



Physiological determinants of mechanical efficiency during advanced ageing and disuse

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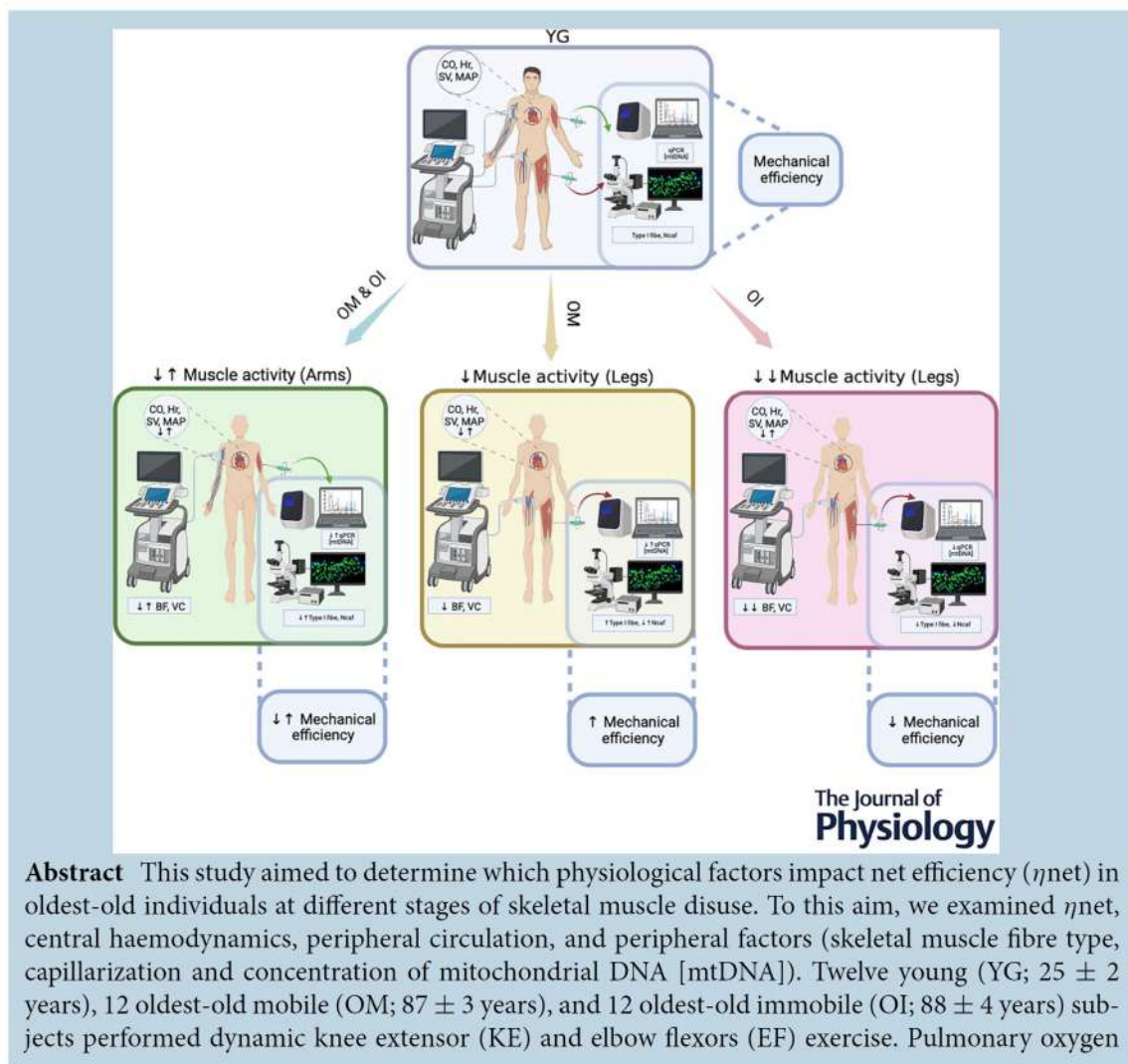
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uptake, photoplethysmography, Doppler ultrasound and muscle biopsies of the vastus lateralis and biceps brachii were used to assess central and peripheral adaptations to advanced ageing and disuse. Compared to the YG ($12.1 \pm 2.4\%$), the η_{net} of lower-limb muscle was higher in the OM ($17.6 \pm 3.5\%$, $P < 0.001$), and lower in the OI ($8.9 \pm 1.9\%$, $P < 0.001$). These changes in η_{net} during KE were coupled with significant peripheral adaptations, revealing strong correlations between η_{net} and the proportion of type I muscle fibres ($r = 0.82$), as well as [mtDNA] ($r = 0.77$). No differences in η_{net} were evident in the upper-limb muscles between YG, OM and OI. In view of the differences in limb-specific activity across the lifespan, these findings suggest that η_{net} is reduced by skeletal muscle inactivity and not by chronological age, *per se*. Likewise, this study revealed that the age-related changes in η_{net} are not a consequence of central or peripheral haemodynamic adaptations, but are likely a product of peripheral changes related to skeletal muscle fibre type and mitochondrial density.

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Abstract figure legend Illustration of causal mechanisms linking advanced ageing, disuse and mechanical efficiency. The level of muscle activity of the arms in oldest-old mobile (OM) and immobile (OI) subjects is not reduced in comparison to young (YG) subjects. This lack of difference in muscle activity during the lifespan is reflected in unchanged ($\uparrow\downarrow$) central (cardiac output (CO); heart rate (HR); stroke volume (SV); mean arterial pressure (MAP)), and peripheral (blood flow (BF); vascular conductance (VC)) haemodynamic responses during exercise. Accordingly, no evidence of age-induced peripheral adaptations (percentage of type I fibres, number of capillaries around a fibre (Ncaf); concentration of mitochondrial DNA [mtDNA]) were retrieved from the arms of both OM and OI. These similarities in the physiological factors related to net efficiency of the arms from YG to OM to OI, implicate similar net efficiency during dynamic arm exercise. In contrast, the level of muscle activity of the legs in OM subjects is reduced (\downarrow) in comparison with YG. Advanced age and decreased high intensity locomotor muscle activity implicates a BF and VC reduction, and a peripheral increase (\uparrow) in the content of type I fibres. This change in the skeletal muscle phenotype of the OM likely affects mechanical efficiency during dynamic knee extensors (KE) exercise. The level of muscle activity of the legs in OI subjects is further reduced ($\downarrow\downarrow$) in comparison with YG and OM. The advanced age and the lack of locomotor muscle activity in OI implicates a further BF and VC reduction coupled with reduced content of type I fibre, Ncaf and [mtDNA]. These dramatic peripheral adaptations of the OI legs were the key factors that affect net efficiency during KE. Created with BioRender.com.

Key points

- Although the effects of ageing and muscle disuse deeply impact the cardiovascular and skeletal muscle function, the combination of these factors on the mechanical efficiency are still a matter of debate.
- By measuring both upper- and lower-limb muscle function, which experience differing levels of disuse, we examined the influence of central and peripheral haemodynamics, and skeletal muscle factors linked to mechanical efficiency.
- Across the ages and degree of disuse, upper-limb muscles exhibited a preserved work economy. In the legs the oldest-old without mobility limitations exhibited an augmented mechanical efficiency, which was reduced in those with an impairment in ambulation. These changes in mechanical efficiency were associated with the proportion of type I muscle fibres.
- Recognition that the mechanical efficiency is not simply age-dependent, but the consequence of inactivity and subsequent skeletal muscle changes, highlights the importance of maintaining physical activity across the lifespan.

Introduction

In the last few decades, the effect of ageing on mechanical efficiency has been debated (Ortega, 2013; Venturelli &

Richardson, 2013a, b). Some studies, including relatively old subjects (60–75 years), have reported a preserved (Gaesser et al., 2018) or reduced mechanical efficiency

(Bell & Ferguson, 2009; Hortobagyi et al., 2011; Mian et al., 2006; Ortega & Farley, 2007, 2015) with ageing. In contrast, our previous study, in centenarians (99–101 years) (Venturelli et al., 2013), suggested that advanced age is actually associated with increased mechanical efficiency, likely a consequence of the age-dependent shift toward a more slow-aerobic muscle phenotype (Conley et al., 2007; Lexell, 1995; Lexell et al., 1988; Purves-Smith et al., 2014). With advancing age, the percentage of type I fibres becomes greater in old compared to young muscle, as ageing is typically associated with selective atrophy and even the rarefaction of type II fibres (Lexell, 1995; Lexell et al., 1988; Murgia et al., 2017; Schiaffino & Reggiani, 2011; Venturelli et al., 2018). Indeed, this combination of atrophy and rarefaction of the type II fibres results in a greater proportion of type I fibres in old individuals, which likely alters the whole muscle efficiency. Accordingly, by using an animal model, Hepple et al. (2004) documented during isometric contractions an increased mechanical efficiency in old rats that had a significant decrease in type II fibre content, which was not evident in the middle-aged animals. These observations suggest that, with advancing age, myocellular changes in fibre-type composition may help to counteract reductions in whole muscle function. However, the relative contributions of fibre-type composition and/or other physiological factors to mechanical efficiency in oldest-old humans (≥ 80 years of age, Wu & Gu, 2021), has not been fully elucidated.

It should also be noted that, independent of age, mobility restriction typically results in skeletal muscle atrophy and an associated shift to a less-efficient, glycolytic muscle fibre phenotype (Blaauw et al., 2013; Schiaffino & Reggiani, 2011). Due to mobility limitations, primarily caused by a decline in ambulatory capacity, the oldest-old humans are more predisposed to chronic periods of lower-limb muscle disuse (Buford et al., 2012; Dufour et al., 2013; Pojednic et al., 2012), resulting in muscle atrophy and shift to a less-efficient, fast-glycolytic muscle fibre phenotype in the legs, that may affect mechanical efficiency during locomotion. In a previous work, utilizing a human model of advanced ageing and varying stages of limb disuse, our group demonstrated that the progressive decrease in skeletal muscle use, as seen in the lower-limb muscles, plays a significant role in the exacerbation of muscle ageing and the loss of muscle mass (Venturelli

et al., 2014). In this scenario, we also revealed that the physiological cause of the reduction in the maximal voluntary force of upper- and lower-limb muscles is primarily due to neuromuscular factors (Venturelli et al., 2015, 2018). Although there are some studies evaluating older subjects (60–80 years) and the impact of physical activity on mechanical efficiency, skeletal muscle, mitochondrial content and function (Broskey et al., 2015; Hopker et al., 2013; Kent-Baum & Fitzgerald, 2016), still very little is known about the physiological determinants of the change in mechanical efficiency with advanced ageing and disuse in the oldest-old humans.

To investigate how advanced ageing and long-term disuse affect the physiological determinants of mechanical efficiency, assessments of the upper- and lower-limb muscles of the oldest-old still able to walk independently (OM) and the oldest-old immobile (wheelchair bound, OI) subjects were compared with measurements in healthy young (YG) subjects. We tested the following hypotheses: (1) compared with the YG, the lower-limb muscle of the OM subjects would exhibit increased mechanical efficiency, while in OI subjects these muscles would demonstrate decreased mechanical efficiency; (2) these differences in mechanical efficiency of lower-limb muscle could be explained by the adaptations and interactions between central haemodynamics (cardiac output (CO), heart rate (HR), stroke volume (SV), mean arterial pressure (MAP)), peripheral circulation (blood flow (BF), vascular conductance (VC)) and peripheral factors (skeletal muscle fibre type, fibre cross-sectional area (CSA), number of capillaries around a fibre (Ncaf) and concentration of mitochondrial DNA ([mtDNA])); and (3) again compared with their young counterparts (YG), the upper-limb muscle of the OM and OI subjects would not be different in terms of mechanical efficiency, which could be attributed to the, generally, better maintained physical activity of the arms across the lifespan.

Materials and methods

Ethical approval

In accordance with the 2013 version of the *Declaration of Helsinki*, all participants were made aware of the purpose and the risks of the study, and provided their informed

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written consent. The protocol (IRB #27111) was approved by the Institutional Review Board of the University of Verona. The study was not registered in a clinical trials database.

Subject characteristics

Twelve healthy young individuals (YG; 25 ± 2 years old), 12 oldest-old still able to walk independently (OM; 87 ± 3 years old) and 12 oldest-old individuals with severe mobility limitation affecting ambulation (88 ± 4 years old) sex-matched controls, took part in this study. The OM and OI were matched for age. A baseline screening included health history, physical examination, evaluation of lower-limb mobility (Tinetti, 1986), blood pressure assessment, and venous blood sample collection. The young subjects were normally physically active college students. The OM subjects were all community dwelling, and able to walk independently (Tinetti, 1986). The OI subjects were selected from among residents of the Mons. Mazzali Foundation, Geriatric Institute of Mantua, Italy. The medical staff recruited oldest-old subjects that, at the time of the experiments, were mobility restricted in the lower limbs (2 years of being wheelchair bound) and unable to walk independently (Tinetti, 1986).

Muscle mass

The assessment of total body fat and lower- and upper-limb mass was determined by dual-energy X-ray absorptiometry (QDR Explorer W, Hologic, MA, USA; fan-beam technology, software for Windows XP version 12.6.1).

Daily energy expenditure

Each participant was equipped with an Actiheart device (CamNtech Ltd, Cambridge, UK) allowing HR and acceleration data to be simultaneously recorded for five consecutive days. Energy expenditure recorded with the Actiheart was calibrated with indirect calorimetry measured with a gas analysis system (K4b² Cosmed Inc., Rome, Italy) (Spierer et al., 2011).

Exercise experimental setup

Before testing, each subject completed a familiarization session on a specially designed knee extensor (KE) and elbow flexors (EF) exercise ergometer consisting of an adapted Monark (Varberg, Sweden) 881 Rehab Trainer ergometer that was used for both exercise trials (Fig. 1A and B). This specially designed ergometer has been previously described in detail elsewhere (Andersen et al., 1985; Ferguson et al., 2000). During the familiarization and the following exercise trials, subjects performed

graded incremental exercise toward maximum and sub-maximal exercise bouts with the dominant arm and leg. During the familiarization, the subject's exact placement in relation to pedal/crank position on the ergometer was determined.

On two separate days participants performed two graded maximal exercise tests for each limb. Specifically, the participants performed 5 min of very light exercise without external resistance followed by progressive increases of the ergometer workload (initial workload of 5 W increased by 5 W/min) for the leg and (initial workload of 0 W increased by 1 W/min) for the arm. The exercise cadence was maintained at 60 rpm and when cadence dropped to 50 rpm for more than 10 s the exercise was terminated.

On the experimental day (Fig. 1C), subjects rested, supine, for 30 min prior to performing 6 min of KE and EF, again at a cadence of 60 rpm. During the exercise, subjects performed at a work rate of 10 W for both KE and EF. For YG, this work rate corresponded to 22% and 34% of the peak work rate achieved during the graded maximal exercise tests for KE and EF, respectively. For the OM this work rate corresponded to 36% and 48% of the peak work rate achieved during KE and EF, whereas for the OI it was 55% and 53%, respectively. A 60 min rest period separated the KE and EF trials, and the order of these trials was balanced. During KE, the seat and the connection to the ergometer were positioned such that the range of motion, at the knee, was $\sim 80^\circ$ (Fig. 1A). During EF, the bed and the connection to the ergometer were positioned such that the range of motion, at the elbow, was $\sim 80^\circ$ (Fig. 1B).

Metabolic measurements

Breath-by-breath gas exchange was continuously recorded and digitized (Quark *b*², Cosmed, Rome, Italy) during the KE and EF trials. Pulmonary oxygen uptake (\dot{V}_{O_2}) was calculated as the average of the last 60 s of exercise (Fig. 1C).

Blood flow

Measurements of arterial blood velocity and vessel diameter were performed in the exercising limbs (right leg and right arm) using a Siemens Acuson P50 ultrasound system (Siemens Medical Solutions, Henkestrabe, Erlangen, Germany). The ultrasound system was equipped with a 10–14 MHz linear array transducer. Artery diameter was determined at a 90° angle along the central axis of the scanned area. Blood velocity (*V*) was measured using the same probe at a frequency of 5 MHz. Measurements of blood velocity were obtained with the probe positioned to maintain an insonation angle of 60° or less, and the sample volume was

centred and maximized according to vessel size. Arterial diameter was measured at rest and during the exercise, and blood velocity was automatically calculated using the Acuson P50. In the leg, blood velocity (V_{mean}) and artery diameter were determined distal to the inguinal ligament and proximal to the bifurcation of the deep and superficial femoral arteries. In the arm, blood velocity was determined in the axillary artery, in the axillary fossa. Utilizing arterial diameter and V_{mean} , BF was calculated as:

$$\text{Blood flow} = V_{\text{mean}} \cdot \pi \cdot (\text{vessel diameter}/2)^2 \cdot 60, \quad (1)$$

where BF is in ml min^{-1} , V_{mean} is reported in cm s^{-1} and vessel diameter is reported in cm.

Central haemodynamics

HR, SV, CO and MAP were determined using a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands). The photoplethysmographic cuff of the finger pressure device was placed on the third finger of the left hand (non-exercising arm). The Finometer signal was calibrated utilizing the procedure indicated by the manufacturer. The height adjustment sensor and reference were positioned according to the manufacturer's instructions. SV was estimated using the Modelflow algorithm (Beatscope version 1.1a; Finapres Medical Systems) (Bogert & van Lieshout, 2005). CO was then calculated as the product of HR and SV. The same method has been documented to accurately track CO during exercise (Azabji Kenfack et al., 2004; Tam et al., 2004).

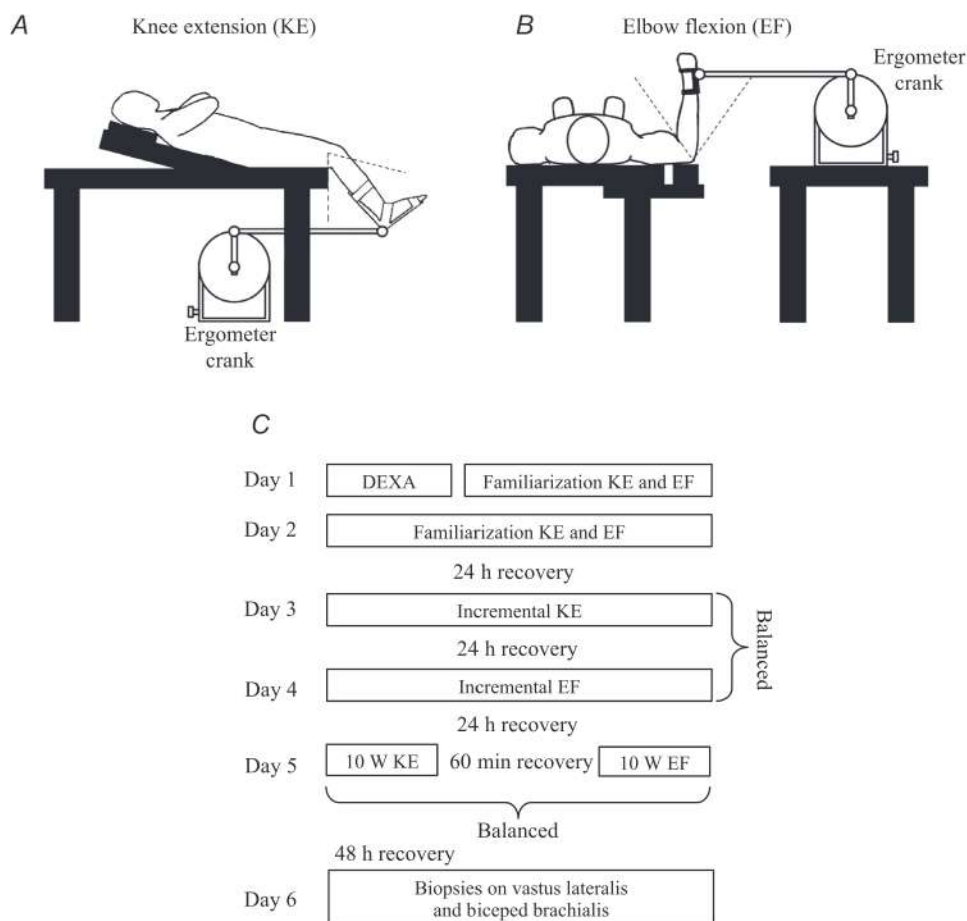


Figure 1. Experimental setup and design

A, the position between the lower limb and connecting bar attached to the crank arm of the modified cycle ergometer utilized for the dynamic knee extensors (KE) exercise. *B*, the position between the upper limb and connecting bar attached to the crank arm of the modified cycle ergometer utilized for the dynamic elbow flexors (EF) exercise. On day 1 dual-energy x-ray absorptiometry (DEXA) was performed and the participants familiarized with KE and EF. Familiarization with the exercise was repeated on day 2. On days 3 and 4 the participants performed graded maximal exercise tests with the with KE and EF. On day 5 the participants performed 2 min of unloaded exercise (ergometer with no tension applied) and 6 min of constant workload exercise at absolute workloads corresponding to 10 W with KE and EF. A 60 min rest interval separated exercise trials executed with upper and lower limbs. The order of KE and EF was balanced. On day 6, samples of skeletal muscle were obtained from both the vastus lateralis and biceps brachii.

Muscle biopsies

On a separate day a series of skeletal muscle samples were obtained with a 14-gauge tru-cut needle (Paoli et al., 2010) from both the vastus lateralis and biceps brachii. Muscle samples were immersed in an isopentane solution dipped in liquid nitrogen, and subsequently stored at -80°C .

Muscle preparation

Biopsy series were prepared in the following manner. Large pieces of muscle (~ 10 mg) were embedded in O.C.T. compound (Tissue-Tek), frozen in liquid nitrogen-cooled isopentane, stored at -80°C . At a later date, biopsies were cut into $10\ \mu\text{m}$ thick cryosections with a cryostat (Thermo scientific) maintained at -20°C and mounted on glass slides.

Type I, type II, and laminin staining

BA-F8 and SC-71 antibodies were purchased from Developmental Studies Hybridoma Bank (DSHB, IOWA, USA). Lamina and capillary antibodies were purchased from abcam (Cambridge, UK). Secondary antibodies were purchased from abcam (Cambridge, UK) and invitrogen (ThermoFisher scientific, Massachusetts, USA). Muscle sections were air-dried at room temperature rinsed three times for 5 min in 10% phosphate-buffered saline (PBS) and incubated for 60 min in goat serum at room temperature. Sections were then incubated for 1 h at room temperature with the primary antibody, BA-F8 [1:50] against major histocompatibility complex (MHC) type I. Following incubation with the primary antibody, the sections were rinsed three times for 5 min with PBS buffer and incubated for 1 h at room temperature with secondary antibody, A21140 Alexa Fluor 350 goat anti-mouse [1:800]. Again, the sections were rinsed three times for 5 min with PBS. Subsequently, the sections were incubated for 1 h at room temperature with primary antibodies, SC-71 [1:200] against MHC type II, and ab11575 [1:200] against laminin. Following incubation with the primary antibodies, the sections were rinsed three times for 5 min with PBS buffer and incubated for 1 h at room temperature with secondary antibodies, A21121 Alexa Fluor 488 goat anti-mouse [1:800], and Ab96884 Dylight 550 goat anti-rabbit [1:800]. Finally, the sections were rinsed three times for 5 min with PBS buffer and washed with dH_2O .

Type I, laminin, and capillary staining

Muscle sections were air-dried at room temperature rinsed three times for 5 min in 10% PBS and incubated for 60 min in goat serum at room temperature. Sections

were then incubated for 1 h with the primary antibody. The following primary antibodies were used: ab14055 [1:1000] against laminin, ab32457 [1:1000] against capillary, and BA-F8 [1:50] against MHC type I. Following incubation with the primary antibody, the sections were rinsed three times for 5 min with PBS buffer and incubated with secondary antibodies: Ab96947 (Dylight 488) goat anti-chicken [1:800], Ab96884 (Dylight 550) goat anti-rabbit [1:800], A21141 Alexa Fluor 350 goat anti-mouse [1:800], and incubated for 1 h at room temperature. Subsequently, the sections were washed three times for 5 min with PBS buffer and rinsed with dH_2O .

Morphometric analysis

Slides were visualized with an Axio Observer Z1 microscope (Carl Zeiss) using conventional wide-field fluorescence microscopy, as well as optical sectioning via structured illumination fluorescence microscopy (Apotome, Carl Zeiss). The microscope was equipped with Green (Excitation: BP 470/40 nm; Emission BP 525/50 nm) and Blue (Excitation: BP 365/12 nm; Emission LP 397 nm) filters, an AxioCam HRm camera and AxioVision software (Carl Zeiss). For fibre-type composition, all muscle fibres on the cross-section were analysed and a mean of 277 fibres was counted for each subject. Selected areas, corresponding to a mean of 91 fibres, were photographed and used for determination of fibre CSAs using the public domain image-processing software, Image-J v1.46r (National Institute of Health, Bethesda, Maryland, USA). Capillaries were quantified manually under the microscope for each fibre, using the following indices: (1) the number of capillaries around a single fibre (Ncaf) and Ncaf relative to the fibre type, (2) the capillary-to-fibre ratio on an individual fibre basis (C:F_i) and the number of fibres sharing each capillary (sharing factor) (Hepple & Plyley, 1997).

Mitochondrial DNA concentration [mtDNA]

Total DNA was isolated and purified from relatively small pieces of skeletal muscle (~ 15 mg) using a QIAamp mini-DNA kit (QIAamp DNA Mini Kit: catalogue number-51306; Qiagen, Inc., Valencia, CA, USA) in accordance with the manufacturer's protocol. Sequence-independent qPCR with SYBR Green was performed with purified DNA to determine mitochondrial DNA concentration [mtDNA] per cell (RT² SYBR Green qPCR Mastermix; catalogue number-330509, Qiagen, Inc.). mtDNA content and albumin content, used as single copy gene to control cell concentration in samples, were determined by standard

curve. Mean mtDNA per cell was expressed as the ratio of mtDNA signal to albumin signal.

Primer sequences were as follows:

mtDNA
 fwd-CCC GGT AAT CGC ATA AAA CTT AAA
 ACT T
 rev-TAA GAA GAG GAA TTG AAC CTC TGA CTG
 TAA
 albumin
 fwd-AAATGCTGCACAGAATCCTTG
 rev-GAAAAGCATGGTCGCCTGTT

Data collection and analysis

HR, SV, CO and MAP underwent analogue-to-digital conversion and were simultaneously acquired using commercially available data acquisition software PowerLab-16/35 data acquisition system (ADInstruments, Bella Vista, NSW, Australia). The data acquisition software allowed high-resolution analysis of HR, SV, CO and MAP throughout the exercise protocols. Mean HR, SV, CO and MAP were calculated during the last 60 s of exercise (Fig. 1C). Mean blood velocity was analysed with 1 Hz resolution on the Doppler ultrasound system (Siemens Acuson P50) for 60 s during steady state exercise (Fig. 1C).

Net efficiency (η_{net}) was calculated for both KE and EF as the ratio of mechanical work (i.e. Watts converted to kcal \cdot min $^{-1}$) to energy expenditure above the resting metabolism reported in the following formula:

$$\eta_{\text{net}} = \left(\text{power output} \cdot \text{energy expended above resting} \right) / \left(\text{metabolism}^{-1} \right) \cdot 100 \quad (2)$$

Both power output and \dot{V}_{O_2} recorded during the last 120 s of KE and EF were expressed as kcal \cdot min $^{-1}$ for all the above calculations (Gaesser & Brooks, 1975; Poole et al., 1992).

Statistical analyses

Sample size calculations were performed using the effect size from a previous study using η_{net} , calculated during single leg dynamic exercise as the main outcome variable (Venturelli et al., 2021), while aiming for a power of 0.8 and alpha level set at 0.05. Normal distribution of the data was assessed with a Shapiro–Wilk test. Student's paired *t* tests were implemented to determine within-group and limb differences in type I and II fibre proportion, fibre CSA, Ncaf and capillary sharing factor. One-way repeated measures ANOVA with Tukey-B *post hoc* analysis was implemented for group characteristics (Table 1): age, BMI, body fat, thigh muscle mass, arm muscle mass, daily

energy expenditure, glucose, RBC, Hb, HDL, LDL, SBP, DBP.

Two-way ANOVA with Tukey-B *post hoc* analysis was performed for group (YG, OM, OI), limb/exercise modality (KE, EF) and group \times exercise modality interaction for: η_{net} , CO, HR, SV, MAP, BE, VC, fibre type, fibre CSA, Ncaf, capillary sharing factor and [mtDNA].

Pearson's Product Moment Correlations were used to assess relationships between continuous variables. Single Pearson's correlation analysis was performed to investigate correlations between continuous variables and the η_{net} measured during KE or EF. The variables significantly correlated with the η_{net} were included in a stepwise multiple linear regression to calculate the variance explained by the best combination of variables. For inclusion in the regression equation each additional variable had to significantly increase the explained variance (adjusted r^2 , $P < 0.05$). In order to control the effect of multicollinearity, variables were excluded if the variance inflation factor was > 2.5 . Data are presented in the tables and text as means \pm standard deviation, while individual data (36 participants, mean values and the 95% confidence interval) are presented in the figures. Statistical analysis was performed using SPSS for Macintosh, version 29.

Results

Subject characteristics

The physical characteristics of the participants are summarized in Table 1. Percentage body fat was significantly greater in the OM and OI than in the YG. As reflected by the differences in daily energy expenditure, the YG were normally active, the OM were relatively inactive and, being wheelchair bound, the OI were significantly more inactive. This graded level of inactivity from YG to OM to OI was reflected by a progressive reduction in thigh muscle mass in these three groups. While in contrast, lean mass of the arms was not different between the YG, OM and OI. Compared with the YG, both the OM and OI exhibited higher systolic blood pressure, blood glucose, low-density lipoprotein, and a lower blood haemoglobin concentration.

\dot{V}_{O_2} and η_{net}

Pulmonary \dot{V}_{O_2} measured at rest was 158 ± 20 ml min $^{-1}$ in the OM and 157 ± 26 ml min $^{-1}$ in the OI (between group $P = 0.9$), while YG exhibited significantly higher values 185 ± 27 ml min $^{-1}$ than the OM ($P = 0.006$) and the OI ($P = 0.006$). During the sixth minute of KE, pulmonary \dot{V}_{O_2} was significantly lower in the OM (444 ± 91 ml min $^{-1}$) than in both the YG (532 ± 68 ml min $^{-1}$; $P = 0.04$)

Table 1. Subject characteristics

	YG = 12	OM = 12	OI = 12
Age (yrs)	25 ± 2	87 ± 3* (<i>P</i> < 0.001)	88 ± 4* (<i>P</i> < 0.001)
Sex (F/M)	9/3	9/3	9/3
BMI (kg·m ⁻²)	23 ± 4	21 ± 3	22 ± 4
Body fat (%)	22 ± 4	33 ± 5* (<i>P</i> < 0.001)	38 ± 6* (<i>P</i> < 0.001); † (<i>P</i> = 0.03)
Thigh muscle mass (kg)	5.38 ± 0.27	4.47 ± 0.17* (<i>P</i> = 0.04)	3.49 ± 0.29* (<i>P</i> = 0.02); † (<i>P</i> < 0.001)
Arm muscle mass (kg)	1.19 ± 0.09	1.18 ± 0.08	1.17 ± 0.03
Daily energy expenditure (Kcal/d)	1985 ± 76	1605 ± 88* (<i>P</i> < 0.001)	1369 ± 56* (<i>P</i> < 0.001); † (<i>P</i> < 0.001)
Glucose (mg·dl ⁻¹)	86 ± 3	95 ± 8* (<i>P</i> = 0.02)	96 ± 7* (<i>P</i> = 0.03)
RBC (10 ⁶ ·μl ⁻¹)	4.2 ± 0.1	4.0 ± 0.2	3.9 ± 0.3
Hb (g·dl ⁻¹)	13.6 ± 0.4	11.6 ± 0.5* (<i>P</i> = 0.04)	11.2 ± 0.8* (<i>P</i> = 0.03)
HDL (mg·dl ⁻¹)	51 ± 8	52 ± 9	54 ± 6
LDL (mg·dl ⁻¹)	96 ± 10	107 ± 11* (<i>P</i> = 0.01)	110 ± 14* (<i>P</i> = 0.01)
SBP (mmHg)	118 ± 4	135 ± 5* (<i>P</i> = 0.001)	133 ± 6* (<i>P</i> = 0.001)
DBP (mmHg)	82 ± 4	86 ± 6	87 ± 8
Comorbidity N (%)			
Cardiovascular diseases	-	2 (17)	1 (8)
Diabetes	-	2 (17)	2 (17)
COPD	-	1 (8)	1 (8)
Pharmacological treatments N (%)			
Trazodone	-	4 (33)	3 (25)
Thiazolidinedione	-	2 (17)	3 (25)
Bisoprolol	-	1 (8)	1 (8)
Etofilline	-	1 (8)	1 (8)
Etinil-estradiol	4 (33)	-	-

F: female, M: male, BMI: body mass index, RBC: red blood cells, Hb: haemoglobin, HDL: high-density lipoprotein, LDL: low-density lipoprotein, SBP: systolic blood pressure, DBP: diastolic blood pressure, COPD: chronic obstruction pulmonary disease.

*Significantly different from YG.

†Significantly different from OM.

and OI ($658 \pm 93 \text{ ml min}^{-1}$; $P < 0.001$). Moreover, the \dot{V}_{O_2} values of OI were significantly higher with respect to YG ($P = 0.004$). No differences were observed between groups in pulmonary \dot{V}_{O_2} measured during the EF exercise (YG = $578 \pm 94 \text{ ml min}^{-1}$; OM = $630 \pm 97 \text{ ml min}^{-1}$; OI = $632 \pm 95 \text{ ml min}^{-1}$; all $P > 0.16$).

Net efficiency calculated during KE and EF in YG, OM and OI subjects are illustrated in Fig. 2. Specifically, η_{net} calculated during KE was $12.0 \pm 2.5\%$ in the YG, it was greater in the OM ($17.6 \pm 3.5\%$, $P < 0.001$), and lower in the OI ($8.9 \pm 2.7\%$, $P < 0.0001$) (Fig. 2A). There were no such differences in the η_{net} calculated during EF between groups ($\sim 10\%$; Fig. 2B).

Central haemodynamics

HR, CO and SV measured during dynamic KE and EF in YG, OM and OI are summarized in Fig. 3. All the three groups exhibited a similarly significantly higher CO and HR during 10 W EF in comparison to 10 W KE (all $P < 0.0001$). No differences in CO and HR were observed between groups during 10 W EF (all $P > 0.3$; Fig. 3B).

While during KE, CO and HR were significantly lower in the YG than in both the OM and OI. During the sixth minute of KE and EF, SV was not different between the three groups ($\sim 104 \text{ ml}$).

Peripheral haemodynamics

BF, VC and MAP measured at rest, during dynamic KE and EF in the YG, OM and OI are summarized in Fig. 4. All three groups exhibited similarly higher MAP values during 10 W EF in comparison to 10 W KE (Fig. 4E and F). No differences in MAP were observed between the groups during 10 W EF and KE.

During the sixth minute of KE, femoral BF and VC were significantly lower in the OM than the YG, and lower again in the OI than the OM. Interestingly, and contrary to the leg peripheral haemodynamic responses, no differences in axillary artery BF and VC were observed between groups at rest and during 10 W EF (Fig. 4B and D). However, the three groups exhibited significantly lower axillary artery BF and VC in comparison to femoral BF and VC (Fig. 4B and D).

Muscle biopsy data

The skeletal muscle characteristics determined from the biopsy samples are presented in Fig. 5. There were significant differences in the proportions of vastus lateralis muscle fibre type, with, compared with the YG, the OM exhibiting an attenuated proportion of type II fibres, and the OI exhibiting a lower proportion of type I fibres (Fig. 5A–C and G). No difference was observed between groups in the proportion of type I and II fibres in the skeletal muscle of the biceps brachii (Fig. 5D–F and H). Muscle fibre CSA was not different between the three groups. However, all three groups exhibited smaller muscle fibre CSA of the biceps brachii in comparison to the vastus lateralis.

OM subjects exhibited attenuated capillarization in type II fibres in the vastus lateralis when expressed as Ncaf and capillary sharing factor (Fig. 5A, B, M, and O). The capillary-to-fibre ratio was 1.01 ± 0.21 , 1.06 ± 0.23 and 1.58 ± 0.29 in the type I fibres of vastus lateralis of the YG, OM and OI, respectively. The C/F_i ratio of OI was significantly higher ($P < 0.001$) than in the YG and OM. A similar trend was observed in the C/F_i ratio of the type II fibres of the vastus lateralis: YG = 0.98 ± 0.13 , OM = 1.11 ± 0.12 and OI = 1.29 ± 0.28 . Assessments of capillarization in the biceps brachii for both type I and II fibres were not different between groups (Fig. 5D–F and P). The C/F_i ratio was 1.02 ± 0.18 , 1.05 ± 0.11 and 1.08 ± 0.24 in the type I fibres of biceps brachii of the YG, OM and OI, respectively. Similarly, for the type II fibres of biceps brachii the C/F_i ratio was: YG = 1.03 ± 0.17 , OM = 1.04 ± 0.14 and OI = 1.01 ± 0.11 . The [mtDNA],

used as surrogate of skeletal muscle mitochondrial density, was significantly lower in the vastus lateralis of OI subjects (Fig. 6A), whereas no such difference in [mtDNA] was evident in the skeletal muscle of the biceps brachii (Fig. 6B).

Correlations among variables

The single Pearson's correlation analysis revealed that the η_{net} measured during the KE was significantly correlated with the percentage of type I fibres and the [mtDNA] of the vastus lateralis muscle ($r = 0.820$, $P < 0.001$) and ($r = 0.771$, $P < 0.001$), respectively. These positive correlations are reported in the Fig. 7A and C, and Table S1. It is important to note that the percentage of type I fibres and the [mtDNA] of the vastus lateralis muscle were also significantly positively correlated to each other ($r = 0.855$, $P < 0.001$). Both type I fibre percentage and the [mtDNA] of the vastus lateralis were utilized for the calculation of a stepwise multiple linear regression for the estimation of the η_{net} during the KE. The regression model found that 66.3% of the η_{net} variance was explained by the type I fibre alone ($P < 0.001$); the other variable included in the regression analysis did not reach statistical significance ($P = 0.722$).

Among the variables measured during the EF and biceps brachii morphology, η_{net} was not correlated with the percentage of type I fibres ($r = -0.412$, $P = 0.12$), but positively correlated with the [mtDNA] ($r = 0.575$, $P < 0.001$). These correlations are reported in Fig. 7B and D, and Table S2. Interestingly, type I fibre percentage

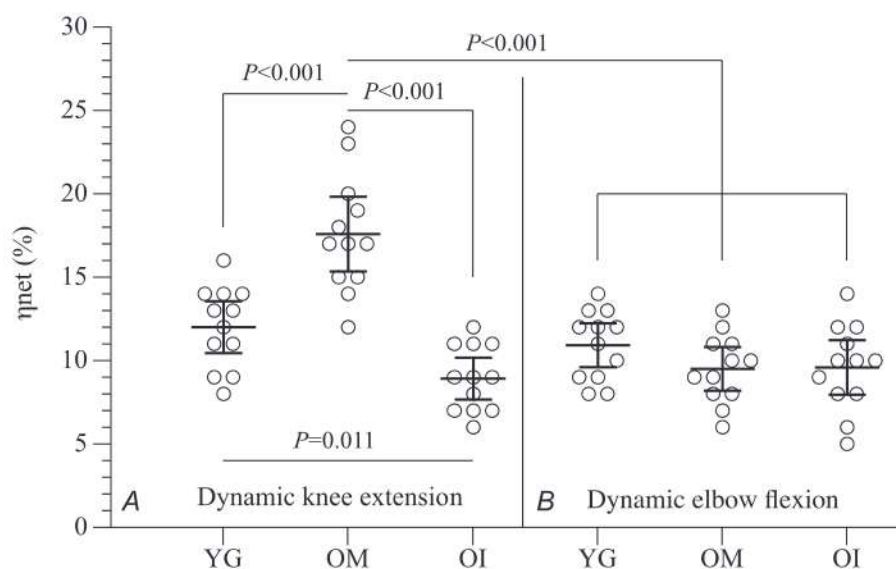


Figure 2. Net efficiency

A and B, net efficiency (η_{net}) calculated during dynamic knee extensors (KE) and elbow flexors (EF) exercise, respectively, of healthy young (YG), mobile oldest-old (OM), and immobile oldest-old (OI). Individual data (36 participants), mean values, and the 95% confidence interval are presented.

and the [mtDNA] of the biceps brachii muscle were not correlated ($r = -0.236$, $P = 0.164$). In this specific case, the regression model found that 31.1% of the η_{net} variance was explained only by the [mtDNA] ($P < 0.001$).

Discussion

This study sought to examine the influence of advanced ageing and long-term disuse on the physiological determinants of mechanical efficiency during exercise. Specifically, the contribution of central haemodynamics, peripheral circulation and skeletal muscle factors linked to η_{net} were assessed in combination with the comprehensive approach of measuring both upper- and lower-limb muscle function, which experience

differing levels of disuse, in YG, OM and OI subjects. In agreement with our first hypothesis, η_{net} of the lower-limb muscle of the OM was increased, while the OI demonstrated decreased η_{net} during KE. Similarly, our second hypothesis, regarding central and peripheral adaptations associated with the degree of changes in η_{net} , was also partially confirmed, revealing strong correlations between the proportion of type I fibres and [mtDNA] with η_{net} . In agreement with our third hypothesis, there were no differences in η_{net} of the upper-limb muscles between YG, OM and OI subjects. Furthermore, independent regression analyses revealed that the proportion of type I fibre of the vastus lateralis explained the 66.3% variability in the η_{net} for the exercise executed with the KE. While the amount of the [mtDNA] in the biceps

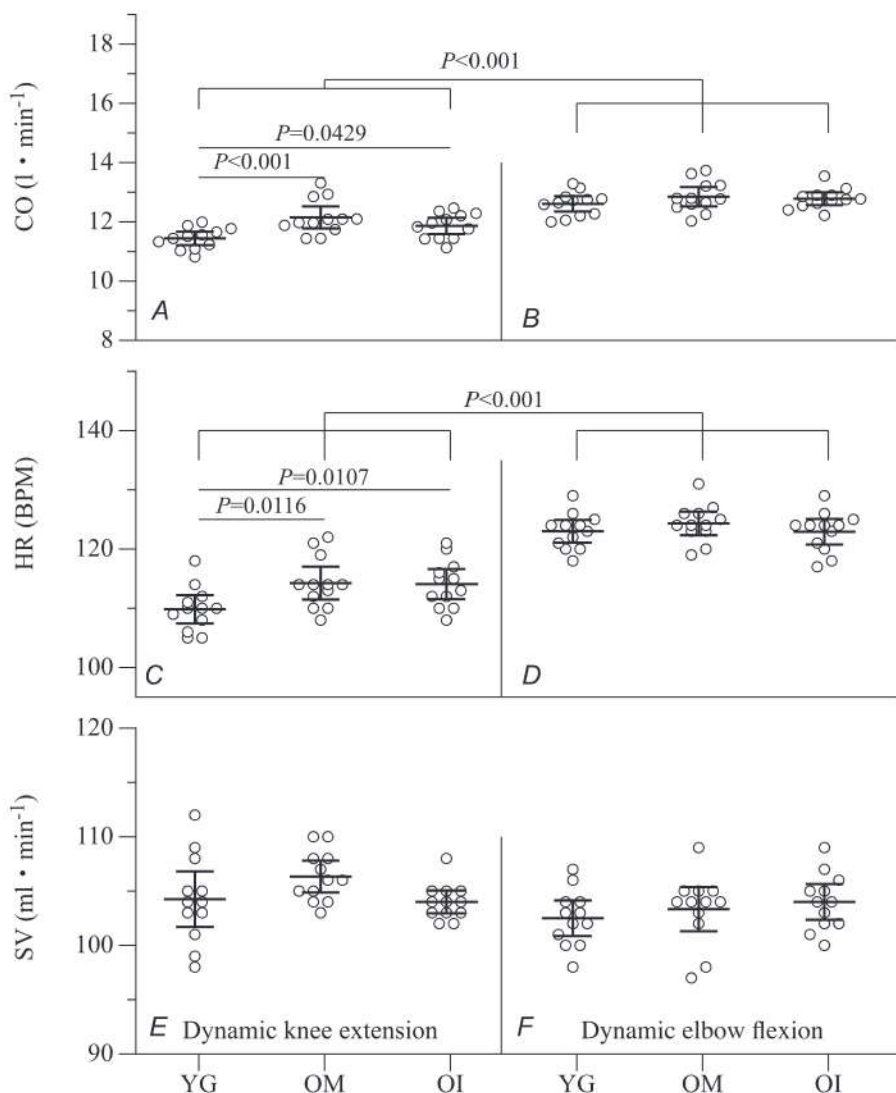


Figure 3. Central haemodynamics

A and B, values of cardiac output (CO) calculated during the last 30 s of 10 W dynamic knee extensors (KE), and elbow flexors (EF) exercise, respectively, of healthy young (YG), mobile oldest-old (OM), and immobile oldest-old (OI). Similarly, C–F, the values of heart rate (HR) (C, D) and stroke volume (SV) (E, F). Individual data (36 participants), mean values, and the 95% confidence interval are presented.

brachii explained the 31.1% variability in the η_{net} for the exercise executed with the EF. Recognizing the differences in limb-specific activity across the lifespan, these findings suggest that η_{net} is attenuated by the muscle inactivity and not by chronological age, *per se*. Likewise, this study revealed that the age-related changes in η_{net} are not a consequence of central and peripheral haemodynamic adaptations, but are likely a product of peripheral changes related to skeletal muscle fibre type in the legs and the mitochondrial density in the arms. Recognition that the η_{net} of skeletal muscle is not simply age-dependent, but the consequence of inactivity and subsequent skeletal muscle changes, highlights the importance of maintaining physical activity across the lifespan.

Evidence that η_{net} is affected by muscle activity and not by ageing

In the literature, the effects of ageing on mechanical efficiency (Ortega, 2013; Venturelli & Richardson, 2013a, b) have been discussed, and emerging evidence suggests a preservation (Gaesser et al., 2018) or reduction of mechanical efficiency in relatively old subjects (60–75 years) (Bell & Ferguson, 2009; Conley et al., 2013; Hortobagyi et al., 2011; Mian et al., 2006; Ortega & Farley, 2007, 2015). In contrast, other outcomes from oldest-old humans (Venturelli et al., 2013), and animals (Hepple et al., 2004) support the view that advanced ageing is associated with an increased mechanical efficiency. Data from the current study of the oldest-old are partially

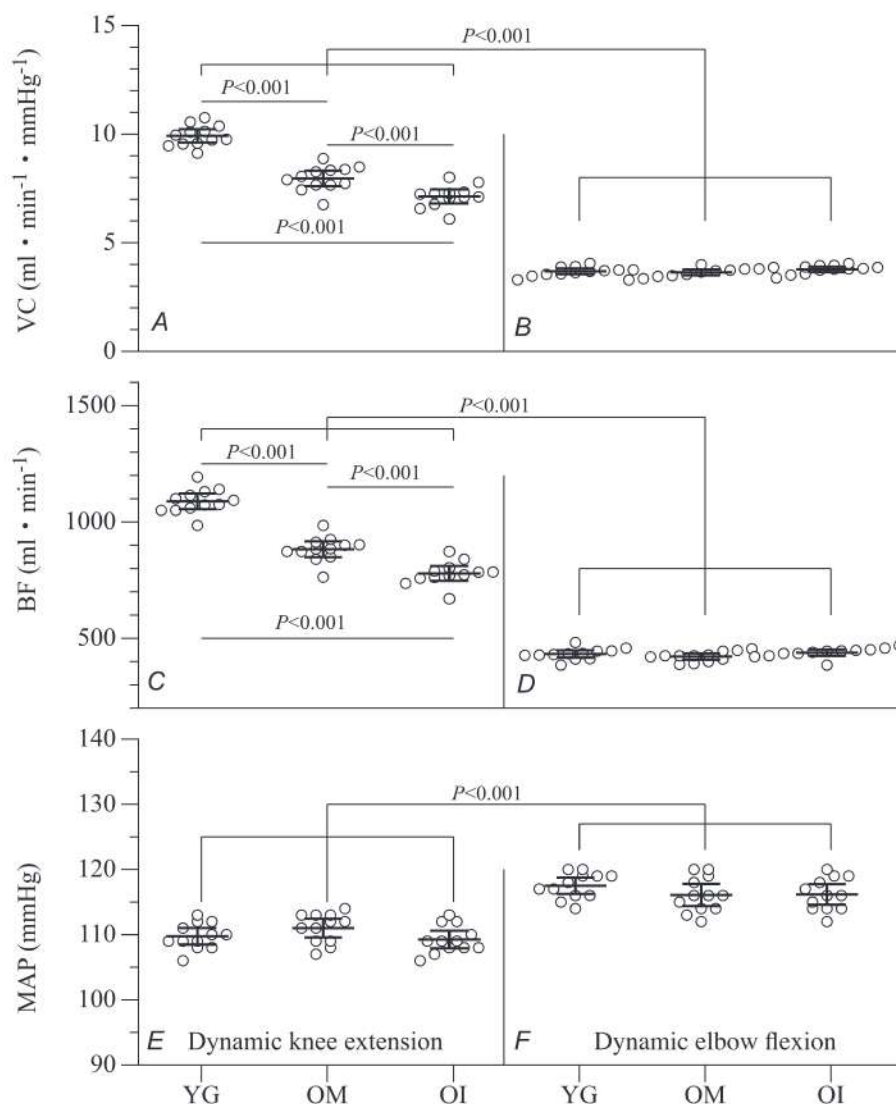


Figure 4. Peripheral haemodynamics

A and B, vascular conductance (VC) values calculated during the last 30 s of 10 W dynamic knee extensors (KE) and elbow flexors (EF) exercise, respectively, of healthy young (YG), mobile oldest-old (OM), and immobile oldest-old (OI). C–F, the values of blood flow (BF) (C, D) and mean arterial pressure (MAP) (E, F). Individual data (36 participants), mean values, and the 95% confidence interval are presented.

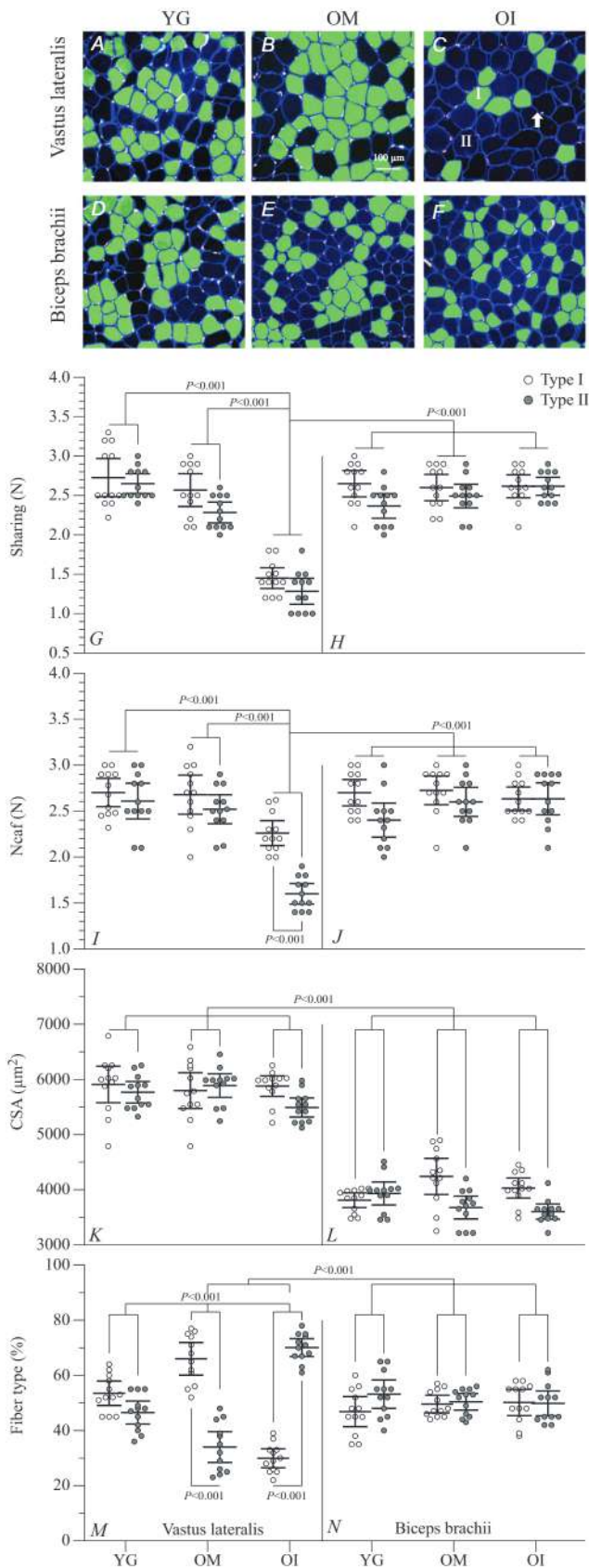


Figure 5. Fibre type, cross-sectional area (CSA) and capillarization

A–F, three muscle cross-sections of vastus lateralis and three muscle cross-sections of biceps brachii images of representative healthy young (YG), mobile oldest-old (OM), and immobile oldest-old (OI). Green and black represent type I and II fibres (see examples on C). Bright dots represent capillaries (see arrow on C). G and H, capillary sharing factor of vastus lateralis and biceps brachii, respectively, of YG, OM and OI subjects. I and J, number of capillaries (Ncaf) per fibre of vastus lateralis and biceps brachii, respectively, of YG, OM and OI subjects. K and L, CSA of vastus lateralis and biceps brachii, respectively, of YG, OM and OI subjects. M and N, proportion of fibre type of vastus lateralis and biceps brachii, respectively, of YG, OM and OI subjects. Individual data (36 participants), mean values, and the 95% confidence interval are presented.

in agreement with the results reported by both Hepple et al. (2004) and Venturelli et al. (2013). However, in this study our comprehensive approach of measuring both upper- and lower-limb muscle function, which experience differing levels of disuse in YG, OM and OI subjects, revealed a novel and important finding: changes in η_{net} during ageing are a phenomenon primarily associated with the muscle activity and are not a consequence of ageing *per se*. Specifically, the progressive level of lower-limb inactivity from the YG to the OM to the OI was reflected by opposite changes (increase in the OM; decrease in the OI) in η_{net} during KE (Table 1 and Fig. 2). This outcome was likely the result of detraining-induced adaptations at the muscular level. In fact, the OM demonstrated a significant reduction in the daily energy expenditure, which was likely related to their reduced capacity to perform higher intensity activities of daily living, such as stair climbing and uphill walking. In fact, as recently reported (da Silva et al., 2019), the mobility of oldest-old individuals is limited to slow ambulation at best, and is the only preserved activity of the lower limbs.

Muscle activity-induced adaptations during ageing

The changes in locomotor and high-intensity muscle activities during advanced ageing generates a sort of pure aerobic stimulus, slow walking, likely contributing to a shift toward the aerobic muscle phenotype in the lower limbs (Coffey & Hawley, 2017). Certainly, this phenomenon is coupled with other intrinsic factors, which cause atrophy and the disappearance of fast-glycolytic fibres (Lexell, 1995; Lexell et al., 1988; Schiaffino & Reggiani, 2011). Indeed, a greater type I fibre proportion of the total skeletal muscle volume in oldest-old individuals likely plays a role in altering the whole muscle efficiency as suggested by the correlation documented in Fig. 7 and by the regression analyses. In fact, the proportion of type I fibre explained the 66.3% variability in the η_{net} for the exercise executed with the KE. Here it is worth mentioning that when the muscle activity is dramatically reduced or even absent, the atrophy of the whole skeletal muscle is exacerbated (Buford et al., 2012;

Dufour et al., 2013; Pojednic et al., 2012). Interestingly, the literature suggests that this chronic period of muscle disuse results in a shift to less-efficient glycolytic fibres, which are characterized by worse energy economy in isometric contraction and by relatively poor mitochondrial support for ATP regeneration. The results of the current study are in agreement with this physiological scenario and highlight that peripheral changes in skeletal muscle fibre phenotype play a pivotal role in the attenuated η_{net} exhibited by OI subjects during KE. In contrast to the lower limbs, η_{net} of the arms was not different between the YG, OM and OI (Table 1 and Fig. 2). These data support our experimental hypothesis that with advanced ageing, and disuse superimposed on this process, the predominant impact of increased inactivity is confined to the locomotor muscles and not observed in the upper limbs (Janssen et al., 2000). It is important to note that the reduction in the use of the upper-limb muscles with age is less pronounced in comparison to locomotor muscles as they are not relied upon for ambulation.

Evidence that in the oldest-old η_{net} is not influenced by central and peripheral haemodynamics

Several studies documented that at light and KE intensities, peripheral haemodynamics appear consistently attenuated in healthy old (65–70 years) subjects (Lawrenson et al., 2003, 2004; Poole et al., 2003; Richardson et al., 2006; Sidhu et al., 2015; Wray & Richardson, 2015), while central haemodynamics do not seem to be affected (Poole et al., 2003; Richardson

et al., 2006; Sidhu et al., 2015). Moreover, this age-related reduction in BF to the exercising muscles appears confined to the knee extensor muscle and not to the plantar flexors or forearm muscles (Donato et al., 2006; Hart et al., 2014; Wray & Richardson, 2006). Data from the current study are in agreement with these previous investigations. Specifically, the measurements of central haemodynamics in this study (Fig. 3) support the notion that even at an advanced age, η_{net} calculated during exercise involving small muscle mass is not influenced by central factors. Also, the peripheral haemodynamic data collected in this study (Fig. 4) are in agreement with the previously referenced studies and suggest that age-related limitations to BF during KE exercise are exacerbated by lower-limb inactivity (Fig. 4). Interestingly, this attenuated BF and VC in the OM and OI seem not to be matched with changes in η_{net} during KE, implicating a potential difference in oxygen extraction in the OM and OI. This hypothesis seems to be corroborated by the different fibre types and [mtDNA] found in the quadriceps muscle of the OM and OI (Figs 5 and 6). Therefore, it seems reasonable to assume that the greater proportion of type I fibres and mitochondrial content may be a response to the reduction in oxygen delivery, mediating greater oxygen extraction. In contrast, both the reduction in oxygen delivery and the smaller portion of type I fibre coupled with limited mitochondrial content may well contribute to the reduction in η_{net} during KE in the OI subjects. Furthermore, the lack of difference between the YG, OM and OI in central and peripheral haemodynamics during EF supports the idea of limb vascular heterogeneity during ageing in humans (Wray & Richardson, 2006).

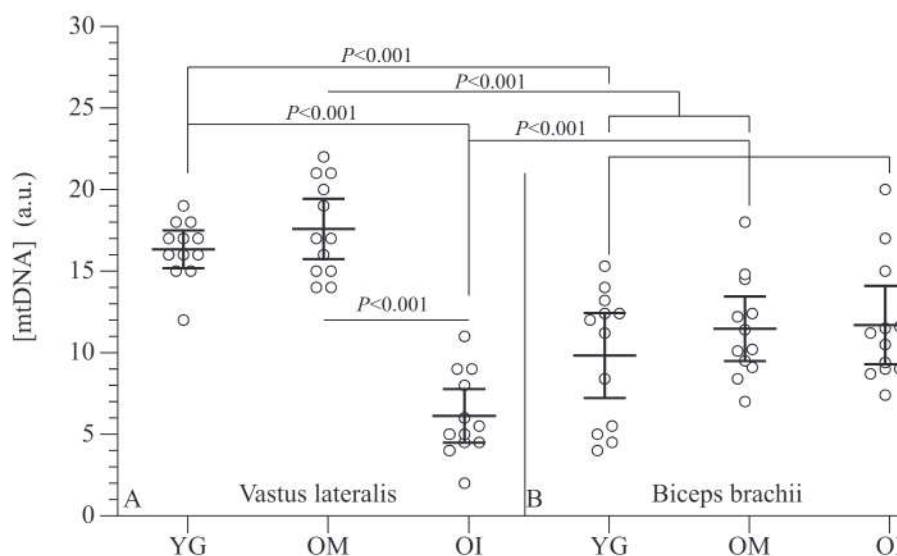


Figure 6. Mitochondrial DNA concentration [mtDNA]

A and B, [mtDNA] of vastus lateralis and biceps brachii of healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). Individual data (36 participants), mean values, and the 95% confidence interval are presented.

Evidence that in the oldest-old η_{net} is influenced by skeletal muscle fibre type and mitochondrial density

The influence of skeletal muscle fibre-type distribution on whole-body exercise efficiency is controversial. Indeed, a significant positive relationship has been reported between the percentage of type I fibres and mechanical efficiency in some (Coyle et al., 1992; Horowitz et al., 1994; Mogensen et al., 2006), but not all studies (Pedersen et al., 2002). Moreover, in another study by Hopker et al. (2013) examining young and old subjects with differing training levels, the relationship between mechanical efficiency and

percentage of type I fibres was not evident. Despite the heterogeneity of results provided by previous studies, data from this current investigation suggest that the percentage of type I fibres of the muscle utilized during exercise is, in fact, correlated with η_{net} (Fig. 7). Moreover, the differences in η_{net} exhibited during the KE performed by the OM and OI seem tightly linked to the dramatic shift toward a slow-aerobic and fast-glycolytic fibre phenotype, respectively, observed in the biopsy samples of the quadriceps. However, this positive correlation between skeletal muscle fibre-type distribution and η_{net} was not evident with EF (Fig. 7). Interestingly, the lack of changes

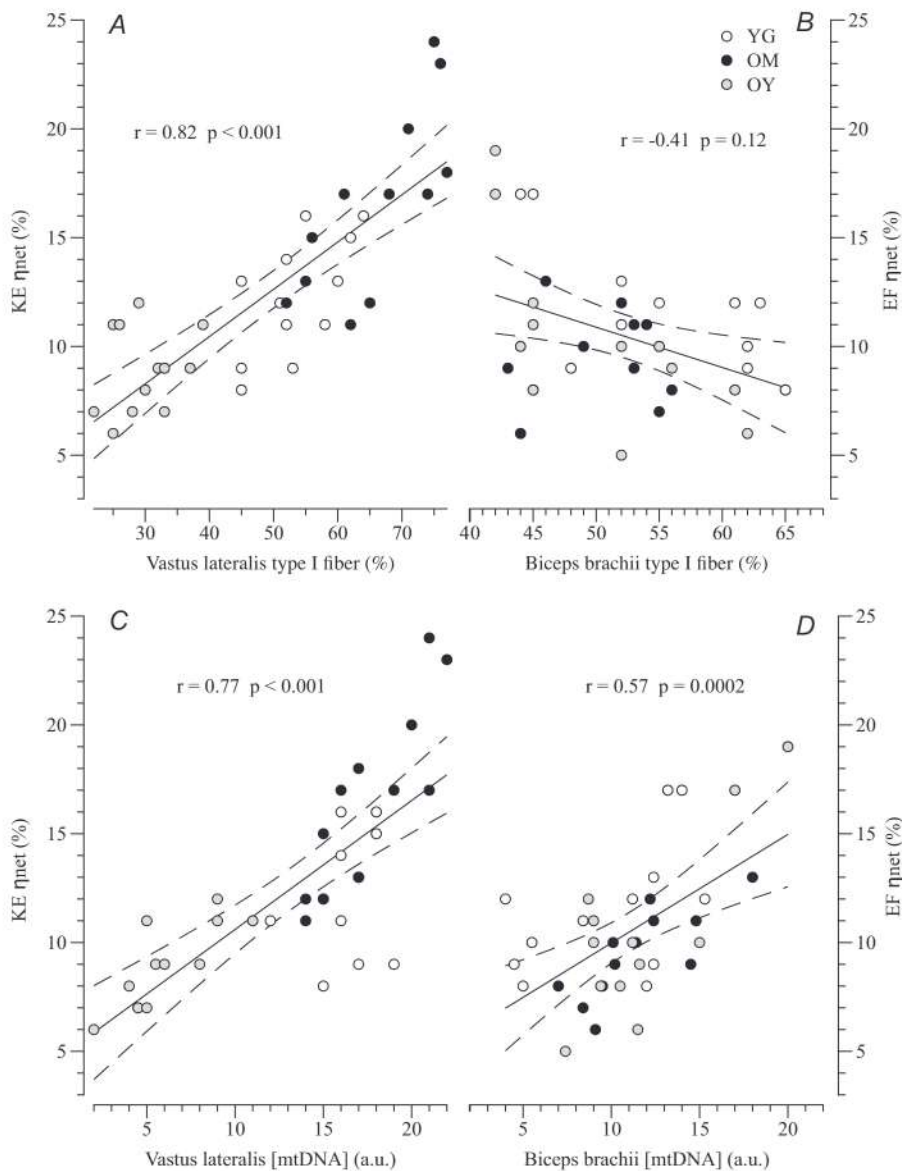


Figure 7. Correlations with net efficiency

The relationship between net efficiency calculated during dynamic knee extensors exercise (KE η_{net}), elbow flexors exercise (EF η_{net}), the concentration of mitochondrial DNA [mtDNA] and the percentage of skeletal muscle fibre type I of vastus lateralis and biceps brachii of healthy young (YG), mobile oldest-old (OM) and immobile oldest-old (OI). Open circles correspond to YG; filled circles correspond to OM; grey circles correspond to OI.

in mechanical efficiency and fibre-type distribution in the upper limbs of the OM and OI, suggests that these changes are likely affected by the level of muscle activity instead of age *per se*. The difference in mechanical efficiency between young and old adults and the extent to which mitochondrial content and function may contribute to these peripheral adaptations has only been partially elucidated (Kent-Baum & Fitzgerald, 2016). For instance, in young healthy subjects a moderate correlation between mechanical efficiency and mitochondrial content, measured by citrate synthase activity, was reported by Mallory et al. (2002) and Mogensen et al. (2006). While Conley et al. (2013) and more recently Broskey et al. (2015) demonstrated that changes in mitochondrial content and efficiency correlate with age-related changes in mechanical efficiency. The current data are partially in agreement with Conley et al. (2013) and Broskey et al. (2015), and support the concept that mitochondrial content, as the last step of the oxygen cascade, is extremely important for oxidative metabolism at rest and during exercise. Indeed, the stepwise multiple linear regression analyses utilized in the current study revealed that the proportion of type I fibre explained the 66.3% variability in the η_{net} for exercise executed with the KE, while in the EF the η_{net} was primarily associated (31.1%) with the proportion of [mtDNA].

Experimental limitations

The values of pulmonary $\dot{V}O_2$ reported in the present study are, likely, a reasonable surrogate for $\dot{V}O_2$ in the exercising muscle, but with such an approach there is the potential for a contribution from other muscles, such as those used for stabilization. The direct Fick measurement of $\dot{V}O_2$ across the leg and arm would have been a better approach to determine O_2 extraction in the exercising limb. However, due to the invasive nature of such an approach in oldest-old individuals, pulmonary $\dot{V}O_2$ was utilized instead. It is important to note that a previous investigation reported parallel pulmonary and limb $\dot{V}O_2$ at low- and moderate-intensity exercise domains (Richardson et al., 1993). Therefore, pulmonary $\dot{V}O_2$ could be used for the calculation of mechanical efficiency in the current study, taking into consideration an over-estimation of this value at muscular level.

Conclusions

This study suggests that η_{net} is attenuated by muscle inactivity and not by chronological age, *per se*. Likewise, this study revealed that the age-related changes in η_{net} are not a consequence of central, and peripheral haemodynamic adaptations, but are likely a product of peripheral changes related to skeletal muscle fibre type

and mitochondrial content. Therefore, recognition that the η_{net} of skeletal muscle is not simply age-dependent, but the consequence of inactivity and subsequent skeletal muscle changes, highlights the importance of maintaining physical activity across the lifespan.

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Additional information

Data availability statement

The data supporting this study’s findings are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

Author contributions

M.V., R.S.R., C.R. and F.S. conceived and designed the research. M.V., G.R.M., C.T., J.Z. and F.N. conducted the experiments. M.V., C.R., J.Z., G.R.M. and F.N. analysed the data. M.V., G.R.M., C.T., F.N., C.R., A.J.D., R.S.R. and F.S. interpreted the results of the experiments. MV prepared the figures. M.V., G.R.M., C.R., R.S.R. and F.S. drafted and edited the manuscript. All authors revised and approved the final version of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

fibre type, mechanical efficiency, mitochondrial content, oldest-old

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History

Supplementary Table 1

Supplementary Table 2