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Physiological and Functional Evaluation of Healthy Young and Older Men and Women: Design of the European MyoAge Study

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KEY WORDS

Ageing, skeletal muscle, mobility, sarcopenia, MyoAge

ABSTRACT

Within the European multi-centre MYOAGE project, one workpackage was designed to investigate the contribution of age-related changes to muscle mass, contractile characteristics and neural control in relation to reductions in mobility in older age. The methodology has been described here. Test centres were located in Manchester, UK; Paris, France; Leiden, The Netherlands; Tartu, Estonia and Jyväskylä, Finland. In total, 182 young (18-30 years old, 52.2% female) and 322 older adults (69-81 years old, 50% female) have been examined. The participants were independent living, socially active and free from disease that impaired mobility levels. The older participants were selected based on physical activity levels, such that half exceeded current recommended physical activity levels and the other half had lower physical activity levels than is recommended to maintain health. Measurements consisted of blood pressure; anthropometry and body composition (dual-energy x-ray absorptiometry and magnetic resonance imaging); lung function; standing balance and cognitive function (CANTAB). Mobility was assessed using the Timed Up and Go, a 6-min walk, activity questionnaires and accelerometers to monitor habitual daily activities. Muscle strength, power, fatigue and neural activation were assessed using a combination of voluntary and electrically stimulated contractions. Fasting blood samples and skeletal muscle biopsies were collected for detailed examination of cell and molecular differences between young and older individuals. The results from this study will provide a detailed insight into "normal, healthy" ageing, linking whole-body function to the structure and function of the neuromuscular system and the molecular characteristics of skeletal muscle.

INTRODUCTION

The elderly population is the fastest growing section of society in developed countries and many elderly people develop mobility problems that restrict their daily activities. In the light of these demographic developments it is important to fully determine the physiological changes that occur with increasing age and to understand how these impact on health, mobility, independence and, ultimately, quality of life. Such work may highlight lifestyle or other factors associated with health and independence in older age and identify risk factors to predict the onset of deterioration so that preventative, rather than remedial, action can be taken.

Reduced mobility in older age is a critical factor impacting on quality of life. Epidemiological research has associated reduced mobility with a loss of muscle mass in older age, termed sarcopenia (Rosenberg, 1997), and muscular weakness (Janssen, 2006; Hairi *et al.*, 2010). The term sarcopenia generally describes the loss of muscle mass that occurs with ageing, where individual fibres become smaller in cross section and there may also be a loss of fibre numbers (Lexell *et al.*, 1988). Several large-scale studies have examined the prevalence of sarcopenia, estimating around 50% of people aged > 80 yrs suffer low muscle mass (Baumgartner *et al.*, 1998; Janssen *et al.*, 2000; Iannuzzi-Sucich *et al.*, 2002; Janssen *et al.*, 2002).

With loss of muscle mass comes reduced muscle strength, but older people also experience an apparent loss of strength per unit muscle mass (Bruce *et al.*, 1989; Rutherford & Jones, 1992; Goodpaster *et al.*, 2006), which might help to explain why muscle weakness in older age is a better predictor of mortality than muscle mass alone (Newman *et al.*, 2006). The reduction in force generated per unit cross sectional area (specific force, Po/CSA) may in part be due to a lower Po/CSA of individual fibres (Larsson *et al.*, 1996; Larsson *et al.*, 1997) which in sedentary older people was associated with a decrease in myosin concentration (D'Antona *et al.*, 2003; Borina *et al.*, 2010). However, the generation of skeletal muscle force *in vivo* during a single contraction is complex and depends on appropriate cognitive and neural control, muscle architecture, contractile properties, force transmission within muscle and from muscle to bone across a joint. During sustained activity, such as ambulation or other activities of daily living, age-related changes in muscle energetics and cardiorespiratory adjustments also have a role to play. Modification of proteins and morphological changes to muscle and surrounding tissue occur with ageing and could impact on function. Structural and morphological changes can be seen in the magnetic resonance images of thigh muscles and the histological sections stained for myosin-ATPase from a young and older man shown in Figure 1.

To gain a more detailed understanding of neuromuscular changes and their impact on mobility in older age requires a detailed integrative physiological approach. Research using integrative physiology to study human ageing relies on minimally invasive procedures and tends to be relatively small scale, often including only tens of subjects, rather than the hundreds of observations seen in epidemiological research. Research is often restricted to a single data-collection centre, which limits the generalisation of results and many large-scale studies of older people have not included a cohort of younger adults as a reference comparison population. There is a need for larger, but detailed multi-centre and international physiology-based studies into the neuromuscular system and mobility in human ageing.

Aims and objectives

The MyoAge project was designed to understand and combat muscle weakness in older age, with the goal to inform the development of interventions to prolong the healthy lifespan by preventing the dependency associated with immobility in older age. Here we describe the methodology and participant characteristics of Workpackage 2. The primary aims of this workpackage were to characterise differences between young and healthy, medically stable and independent living older men and women in Europe for muscle mass, contractile characteristics and neural control; to relate neuromuscular function to mobility; and to determine the extent of differences between young and old in muscle "quality", measured as changes to contractile characteristics and force per unit muscle mass.

METHODS

Participant Recruitment

Participants were recruited and tested using similar procedures in five sites across Europe, including: Manchester, UK; Paris, France; Leiden, Netherlands; Jyväskylä, Finland and Tartu in Estonia. Data have been collected from 504 participants. Table 1 shows participant characteristics consisting of 84 recruited in the UK, 105 in France, 110 in the Netherlands, 100 in Estonia and 105 in Finland. Ethical approval was received from the local ethics committees at each of the five research centres. All participants provided written informed consent and were medically screened prior to participation.

The study population in each test centre was made up of three groups: *Young* men and women who were healthy, but not engaged in athletic competitions; *Physically Active* older men and women (but not competing in athletic events); and *Less Active* older men and women. The recruitment of active and inactive older participants allows for the examination of the effects of habitual physical activity levels on age-related health outcomes. Young were aged between 18 – 30 years and older were aged between 69 – 81 years. All participants regularly attended social or group activities to improve their knowledge or skills. Thus, younger participants were university students (but not enrolled on sports or exercise-related studies) and older participants were recruited from amongst those attending local groups such as University of the Third Age, further learning, history, teaching children, church organisations or arts and crafts. As far as possible, equal numbers of men and women were included and all were medically stable, community dwelling individuals free from major diseases and other factors indicated in the exclusion criteria. These criteria were assessed during a telephone-screening interview and again during a screening visit to the study centre.

Exclusion Criteria

Young women who were pregnant or were breast feeding a child were excluded. Also excluded were those who were participating in other on-going research projects; competing in high-level sports or athletic competitions; did not have social security or medical cover (National Health Service or other) or a fixed abode; were not deemed capable of making their own choices (such as those who had a legal guardian); had a BMI < 18 $kg \cdot m^2$ or > 35 $kg \cdot m^2$; were unable to walk more than 250 metres without assistance (walking aids were permitted); were institutionalised; were suffering any of the following co-morbidities (as assessed from the medical screening): neurological disorders, metabolic diseases (insulin dependent), rheumatoid arthritis or osteoarthritis in the hip and/or knee causing pain and functional limitation; diagnosis and treatment of cancer within the last year: polymyalgia rheumatica; cardiac or heart failure (NYHA 3-4); had a pacemaker; had myocardial infarction or angina

pectoris complaints in last 3 yrs; had peripheral arterial disease; cerebral event (TIA/CVA), deep vein thrombosis within previous 3 yrs; chronic obstructive pulmonary disease (Gold 3-4; (Pauwels *et al.*, 2001)); chronic locomotor system related pain syndrome (fibromyalgia, i.e. lower back pain or other complex regional pain syndrome that could interfere with mobility); patients with haemocoagulative syndromes in which biomaterial collection could cause strong bleeding; daily use of painkillers, except paracetamol (daily use of paracetamol was allowed); immunosuppressive drugs (eg prednisone, methotrexat, biologicals (TNF-alpha antagonists etc); insulin; anticoagulants (eg coumarines, carbaspirin calcium); fracture within the previous year; hip, knee and/or spinal stenosis surgery in medical history during the last 2 years; hip / knee replacement or spinal stenosis causing pain and physical limitation; amputation; immobilisation for 1 week during the previous 3 months; Mini Mental State Examination score of 23 points and lower (assessed when participant attended the laboratory); Geriatric Depression Scale (Yesavage *et al.*, 1982) score of 5 points or higher (assessed when participant attended the laboratory); severe visual or hearing impairment.

Physically Active was defined as those individuals involved in moderate or vigorous activities where the intention was to improve health and fitness. Activity sessions should work up a sweat and last $\geq$ 30 min per session, for $\geq$ 3 sessions per week and individuals must have consistently maintained such activities for the majority of the year and for the past 3 years or more.

Less Active was defined as those individuals who, for the past three months or more, had completed only one session or less per week in any physical activities designed to improve health and fitness other than their usual activities of daily living, and were rarely active in their daily lives to such an extent as to work up a sweat.

Data collection, management and quality control

The research centres involved in MyoAge have a long tradition of measuring mobility and functional capacity among populations of older men and women. A single standard operation procedure (SOP) was developed and agreed by senior research staff. All study centres adopted these same SOPs to ensure consistency across countries in data collection procedures. Whenever possible, the same manufacturer's equipment was used for testing in each centre. If this was not possible, a careful evaluation was made to be sure that the different research centres produced comparable data. Adherence to SOP was monitored carefully throughout the study. A senior researcher visited each test centre and took care to instruct and train the personnel performing the measurements at each test centre. Meetings were held periodically to ensure consistency and quality of data collection. The same staff in each study centre engaged in the data collection throughout the full duration of the study. Data quality checking and analysis of data from all countries was performed in one centre.

The data relating to the 504 participants (Table 1) were collected between 2010 – 2013. The testing sequence and procedures were carried out following the SOP (described below in Testing Procedures) and data were recorded on a standardised Case Report Form and digital copies were kept. Data of each participant were entered into a dedicated Microsoft Access database. The separate databases for each of the participating study centres were combined and stored on the server at the Leiden University Medical Centre, with daily back-ups. Access to the database and to the back-ups was restricted to the staff directly included in the study. The server was not accessible via the web. A second researcher screened all data entered to the database for appropriate use of in- and exclusion- criteria, consistency between study

centres, missing items and outliers. Queries were raised against any data that were not clear or consistent and the local study centres acted on the query to evaluate and resolve all issues.

To minimise the impact of possible differences between study centres that might occur due to the use of different equipment, all data obtained with different equipment (e.g. outcome of Dual Energy X-ray Absorptiometry) were standardised as country-specific Z-scores. To assign the Z-score, the mean ± standard deviation (SD) of the dataset was calculated for each country separately for the particular variable and the datapoint replaced with a Z-score representing the difference between that point and the mean of the country-specific dataset divided by the SD of the country dataset. For instance, a datapoint that matched precisely the mean of the country sample was assigned a "0", one that was 2 SD higher than the mean was assigned a "2" and one that was 1.5 SD lower than the mean was assigned -1.5. The data contained in the Database were imported into SPSS (IBM, SPSS Inc, Chicago, IL, USA) for further statistical analysis.

Testing procedures

Participants arrived in the morning at around 8:00 or 9:00 am in a fasted state having not eaten anything for 12 hours, but having had a glass of water before attending the laboratory. Alcohol intake and sauna use were not allowed for at least 24 hr prior to the visit and heavy or strenuous exercise was avoided for 48 hr prior to the visit. Smoking was not allowed in the 2 hrs prior to muscle function measurements.

The morning session included providing the written informed consent after the procedures had been fully explained and questions answered, issue of unique participant identification code to anonymise the data, completion of questionnaires, blood pressure, blood sampling, body anthropometry and composition, a small snack, drink and a rest, lung function, balance and cognition tests. After a 60 min light lunch, usually consisting of a sandwich or soup, a drink and 30 min rest, all participants completed the Timed Up and Go, a 6-min walk and measures of muscle strength, power and fatigue. In all tasks, the investigators provided verbal instructions and physical demonstration and measurements only commenced when the participant was clear of the requirements.

General characteristics and food intake

Participants provided information on smoking history as number of pack years; alcohol intake in units per week, medical history was assigned a numerical value as the sum of diagnosed conditions, and on-going medication usage was recorded. The Simplified Nutrition Appetite Questionnaire (Kruizenga *et al.*, 2005) was used to provide information on food intake.

Habitual physical activity

To estimate physical functioning, a questionnaire of activities of daily living was used (Katz *et al.*, 1970). The extent of habitual physical activity was estimated using the Voorrips physical activity questionnaire (Voorrips *et al.*, 1991) in older participants and the Baecke physical activity questionnaire (Baecke *et al.*, 1982) in young participants.

A sub-group of volunteers from each test centre were supplied with a tri-axial accelerometry-based physical activity monitor (MiBand, MiLife, Bedfordshire, UK). The participants were instructed to maintain their usual habitual physical activity levels for the duration of monitoring. The monitors were water resistant and capable of data collection for 15 consecutive days. They were worn on the wrist of the non-dominant hand for 10 consecutive

full days (not including the day that the monitor was issued) and were worn day and night. In Leiden and in Paris, physical activity was additionally monitored in all participants by use of another tri-axial accelerometer (GENEA, ActivInsights Limited, Kimbolton, Cambridgeshire, United Kingdom) with a sampling frequency of 75 Hz. In The Netherlands, GENEA accelerometers were worn on the lateral malleolus of the right ankle and on the right wrist. In France, they were worn on the wrist of the non-dominant arm. Subjects were instructed to wear the accelerometer(s) day and night for at least 7 consecutive days, with the exception that they were not to be worn in a sauna and if swimming or bathing. The monitors were returned using the postal service and when received back, the data were immediately downloaded to a computer for off-line analysis.

Blood pressure measurement and resting heart rate

Prior to the blood pressure measurement, the participant remained seated in a quiet room for at least 10 minutes. The correct size cuff was selected and wrapped around the upper arm at the level of the atrium and inflated to make the recording using an automated monitor (Omron Healthcare Europe, B.V, Nijmegen, Netherlands). Readings were taken three times with a 2 min gap between subsequent measurements (Pickering *et al.*, 2005). For each measurement, systolic and diastolic pressures and pulse rate were recorded.

Mental state and cognitive tests

All tests were performed in a quiet room free from distractions. Experimental staff were trained to carry out the tests. Participants completed the Geriatric Depression Scale (Yesavage *et al.*, 1982) and the Mini Mental State Examination (Folstein *et al.*, 1975). In addition, neuropsychological cognition was assessed using a computerised, touch screen CANTAB device (Cambridge Cognition Ltd, Cambridge, UK). Tests were performed according to the CANTAB Guidelines and completed in the following order and the given time limits: *Spatial Span test* (10 min time limit) was a test of working memory capacity in which white squares change colour in a particular sequence and the participant must remember and correctly repeat the sequence in the same order they were displayed by the computer. The *One Touch Stockings of Cambridge* test (20 min time limit) was a spatial planning task in which the participant must alter the arrangement of coloured balls to match that shown in a different part of the screen using as few moves as possible. The *Paired Associate Learning* test (10 min time limit) required the participant to remember the location of different symbols hidden beneath white blocks.

Anthropometry and body composition

Standing height was measured to the nearest mm and body mass was measured to the nearest 0.1 kg with shoes removed and light clothing only. Arm span was measured to the nearest cm from the tip of the middle finger of the left hand to the tip of the middle finger on the right hand with arms outstretched horizontally as wide as possible.

Total body composition was assessed by dual-energy x-ray absorptiometry (DXA) (UK: Lunar Prodigy Advance, version EnCore 10.50.086; France: Lunar Prodigy, version EnCore 12.30; Netherlands: Hologic QDR 4500, version 12.4; Estonia: Lunar Prodigy Advanced, version EnCore 10.51.006; Finland: Lunar Prodigy, version EnCore 9.30). The scan was performed in the morning with the participant in the fasted state and wearing only standard thin cotton clothing, such as a hospital gown. All other clothing and jewellery were removed. The local (site-specific) safety procedures were followed. A whole-body scan was performed with the participant lying supine with legs and arms fully extended. Tape or a thin cotton strap

was used to hold the feet together just below the level of the toes, but the heels remained parted by around 5 cm. The head was flat on the scanning bed. Appendages were isolated from the trunk and head by using DXA regional computer-generated default lines, with manual adjustment. Details of bone mineral density, fat mass and lean mass of the whole body and of the body segments on the left and right sides of the body were recorded.

Participants recruited in UK, France and a sub-group of those recruited in Estonia, completed magnetic resonance imaging of the thigh (UK: Esaote G-scan, Biomedica, Genoa, Italy; France: Tim Trio, Siemens Healthcare, Erlangen, Germany; Estonia: Achieva 3.0T X-series, Philips Healthcare, Best, The Netherlands). Turbo 3D T-1 weighted images were obtained and measurement of cross-sectional area of individual quadriceps muscles, femur cortical and trabecular areas and all other muscles of the thigh were analysed at 2.5 cm intervals along the full femur length from distal to proximal regions, and overall muscle volume was estimated (McPhee *et al.*, 2009).

Lung function

The spirometric lung function test was performed with the participants in a sitting position with knees and hip flexed at around 90° and using a nose clip (UK, France and The Netherlands all used Micro Medical Spiro USB spirometer and Spida 5 software (Cardinal Health, UK); Estonia used a SPIRA Peak Flow for FEV1 (Spira, Finland) and a Spirometer Spiropet for FVC (Nihon Medical Instruments Co Ltd, Japan); Finland used a SpiroStar (Medicro, Finland). The participant gave at least three maximal attempts to blow into the mouthpiece of the spirometer as forcefully and quickly as possible and continued to blow until the lungs were emptied of air as much as possible to record forced expiratory volume in 1 second (FEV1, L) and forced vital capacity (FVC, L) from each of these efforts. The best ratio of FEV1/FVC from the 3 efforts was also recorded. Measurements were repeated until the two highest values for FEV1 or FVC were within 0.15 L of one another (Miller *et al.*, 2005).

Standing balance

Balance was tested during two-leg and one-leg standing, completing trials first with eyes open and then with eyes closed. The participant attempted to stand as still as possible for a maximum of 30 sec or until one of the termination criteria was violated. Shoes were not worn and a visible marker (such as a ‘X’) was placed on a wall 2 metres in front of the participant to provide a fixed point of gaze during eyes open trials. The two-leg standing trials were completed with feet together and arms relaxed. The participants were not allowed to use their arms by moving them in an effort to maintain balance. One-leg balance trials were performed with the contra-lateral leg held around 5 cm off the ground. All trials were repeated twice unless the participant was not able to balance for the full 30 sec, in which case a third trial was included. There was a rest of 30 sec between trials and the participant was encouraged to take a few small steps to move the legs between trials. In all centres except UK, measurements were completed on a force platform that could record centre of pressure movements (France: AMTI OR6-7, Watertown, MA, USA; Netherlands: Forcelink B.V., Culemborg, The Netherlands; Estonia: Kistler, Switzerland; Finland: Good Balance, Metitur, Finland). In The Netherlands, additional balance tests included semi-tandem stance, where participants were standing with the medial side of the heel of one foot touching the big toe of the other foot; and tandem stance, where participants were standing with both feet in line while the heel of one foot touched the toes of the other.

Mobility

The *Timed Up and Go* test (Podsiadlo & Richardson, 1991) was performed using a standardised chair without arm rests. Participants started from a seated position and when instructed they walked around a small cone placed on the ground 3 m directly in front of the chair and returned to the original sitting position, moving as quickly as possible whilst taking care not to run and to remain safe. Comfortable, non-slip shoes or trainers were worn and a walking assistive device was allowed if needed. After a practice trial, the participant completed three timed trials. Encouragement was offered to try to improve after each attempt. There was 1 min rest between trials.

The *6 minute walk test* was completed using a 20 m circuit, except in France where a 25 m circuit was used (Enright, 2003). The participant walked around the track as many times as possible during 6 min, attempting to walk as far as possible in the allotted time. One exception was in the Netherlands, where participants were instructed to walk at their usual pace throughout the trial. Running was not allowed and use of a walking assistive device was permitted if normally used for walking. Participants were allowed to stop and rest if they need to, but were encouraged to continue as soon as they felt able. Standardised verbal instructions were given at the end of each minute to inform of progress and the amount of time remaining. The participant was familiarised with the Borg Scale of Perceived Exertion (RPE) (Borg, 1982) and wore a heart rate monitor during the trial (Polar Electro, Oy, Finland). The RPE and heart rate were recorded after 3 min, whilst taking care not to interfere with the task or stop the participant from walking, and again immediately at the end. The exact distance (m) covered in the 6 min walk was recorded.

Muscle function

Grip strength was measured using a Jamar handgrip dynamometer (Sammons Preston Inc, Bolingbrook, IL, USA) in a standing position and arms fully extended vertically along the side of the body. The participant was instructed to squeeze the handle as hard as possible for around 3 sec and the maximum contraction force was recorded. This was repeated 3 times for both hands, alternating between right and left hands and 30 sec rest between trials.

To assess leg extension muscle power, a maximal-effort countermovement vertical jump was performed on a force platform (UK: Leonardo, Novotc Medical, Pforzheim, Germany; France: AMTI OR6-7, Watertown, MA, USA; The Netherlands: Forcelink B.V., Culemborg, The Netherlands; Estonia: Kistler, Switzerland; and Finland: custom built force platform). The test was repeated a further two times with a rest interval of 60 sec. The maximum force (k.N), maximum power of the concentric phase (Watts) and jump height (m) were measured using the vertical component of the ground reaction force (Caserotti *et al.*, 2001).

The knee extensor maximal isometric voluntary contraction (MVC), level of voluntary activation and fatigue resistance were measured using a combination of voluntary and electrically stimulated contractions. Participants were seated on a dynamometer with hip and knees flexed at 90° and tightly secured with straps (UK, Estonia and Finland all used custom-built devices, The Netherlands used a Forcelink B.V. (Culemborg, the Netherlands) and France used a Biodex system 3 Pro isokinetic dynamometer (Biodex Medical Systems, Shirley, New York, USA)). The MVC was assessed on the leg that the participant believed would be strongest. The force transducer attachment was positioned 2 cm above the ankle malleolus. After a standardised warm up, participants were instructed to perform a maximal isometric knee extension. Strong verbal encouragement was given throughout the effort. This was repeated a further two more times with a rest of 90 sec between efforts. Where torque (Nm) was not measured directly (Estonia, Finland, UK), it was estimated as force (N) * lever

length (m). Lever length was estimated as the distance (m) from the centre of rotation of the knee to the point of force application on the force transducer.

To assess the level of voluntary activation, percutaneous electrically stimulated doublets were used (2 pulses of 400 V and pulse width of 1000 μs, separated by 10 ms). Two stimulation electrodes were positioned on the skin over the proximal and distal heads of quadriceps femoris muscles (AmericanImex: Dispersive electrode, ref 00200-800: 4 x 7 inch; CA, USA). A current to elicit 30% MVC was used (Rutherford *et al.*, 1986). A doublet stimulus was first applied to the resting muscle, the participant then performed an MVC and a doublet was applied at the highest point of the MVC. This was repeated a second time after 90 sec rest. The percentage of voluntary activation was calculated as: 100 * (1 – t/T) and maximal torque calculated as: MVC / (1 – t/T). Where t was the amplitude of the superimposed doublet (i.e. the size of the additional peak); T the value of the rest doublet; and MVC was the level of voluntary contraction immediately prior to the doublet stimulation.

Muscle fatigability was assessed as the time-to-task-failure during a sustained contraction at 50% MVC. This test was performed on the same leg as that measured for MVC with the participant sitting in an identical position. A line to indicate the target force was clearly displayed on the computer monitor and the participant received real-time visual feedback of their actual force in comparison to the target line. The test was terminated when force dropped below 5% of the target force for more than 3 sec despite strong verbal encouragement from the investigator urging the subject to recover and continue. Electrically-stimulated doublets of the same amplitude as that used in the test of voluntary activation were superimposed at 10 sec intervals throughout the trial to distinguish between central and peripheral mechanisms of fatigue.

Blood and Muscle Biopsy samples

Whole-blood was collected into vacutainer collection tubes, including a 10 ml serum; two 10 ml EDTA glass tubes and one 6 ml HEPARIN tube (BD, Oxfordshire, UK). Samples were centrifuged at 2,750g at 4 °C for 15 min within 20 min after obtaining the blood. Serum, plasma and the buffy coat were aliquotted and stored at -80°C until analysis. All samples were stored in a Biobank or distributed to *Partner* research groups in Copenhagen, Denmark; Unilever, UK and Bologna, Italy for analysis of cytokines, hormones and growth factors that form work within other MyoAge Workpackages (WP 3 and 5). Additional blood samples were collected in The Netherlands for immunological analyses and during an oral glucose tolerance test.

A subgroup of participants (n = 173) returned on a separate day around 2 hours after breakfast to provide a biopsy sample of the vastus lateralis muscle. The sample was collected under local anaesthesia (2% Lidocaine or Lignocaine) from around the mid-femur with the participant lying supine and relaxed. A small incision was made through the skin, subcutaneous tissue and muscle fascia using a sterile scalpel and the sample was collected using a conchotome (Dietrichson *et al.*, 1980) or Bergstrom needle (modified for application of suction, Maastricht Instruments, Maastricht, The Netherlands). The incision site was closed using surgical tape or Steri-Strips and a compression bandage applied. In Estonia, 30 samples were collected and distributed to *Partners* working in other MyoAge workpackages in Bologna, Italy and UPMC, France for protein, DNA and gene expression analysis. In Leiden, 107 samples were obtained. These were distributed to *Partners* working in other MyoAge workpackages for further analysis; 15 samples were immediately stored in pre-cooled F-10 medium and transported on dry ice to Paris Inserm UPMC, France for cell-culture. The remaining 92 biopsies were divided into three equal pieces, immediately frozen in liquid

nitrogen and stored at -80° until analysis. These were distributed to University of Rome, Italy; UNIBO, Italy; and UPMC, France. A total of 36 samples were collected from Manchester, UK and 27 from Paris, France. These were used to examine differences between young and old in muscle "quality" within the scope of Workpackage 2. Histological assessment was made of muscle fibre type and cross-sectional area, capillarisation and succinate dehydrogenase activity. Western-blotting was used to detect focal adhesion kinase, vinculin, tenascin C, and mitochondrial proteins. Samples from the older men tested in Manchester were also sent to *Partners* in University of Pavia, Italy, for proteomic analysis, single fibre-contractile properties, myosin content and identification of oxidised proteins.

Single fibre characteristics

Small bundles of muscle harvested from the muscle biopsy collected from older men in Manchester (n = 16) were immediately immersed in ice-cold skinning solution and stored at -80°C until analysis. The analysis of these samples was completed in the same way as tests performed on muscle collected from within MyoAge Workpackage 8 from elderly athletes and younger men undergoing experiments of disuse atrophy. Twenty samples from each subject were tested for maximal shortening velocity, cross-sectional area (CSA) and specific force (Po/CSA) as previously described (D'Antona *et al.*, 2003). The technique to determine myosin concentration was adapted to examine whether the myosin content was decreased in healthy older men, as was previously observed in sedentary elderly (D'Antona *et al.*, 2003). Measurements of myosin content were done in duplicate and the average taken for each fibre. In the same gel, a known amount of myosin standard was loaded in order to determine a standard curve. Example bands and a standard curve are shown in Figure 2.

DISCUSSION

The methodology used in the MyoAge Workpackage 2 (WP2) has been described and participant phenotype characteristics presented. The main purpose of this workpackage was to examine the physiological and functional characteristics of older men and women who were free from disability and major chronic disease and compare them with younger, healthy adults. Participant characteristics are shown in Table 1. Muscle biopsy samples were collected for determination of molecular aspects of muscle "quality", relating to the structural and functional changes that affect the force generating capacity per unit muscle mass. Muscle biopsy and blood samples were also distributed to *Partners* in other workpackages within the wider MyoAge framework for analysis on cytokines, hormones, growth factors, gene expression, mitochondrial DNA and oxidative stress.

The measurements used in Workpackage 2 were selected to be physiologically relevant to the cohort of healthy older and young adults. The clear advantage of a multi-centre, Pan-European trial was that detailed measurements could be collected from many more participants than would have been possible in a single-centre trial. Where different manufacturer's equipment was used, such as during DXA scanning, the data can be normalised as Z-scores and the "Country" can be used as a possible co-variate in statistical analyses. The advantage of standardisation into Z-scores is that it ensures the data obtained from different equipment in different countries can be directly compared and therefore adds greater confidence to observations derived from the combined datasets. Data were also retained in their original format and the Z-score was only applied to variables where different equipment was used and the effect sizes (means ± SD for young and older subjects) were not comparable across countries.

The data shown in Table 1 describe the general background and differences between young and older participants in respect to anthropometry, lifestyle, comorbidities and mental status. As expected, the older participants had reduced mobility levels compared with young: walking speed during a 6-minute assessment was 20% slower and the Timed Up and Go was 28% slower. Older people could balance on one leg for less time than young and when eyes were closed the older people could manage on average only around 4 sec, while younger subjects generally maintained the full 30 sec. Concomitant with the reduced mobility levels, older people had 9% higher body fat, 4% lower lean mass in relation to height (ALM/h^2), 22% lower handgrip strength and 35% lower knee extensor strength. Despite the very modest difference between young and old in total muscle mass, the older subjects clearly demonstrated mobility limitations and muscle weakness compared with young. One possibility is that strength is reduced to a greater extent than muscle mass with ageing (Goodpaster *et al.*, 2006), another is that the leg muscles are more susceptible to age-related loss of muscle mass compared with muscles of the upper body (Janssen *et al.*, 2000). Indeed, our preliminary analysis of MRI images suggests the quadriceps muscles are particularly susceptible to loss of muscle mass with ageing (unpublished observations). The physiological relevance and relationship between the measurements used in the present study will be explored in detail in future publications.

It is recommended that older people partake in regular physical activities to maintain their neuromuscular function and health (Chodzko-Zajko *et al.*, 2009; Department of Health, 2011). The older participants in the present study were recruited based on their physical activity levels, with half exceeding physical activity recommendations and the other half not achieving the recommended minimum activity levels for older people. Care was also taken to ensure all participants were socially engaged in a range of activities to develop their

knowledge or cognitive skills. All the participants in Workpackage 2 were relatively healthy and few were smokers, consumed excessive alcohol or were obese (Table 1). This study design allows for examination of the effects of ageing and the impact of regular physical activity without the complication of having a high frequency of risk factors for major chronic disease. Volunteers were asked to self-classify their activity levels during a telephone interview and a physical activity questionnaire. There is a need to develop more sophisticated assessments of habitual physical activity to overcome the limitations of subjective self-report. One possibility is to use accelerometry-based data and to report intensity as low, moderate or high (e.g. (Hansen *et al.*, 2012). Accelerometers were therefore adopted for use in a sub-group of participants from all countries. Master Athletes and those suffering from chronic disease that impacted on mobility were excluded from participation in this study. However, within the wider MyoAge study it will be possible to compare results with those obtained from older adults suffering disease, such as osteoarthritis or rheumatoid arthritis who were studied in Workpackage 1 and the exceptionally healthy and active Master Athletes competing at international standard in track and field events studied in Workpackage 8. This will contribute to the overall aim of the MyoAge project, to understand and combat muscle weakness in older age and to prolong the healthy lifespan.

Limitations: Multi-centre trials have the possible limitation that data collection cannot be identical across different test centres. To minimise such effects, all equipment were calibrated according to manufacturer's and local lab recommendations, personnel attended a training induction held at a single test centre and one experienced researcher visited all study centres prior to the commencement of data collection to train experimental staff and ensure consistency in data collection in line with the SOPs. A further limitation of studies that recruit older volunteers to attend research laboratories to complete measurements is that a relatively healthy population might be inclined to volunteer. However, this was a deliberate strategy in the present study of "healthy" ageing. Our recruitment strategy was primarily based on self-report during a telephone interview and a physical activity questionnaire. Volunteers suffering any disease or disability that impacted on mobility levels were excluded, so the results of the present study represent "healthy ageing" across Europe and are not necessarily representative of the wider population of older people.

Overall, the results from WP2 will provide a detailed insight into "normal, healthy" ageing, linking whole-body function through to structure and function of the neuromuscular system and molecular characteristics of skeletal muscle. This work will build upon large-scale epidemiological studies into ageing and allow for detailed characterisation of the inter-individual variability in neuromuscular ageing that cannot be studied in smaller-scale physiological studies.

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TABLES

Table 1: Participant characteristics, stratified by age (n=504).

	Young (n=182)	Old (n=322)	P-value
Age (years)	23.4 (2.9)	74.4 (3.3)	**<0.0005**
Females, n (%)	96 (52.7)	161 (50.0)	0.554
Living with partner, n (%)[a]	40 (26.8)	173 (64.8)	**<0.0005**
Highly educated, n (%)[b]	134 (87.6)	119 (44.6)	**<0.0005**
Anthropometry			
Height (m)	1.73 (0.09)	1.67 (0.09)	**<0.0005**
Body mass (kg)	68.7 (12.3)	71.6 (12.7)	**0.014**
Body mass index ($kg \cdot m^2$)	22.8 (3.0)	25.6 (3.3)	**<0.0005**
Lifestyle			
High alcohol use [c], n (%)	22 (12.1)	28 (8.7)	0.221
Current smoking, n (%)	24 (13.2)	14 (4.3)	**<0.001**
Comorbidities			
Number of diseases, median (IQR)	0 (0-0)	1 (0-1)	**<0.0005**
Number of medications, median (IQR)	0 (0-1)	1 (0-3)	**<0.0005**
Mental state			
MMSE score (points), median (IQR)	30 (29-30)	29 (28-30)	**<0.0005**
GDS score (points), median (IQR)[d]	0 (0-1)	1 (0-2)	**<0.0005**
Diagnostic criteria for sarcopenia			
Lean mass percentage (%) [e]	72.8 (9.1)	66.6 (8.3)	**<0.0005**
ALM percentage (%) [f]	33.1 (4.7)	28.6 (4.1)	**<0.0005**
ALM/height² (kg/m^2)	7.5 (1.3)	7.2 (1.1)	**0.013**
Total lean mass (kg)	50.1 (11.4)	47.4 (9.9)	**0.008**
Knee extension torque ($N \cdot m$) [g]	196.6 (69.6)	126.5 (46.0)	**<0.0005**
Handgrip strength (Kg) [g]	42.3 (12.3)	33.1 (9.6)	**<0.0005**
Physical performance			
TUG (s) [h]	4.85 (0.91)	6.24 (1.16)	**<0.0005**
Walking speed (m/s)	1.85 (0.30)	1.49 (0.23)	**<0.0005**
One-leg balance with eyes open (sec), median (IQR) [g]	30.0 (30-30)	30.0 (15-30)	**<0.0005**
One-leg balance with eyes closed (sec), median (IQR) [g]	30.0 (20-30)	4.0 (2-6)	**<0.0005**

Variables are presented as mean (standard deviation), unless indicated otherwise. For strength and performance measurements the best effort has been used for analysis. Independent samples t-tests were used to assess differences between young and old. [a] Data available in n=416. [b] Data available in n=420. [c] High alcohol use defined as for males > 21 units/week and females > 14 units/week. [d] Data available in n=411. [e] Total lean mass as percentage of total body mass. [f] ALM (appendicular lean mass) as percentage of total body mass. [g] The highest value from the duplicate measurements has been used for analysis. [h] The fastest time from the duplicate measurements has been used for analysis. MMSE: mini mental state examination. GDS: geriatric depression scale. TUG: Timed Up and Go test.

FIGURES AND LEGENDS

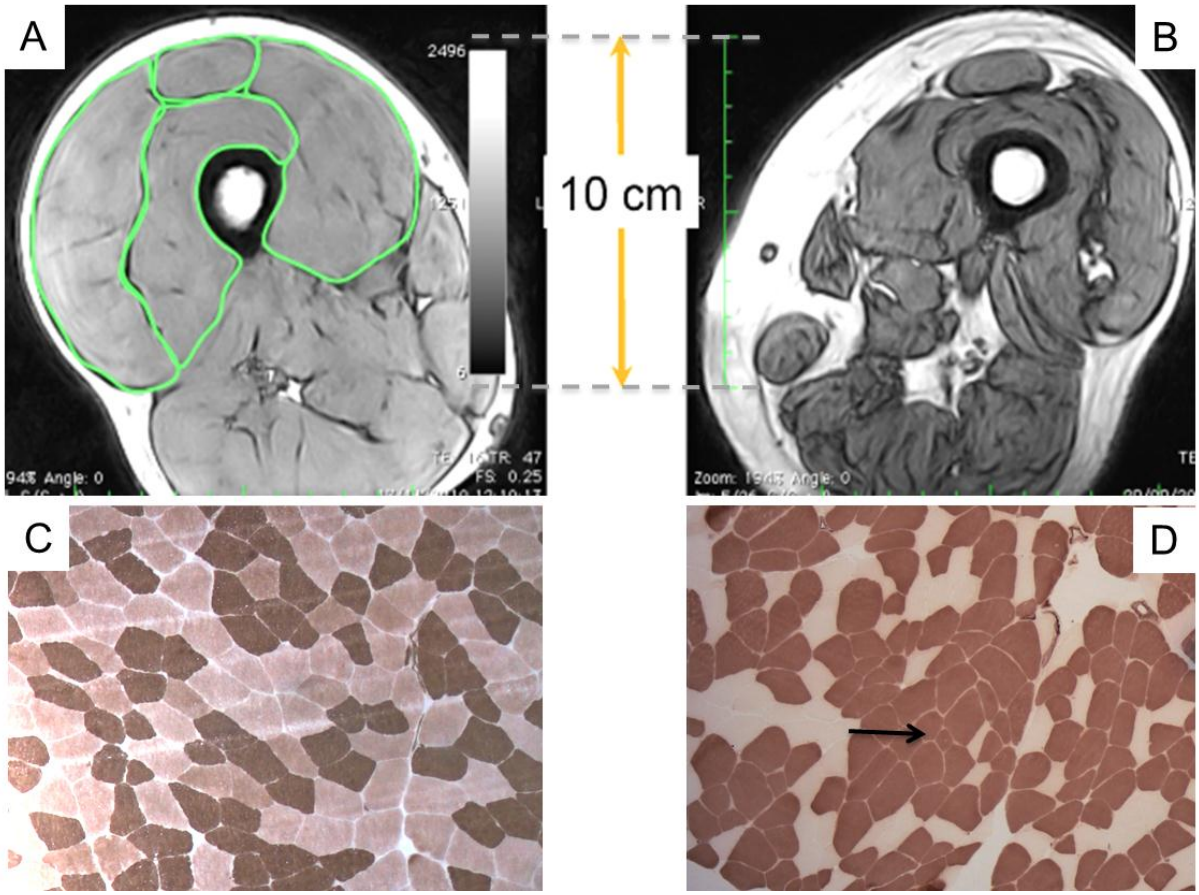

Figure 1. Magnetic resonance images of the thigh and vastus lateralis biopsy sections stained for myosin-ATPase. MRI scans were obtained at the mid-thigh level. It is clear to see that the older thigh (B: 81 yr old man) had smaller muscles and relatively more adipose tissue than the younger thigh (A: 25 yr old man). The quadriceps muscles are highlighted on the scan from the younger subject. The histological sections are 10x magnified and show type I (darker stain) and type II (lighter stain) muscle fibres from a young (C) and older man (D). Fibre-type grouping is sometimes a feature of aged muscle and is evident in the section from the older man, identified by the arrow.

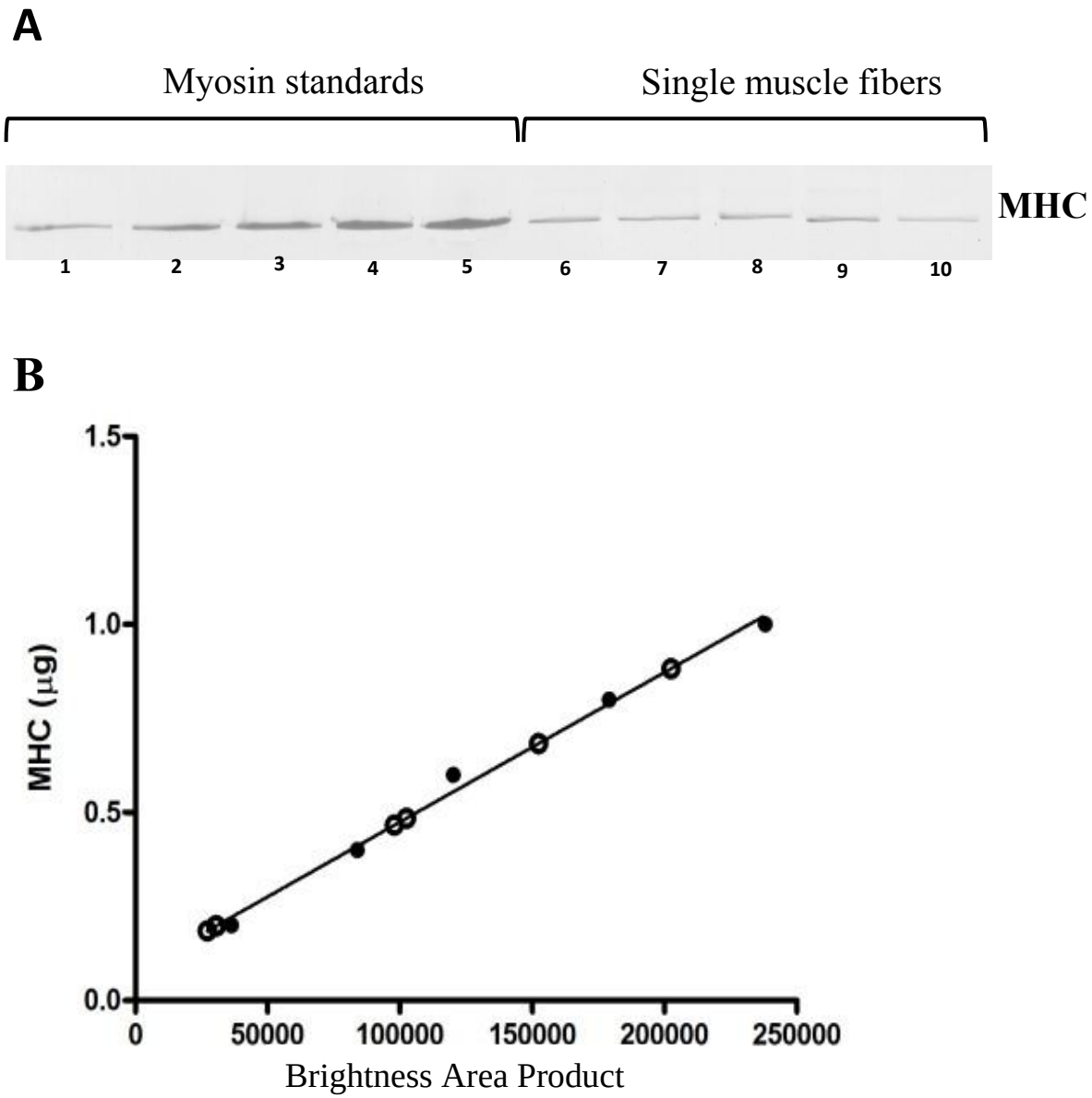

Figure 2: Quantification of myosin concentration in single muscle fibres. An example of the procedure used to determine myosin content in single fibres from young and older men. Five samples of 0.2, 0.4, 0.6, 0.8 and 1 µg of a myosin standard were loaded from left to right in the gel and used to build a standard curve (full circles) in the graph plotting their brightness area product (BAP). Segments of single fibres were loaded in different wells of the same gel and their BAPs (empty circles) plotted in the graph to determine myosin content using the standard curve.