This reduction in muscle voluntary activation was also apparent in the arms, but likely as there was no evidence of disuse in upper limbs both the OM and OI exhibited the same attenuation (Fig. 2, Panel e). Additional evidence of the potential neuromuscular contribution to the reduction of *in vivo* force is the RT-specific force. This variable is an indicator of *in vivo* force without a role for neural drive and PCSA as potentially limiting factors.

With the similar RT-specific force in the arms and legs across the three groups, the reduction in muscle voluntary activation with advanced ageing and disuse, and the good correlation with Po assessed, independently, *in vitro* ( $r = 0.81$ ,  $P \leq 0.05$ ; Fig. 5), it seems reasonable to assume that a deficit in neural drive plays a significant role in the reduction in voluntary force production with age and disuse. Although this study cannot identify exactly which components of the nervous system are responsible for these age- and disuse-related changes, potential candidates include a reduction in the number of motor neurones, alteration of the motor units and the decrease of neuromuscular activation as a consequence of a decline in maximal motoneurone firing rate (Kamen *et al.* 1995, Lexell 1997, Aagaard *et al.* 2010). Moreover, increasing evidence points to a decline in neural influence on skeletal muscle at later ages, and this might also lead to changes in muscle structure which together result in excitation–contraction uncoupling (Delbono 2003). Interestingly, in a recent murine study, it has been revealed that large

changes in the genes associated with neuromuscular junction denervation, such as *Chnrg*, *Chrnd*, *Ncam1*, *Runx1*, *Gadd45a* and *Myog*, appear to be closely linked to the denervation of ageing myofibres (Barns et al. 2014). Taken together, these studies support the concept that *in vivo* force characteristics are largely dependent upon neural activity, including a possible involvement of DHP, and consequently influence excitation–contraction coupling.

## Conclusion

Utilizing a novel human model of advanced age and differential limb disuse, this study has documented that, despite preserved muscle mass in the upper limb, volitional contractile function was attenuated in all limbs of the oldest-old compared to the YG and this difference was exacerbated in the legs by disuse. However, the *in vitro* assessment of single skeletal muscle fibres implied that neither age nor disuse affected intrinsic skeletal muscle function. This finding was supported by electrically evoked twitches *in vivo*, which revealed attenuated muscle voluntary activation with advanced age and disuse, but preserved intrinsic contractile function in all muscles of the oldest-old. In combination, these data implicate factors other than skeletal muscle, such as neuromuscular control, in the *in vivo* attenuation of skeletal muscle function associated with advanced ageing and disuse.

## Conflict of interest

There is no conflict of interest.

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