Plasticity and function of human skeletal muscle in relation to disuse and rehabilitation: Influence of ageing and surgery

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Plasticity and function of human skeletal muscle in relation to disuse and rehabilitation: Influence of ageing and surgery

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1. THE PRESENT THESIS IS BASED ON THE FOLLOWING PAPERS:


Study I, II & V has previously been part of the PhD dissertation “Muscle function in the elderly after hip-replacement surgery – effects of long-term disuse and physical training.” Faculty of Health, University of Copenhagen, June 2004.
2. PREFACE

The present thesis is based on experimental work performed during my employment at Institute of Sports Medicine Copenhagen (ISMC), Bispebjerg Hospital as a research fellow from 2000-2003 and in a Post-doc position from 2005-2008.

A large number of persons have been important to make this possible, but most of all I am deeply grateful to Professor, DMSci Michael Kjær for being an inspiring and supportive supervisor and mentor. His thorough knowledge in physiology and pathophysiology combined with his scientific and human generosity has been an invaluable help and immense inspiration.

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Further, I would like to express my deepest gratitude to all the patients and participants who volunteered to participate in the experiments and dedicated their time and muscle tissue to make our experiments possible.

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4. INTRODUCTION

In humans skeletal muscle tissue accounts for about 40% of the total body mass and, in addition to being a crucial factor for locomotion, skeletal muscle represent a key element in maintaining metabolic function and as an energy reservoir in catabolic conditions. Thus, deteriorations in the contractile and metabolic properties of skeletal muscle have significant negative effects on human health and even short periods of muscle disuse rapidly leads to a number of negative consequences, such as skeletal muscle atrophy (Wall et al., 2014) reduced muscle strength (Deschenes et al., 2008;Hvid et al., 2013;Wall et al., 2014) and a decline in basal metabolic rate (Haruna et al., 1994) in otherwise healthy individuals.

In parallel, the loss of muscle mass observed with ageing i.e. sarcopenia and the concomitant decline in muscle strength and power have extensive consequences for the elderly since associated with an impaired ability to perform tasks of daily living, along with an increased risk of disability and mortality (Metter et al., 2002;Rantanen et al., 2000). Moreover, periods of skeletal muscle disuse due to a higher degree of comorbidity and hospitalisation per se results in a rapid and accelerated loss of skeletal muscle mass (Janssen et al., 2002). In fact, immobilisation due to major surgery and hospitalisation markedly increases the risk of deterioration in muscle function, often leading to onset of disability in frail elderly individuals (Covinsky et al., 2003;Kortebein, 2008). In addition, the recovery of losses in muscle mass and muscle strength is often very slow (Visser et al., 2000;Trudelle-Jackson et al., 2002; Reardon et al., 2001a) and many elderly patients fail to regain the level of function that was present prior to hospital admission (Covinsky et al., 2003;Visser et al., 2000;Hirsch et al., 1990; Sager et al., 1996). Thus attempts to counteract the muscle atrophy associated with surgery and hospitalisation in the elderly seem highly relevant. We therefore set to investigate the mode and magnitude of muscle activity required to effectively counteract the decline in muscle mass and function associated with surgery and hospitalisation (Study I-IV).

The observation that skeletal muscle disuse leads to substantial atrophy is far from new and the negative effects of unloading on skeletal muscle are relatively well described in young individuals (Berg & Tesch, 1996;Berg et al., 1997;de Boer et al., 2007a;Hespel et al., 2001;LeBlanc et al., 1997). In contrast, very little is known about how immobilisation and skeletal muscle disuse affects skeletal muscle size and function in old adults (Urso et al., 2006;Deschenes et al., 2008; Kortebein et al., 2007a). Thus, our present knowledge is primarily based on animal data where hind-limb suspension (HS) has been used as a model of muscle unloading to investigate the underlying mechanisms associated with disuse muscle atrophy in aging (Vandervoort, 2002a;Alway et al., 2001;Alway et al., 2003;Aagaard et al., 2010a;Bodine et al., 2001b;Booth & Seiler, 1979;Booth & Gollnick, 1983;Brown & Hasser, 1996; Brown & Hasser, 1996;Deschenes et al., 2003;Desplanches et al., 1987). Although it is evident that aging leads to a multitude of changes in the neuromuscular system that are similar to those evoked by unloading (Vandervoort, 2002a; Aagaard et al., 2010a), the lack of research into the effect of unloading in elderly humans makes it difficult to ascertain what effects can be attributed to a decreased physical activity per se and which to the aging process, as such. An important question is therefore whether processes responsible for the loss of muscle mass due to acute or chronic disuse are similar to those underlying sarcopenia and additionally, whether skeletal muscle disuse leads to similar effects in old and young individuals. On this background, studies V and VI were carried out, in order to investigate the effects of skeletal muscle disuse in aged individuals, initially in individuals exposed to chronic disuse (Study V) while in Study VI, we intended to discriminate between the differential effects of a defined period of muscle disuse and aging per se.

Moreover, based on previous animal data demonstrating an attenuated recovery response after immobilisation and injury in old compared to young muscle tissue (Brooks & Faulkner, 1990;Chakravarthy et al., 2000;Grounds, 1998; Zarzhevsky et al., 2001) we investigated the ability of young and older individuals to recover muscle mass and mechanical muscle function after 14 days and 4 days of immobilisation, respectively (Study VI and IX).

At the molecular level effective cellular communication is known to play an essential role in skeletal muscle plasticity and in adult skeletal muscle tissue the size of the muscle is, in essence, determined by the relative rates of protein synthesis and protein degradation (Glover et al., 2008). Thus, skeletal muscle atrophy is a consequence of a reduction in muscle protein synthesis and/or an increase in protein degradation. However, despite the existence of several robust candidate pathways (Rennie et al., 2004;Sandri, 2008), the molecular mechanisms responsible for the regulation of skeletal muscle atrophy and the subsequent restoration in skeletal muscle mass in response to exercise based rehabilitation are relatively unknown, particularly in humans. A better understanding of the pathways regulating myofibrillar protein synthesis and protein degradation in humans and their temporal relationship to changes in muscle function and lean mass is hence of considerable clinical importance and has far-reaching implications to counteract muscle wasting during periods of skeletal muscle disuse. In animal models, loss of muscle mass with immobilisation or unloading has been shown primarily to occur through an accelerated degradation of myofibrillar proteins via the ubiquitin-proteasome pathway (Bodine et al., 2001a;Gomes et al., 2001). Somewhat in contrast, studies in young human individuals have suggested that a decline in protein synthesis rather than accelerated protein breakdown is responsible for the atrophy related muscle loss (de Boer et al., 2007b; Gibson et al., 1987;Glover et al., 2008). With aging, loss of muscle has been associated with increased inflammation (Bruinsgaard & Pedersen, 2003) and decreased anabolic signalling (Cuthbertson et al., 2005), increased apoptosis (Dirks & Leeuwenburgh, 2002;Dupont-Versteegden, 2005), impaired myogenic responsiveness (Carlson et al., 2009;Conboy et al., 2005; Grounds, 1998) as well as decreased mitochondrial function (Marcinek et al., 2005). Moreover, aging has been found to affect signalling pathways that regulate myogenic growth factors and myofibrillar protein turnover in skeletal muscle of rodents.
(Alway et al., 2001). In order to investigate some of these cellular and molecular mechanisms suggested being responsible for the age-related changes in skeletal muscle with disuse and recovery, including the differential involvement and time course of such signalling pathways, Study VII and VIII was carried out.

The present thesis provides an overview of the information gathered from Study I-IX and the current knowledge about the plasticity of aging muscle in relation to disuse and re-training.

5. MATERIAL AND METHODS

While relevant details related to Material and Methods are described below, more detailed information can be found in the respective articles.

5.1 SUBJECTS
The patient population recruited for Study I-V consisted of patients scheduled for a primary unilateral hip-replacement operation at Bispebjerg University Hospital, Copenhagen, Denmark from May 2000 to May 2002. Eligibility criteria included; age 60 years or older, unilateral primary hip replacement due to primary hip osteoarthritis in patients without cardiopulmonary, neurological or cognitive problems. In the two immobilisation studies (Study VI – IX) comparable groups of healthy young (20-30 years) and elderly (60-75 years) individuals were recruited. Prior to inclusion, all subjects were screened by a physician to exclude individuals with cardiovascular disease, diabetes, neural- or musculoskeletal diseases, inflammatory or pulmonary disorders or any known predisposition to deep venous thrombosis. Anthropometrical data of the patients and subjects are provided in Table 1.

5.2 IMMOBILISATION PROTOCOLS
Two different immobilisation studies were conducted. In the first experiments (Study VI, VII & VIII), subjects had one lower limb immobilised for 14 days by unilateral whole-leg casting using a lightweight fibre cast applied from just above the malleoli to just below the groin. The cast was positioned in 30 degrees of knee joint flexion to circumvent walking ability of the casted limb and the subjects were carefully instructed to perform all ambulatory activities on crutches and abstain from ground contact as well as performing isometric contractions of quadriceps of the immobilised leg.

In the following short-term study (Study VII & IX), subjects had a randomly assigned leg immobilised for 4 days using a knee brace (DonJoy, Orthopedics, Sunny Vista, CA, US) fixated at a knee angle of 30 degrees (similar to the cast). Using a knee-brace instead of a whole-leg cast enabled us to obtain muscle biopsies during the immobilisation period without removing the brace. Both methods have previously been shown equally efficacious of inducing muscle atrophy in young individuals (de Boer et al., 2007a; Deschenes et al., 2008; Hespel et al., 2001). Similarly to the 14 days immobilisation intervention, subjects were provided with crutches during the immobilisation period.

5.3 RE-TRAINING PROCEDURES

5.3.1 Hip-replacement patients
Patients were stratified by age and sex and randomly allocated to one of three groups: home-based standard rehabilitation (SR), SR plus unilateral lower-limb resistance training (RT), or SR plus unilateral neuromuscular electrical stimulation (ES). The RT and electrical stimulation (ES) groups performed the additional training or received ES on the operated leg, so the non-operated side could serve as a within-subject control.

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<tr>
<th>Table 1. Anthropometrical data of the study participants</th>
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<td>Study</td>
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<td><strong>Retraining after Hip-replacement</strong>&lt;br&gt;Study I-IV</td>
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<td><strong>4 days immobilisation study</strong>&lt;br&gt;Study VII, IX</td>
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<td><strong>14 days immobilisation study</strong>&lt;br&gt;Study VII, VIII</td>
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5.3.1.a Home-Based Standard Rehabilitation
All three intervention groups were provided the same standard rehabilitation procedure for hip-replacement patients at Bispebjerg Hospital. The standard rehabilitation program consisted of 15 functional exercises with no use of external loads. The SR group was instructed to perform the exercises twice a day and attend weekly control sessions in the Physical Therapy department, during which an experienced physical therapist guided them through all the exercises to ensure they were performed correctly. Identical instructions were given to the two other treatment groups.

5.3.1.b Neuromuscular Electrical Stimulation (NMES)
The ES group began the stimulation program on the operated leg the day after hip surgery. Patients were carefully instructed in the use of the stimulator and the placement of the electrodes. The stimulator was a pocket-sized battery-operated unit (Elpha 2000, Biofina, Denmark) that delivered a constant biphasic current (0–60 mA). After careful preparation of the skin, two stimulation electrodes (Bio-Flex, 50 x 89 mm, Biofina A/S; Odense, Denmark) were placed over the quadriceps muscle belly 5 cm below the inguinal ligament and 5 cm above the patella. The pulse rate was 40 Hz, with a pulse width of 250 µs, and stimulation time of 10 s, followed by 20 s of rest (Hartkopp et al., 2003). The amplitude increased and decreased gradually during the first and last 2 s. The intensity of the stimulation was adjusted according to patient tolerance, at maximal tolerable stimulus intensity. The total stimulation time was 1 h/d for 12 weeks, and all patients registered total stimulation time and intensity. After discharge from the hospital, the stimulator was used at home, and weekly controls were conducted.

5.3.1.c Resistance exercise
Resistance training was performed as unilateral progressive exercise for the operated lower limb. The post-operative training was initialized the first day after surgery and consisted of daily knee extension exercises (3 x 10 reps) in a seated position with sandbags strapped around the ankle. As soon as possible (~day 5-7 post surgery) training was performed by use of adjustable leg-press and knee-extension machines (Techno gym International) three times per week. Following a brief 10 min warm-up on a stationary bicycle, knee-extension and leg-press exercises were performed. A trained physical therapist carefully supervised all training sessions. Training intensity was progressively increased in intensity from 20 RM (~50% of 1-RM) the first week, 15 RM (~65% of 1-RM) during week 2-4, 12 RM (~70% of 1-RM) during week 5-6 and 8 RM (~80% of 1-RM) the last six weeks. Training loads were carefully adjusted on a weekly basis, measured by a multiple-RM testing based on goal repetitions, to ensure that all patients exercised at the intended intensity.

5.3.2 RE-TRAINING SUBSEQUENT TO IMMOBILISATION
The re-training protocol for the 2 weeks immobilisation protocol consisted of a 4 weeks supervised resistance exercise for the intervention leg that was fairly similar to the above program (cf. 5.3.1c) during week’s two to six. The program was previously shown to elicit increases in muscle size and maximal muscle strength in elderly individuals (Esmarck et al., 2001). Training sessions were carried out three times per week and after a 10 min warm-up on a stationary bike, subjects performed knee extension, leg press, and knee flexion, with all the machines being adjustable (Technogym International). The subjects were instructed to use moderate (~1-2 s) and slow speed (~3-4s) in the concentric and eccentric contraction phases, respectively. Load intensity was 3-4 sets x 12 reps (at 15

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repetition maximum (RM)) in week 1, followed by 5 sets x 10 reps (at 12 RM) in weeks 2 and 3, and 4 sets x 10 reps (at 12 RM) in week 4. Training loads were determined and progressively adjusted on a weekly basis by use of 5-RM testing. The 7 days re-training protocol following 4 days of immobilisation was designed in a similar way as the first week of the 4 week re-training period with a load intensity of 3-4 sets x 12 reps (at 15 RM) determined by use of 5-RM testing.

5.4 FUNCTIONAL CAPACITY
To evaluate changes in functional performance in the group of hip-replacement patients (Study I) a number of functional parameters were obtained that have previously been shown to correlate significantly with risk of physical disability, dependency and falls (Dargent-Molina et al., 1996; Janssen et al., 2002). Maximal gait speed over a 10-meter course was measured to the nearest 0.1 s and stair-climbing performance was measured as the time to ascend 10 steps (height 20 cm). Both tests were started from a standing position and stopped when both feet were at the determined ending position. The ability to rise from a chair (Sit-to-stand test) was measured on a standardised chair, as the 5-repetition time to the nearest 0.1 s. Furthermore, maximum stair walking power per kg body mass (watt/kg) was calculated as the distance of vertical displacement of the body centre mass times g (9.81 m/s²), i.e. the change in potential energy, divided by the fastest time of stair ascent (Study III). Each subject performed three trials, and the stairs consisted of 10 steps each with a height of 16.5 cm leading to a total vertical displacement of 1.65 m.

All three tests are highly validated showing high test-retest reliability and with strong relationships to muscle strength, frailty and mortality (Guralnik et al., 1995; Rantanen et al., 2000; Rantanen et al., 2003). Moreover, the tests are easy to carry out, cheap and not very time-consuming. There are however, also certain limitations of these tests. The most important being a relatively low sensitivity and an early ceiling effect, which makes them best suitable for frail populations.

5.5 MECHANICAL MUSCLE FUNCTION
5.5.1 Isokinetic muscle strength
To assess mechanical muscle function, isokinetic dynamometry (Kinetic Communicator, Chattanooga, Tennessee, TN, KinCom) was employed in Study I-III, V-VII and IX. Dynamic muscle strength was measured as the maximal voluntary isometric knee extensor moment (peak moment, Nm) during concentric quadriceps contraction performed at slow (60°/s) and fast (180°/s) knee joint angular velocity. Maximal isometric quadriceps and hamstring muscle strength were assessed at a 60° knee joint angle (0° = full knee extension) and the trial with the highest maximal voluntary contraction moment (MVC) was selected for further analyses of rate of force development (RFD) and contractile impulse (Aagaard et al., 2002). Individual settings of the seat, backrest, dynamometer head and lever arm length was registered, so identical positioning was ensured for each subject at all time-points. All measurements were performed on both thighs, and were preceded by a familiarisation trial conducted on a separate day. Verbal encouragement was given and visual feedback was provided as a real-time display of the force output (Kellis & Baltzopoulos, 1997). Successive trials were performed until peak moment could not be improved any further (Aagaard et al., 2002), which typically included 7-9 attempts at each velocity. Reliability and validity of the KinCom dynamometer has been verified in detail by Farrell & Richards and is characterized by a high validity and reliability (Farrell & Richards, 1986). Furthermore, strong test-retest robustness has previously been demonstrated for the use of isokinetic dynamometry to assess maximal strength of the knee extensors, knee flexors and plantar flexors in both young (Sievert & Wenger, 1994; Wennerberg, 1991) and old adults (Holsgaard et al., 2007).

5.5.2 Interpolated twitch technique (ITT)
In Study V and VI maximal evoked muscle force was measured using a custom-made set-up (Harridge et al., 1999) with the subjects seated in an upright position with a rigid back support and the hip and knee joint flexed at 90° (Harridge et al., 1999). A steel cuff was strapped around the lower leg, approximately 2 cm above the medial malleoli and was connected via a rigid steel bar to a strain gauge load cell (Bofors KRG-4, Bofors, Sweden), which was connected to a linear instrumentation-amplifier (Gould 5900, Gould Inc. Valley View, OH USA).

5.5.2.a Resting muscle twitches
Each test session was initiated by determination of the maximal twitch response in the resting muscle. Twitch contractions were evoked in the passive muscle using electrical stimulation consisting of single square wave pulses of 0.1 ms duration delivered by a direct current stimulator (Digitimer Electronics, model DS7). Stepwise increments in the current were delivered until no further increase in twitch amplitude was seen (Harridge et al., 1996).

5.5.2.b Superimposed twitches
To evaluate the ability to activate the quadriceps muscle, i.e. to assess the magnitude of central activation (neuromuscular activation), electrically evoked muscle doublet-twitches were superimposed onto maximal voluntary muscle contraction (Merton, 1954; Strojinik & Komi, 1998). Contractions were evoked using doublet square-wave pulses of 0.1 ms duration and a minimum of two trials was performed with a requirement to reach within ≥95% of the peak MVC force measured in preceding trials. Supra-maximal doublet stimulation (100 ms pulse duration, 10 ms interpulse interval) was manually delivered 5 s before (non-potentiated resting doublet), at the highest attained force plateau (superimposed doublet), and 2 s after (potentiated resting doublet), with the latter response being used as the resting reference twitch.

5.6 MUSCLE SIZE AND ARCHITECTURE
5.6.1 Computed tomography (CT)
Computed tomography (CT, Picker 5000, Ohio, US) was used to obtain anatomical cross-sectional area of the quadriceps femoris muscle in the population of patients undergoing hip-replacement surgery (Study I, II and V). A slice thickness 8 mm was used and a scanning time of 5s with an image matrix of 512 x 512 pixels. Axial scans of the quadriceps muscle were
obtained at the midpoint between the great trochanter and lateral joint line of the knee. A trained radiologist measured cross sectional areas using the Picker VOXEL-Q CT/MR Software Package for real-time Analysis after each scan was blinded for both subject and time point. Each scan was evaluated three times and the mean value was recorded as the result. The coefficient of variation between two consecutive measurements was < 2%.

5.6.2 Magnetic resonance imaging (MRI)
In Study VI muscle cross sectional area and muscle volume of the quadriceps muscle was measured by use of axial Magnetic Resonance Imaging (MRI) (Aagaard et al., 2001). Imaging was performed in a body array coil with the subject in a supine position with both limbs extended and relaxed. Prior to the first scan a localising scan centred mid femur was conducted to ensure the knee joint was included in the field (Field of View, FOV 48). The subsequent scan was centred just below the femur condyles to ensure the same scan position at all time-points. Dependent on the femur length of the subject 7-8 T1-weighted transverse scans with a FOV 42 and matrix 512 + 512 pixel matrix were obtained with a slice thickness of 10 mm and an inter-slice gap of 50 mm. After blinding of each scan the Anatomical Cross Sectional Area (ACSA) of each scan was measured three times using Web1000 imaging software. The mean value of the three measurements was recorded as the result and the coefficient of variation between consecutive measurements was < 5%. Subsequently, Quadriceps muscle volume (Qvol) was calculated by the summation of 6 successive ACSA values (scan 2-7), each multiplied by the sum of the slice thickness and inter-slice gap. Based on cadaver analysis, a high validity has been reported for the non-invasive assessment of human skeletal muscle CSA and volume by means of MRI (Beneke et al., 1991; Mitsiopoulos et al., 1998). Furthermore, high test-retest reliability is reported for the repeated recording of transversal anatomical CSA of the human quadriceps femoris muscle by means of MRI, demonstrating excellent test-retest reliability (Reeves et al., 2004a).

5.6.3 Ultrasound (UL)
To assess changes in muscle architecture measurements of muscle fibre pennation angle and muscle thickness ultrasonography was performed in Study III and VI. Sagittal ultrasound images of the quadriceps femoris muscle were recorded with the use of a Siemens real-time scanner with a 7.5 MHz linear array transducer. Images were obtained with the subject in a seated position (90 deg. flexion in the hip and knee joint) at 50% of femur length over the mid-belly of VL muscle (Aagaard et al., 2001). Vastus lateralis (VL) fibre pennation angle (qP) was measured as the angle between VL muscle fibre fascicles and the deep aponeurosis of the insertion, i.e. the fascia separating VL and the vastus intermedius muscle (Herbert & Gandevia, 1995; Reeves et al., 2006; Rutherford & Jones, 1992). Two images from each limb were obtained from all subjects. Each image was evaluated three times and the mean value was recorded as the average fibre pennation angle. The coefficient of variation between two consecutive measurements was < 5%. In order to obtain ultrasound images from an identical position at the thigh during longitudinal sessions, anatomical landmarks were carefully drawn on a transparent sheet. Moderate-to-excellent test-retest reliability has been reported for the measurement of qP in the human quadriceps femoris muscle in vivo (Reeves et al., 2004b; Raj et al., 2012; Blazevich et al., 2006) as well as in other lower limb muscles (Aggeloussis et al., 2010; Raj et al., 2012).

5.6.4 Dual-energy X-ray absorptiometry (DEXA)
In Study VI, Dual-energy X-ray absorptiometry (DEXA) (Lunar DPX, version 3.6Z software) was used to assess whole body composition and percent body fat. Subjects were measured in supine position on the same time of the day on consecutive measurements. Good-to-excellent test-retest reliability has been reported for the measurement of body composition, body fat and lean muscle mass in young and old women (Madsen et al., 1997).

5.7 NEURAL ACTIVATION
5.7.1 Electromyographic recordings (EMG)
In Study II and V surface EMG-recordings were carried out to measure neuromuscular activity in the quadriceps and hamstring muscles. After careful preparation of the skin, pairs of surface electrodes (Medicotest Q-10-A, 20 mm inter electrode distance) were placed over the belly of vastus lateralis (VL), vastus medialis (VM) and rectus femoris (RF). All electrode positions were carefully measured for each subject to ensure identical recording sites throughout all tests. EMG and dynamometer strain-gauge signals were synchronously sampled with a 1000-Hz analogue-to-digital conversion rate using an external analogue-to-digital converter (dt 2801-A, Data Translation, Marlboro, MA). Subsequent, during later off-line analysis, EMG signals were digitally high-pass filtered with a fourth-order, zero-lag Butterworth filter with a 5-Hz cut-off frequency, followed by a moving root-mean-square filter with a time constant of 50 ms. Maximum EMG amplitude of the RMS-filtered signal was identified for the entire contraction phase and to reflect neural adaptations in the early phase of contraction, integrated EMG (iEMG) and mean average voltage EMG (MADV=iEMG/integration time) were calculated in time intervals of 0-30 ms, 50 ms, 100 ms and 200 ms relative to the onset of EMG integration, which was initiated 70 ms before force onset to account for electromechanical delay (Aagaard et al., 2002). The degree of antagonist co-contraction was calculated by dividing maximum antagonist hamstring EMG by maximum agonist hamstring EMG measured during maximal isometric knee flexion. For the quadriceps muscle acceptable reproducibility has been observed for the surface EMG recorded during static as well as dynamic contraction conditions, including isokinetic knee extension (Moritani & deVries, 1979; Viitasalo et al., 1980).

5.8 CELLULAR AND MOLECULAR ANALYSES
In Study III, IV and VII-IX muscle samples were obtained from the middle portion of m. vastus lateralis utilizing the percutaneous needle biopsy technique of Bergström (Bergström, 1962) in order to perform cellular and molecular analyses.
5.8.1 Muscle fibre CSA and fibre type composition

After dissecting the muscle samples of all visible blood, adipose and connective tissue, the muscle samples were oriented in embedding medium (Tissue Tec) frozen in isopentane cooled with liquid nitrogen and stored at -80°C. Subsequently serial transverse sections (10 mm) were cut in a cryotome at -20°C and stained for myofibrillar ATPase at pH 9.4 after both alkaline (pH 10.3) and acid (pH 4.3 and 4.6) pre-incubations (Brooke & Kaiser, 1970). All samples of each individual person were stained in the same batch to avoid inter-assay variation. Fibre type distribution (fibre number percentage, fibre area percentage) and fibre cross-sectional area for each of the three major fibre types (I, IIA, IIX) were analysed in a blinded fashion using computerized digital image analysis techniques. Since myofibre area is known to vary in a systematic way along the length and depth of the human VL muscle (Lexell & Taylor, 1989), all biopsies were obtained by the same investigator, and careful efforts were made to extract tissue from the same depth and with ~2 cm distance between each biopsy, which has been shown to be sufficient to avoid the influence of muscle damage from repeated biopsies (Guerra et al., 2011). Using comparable procedures, relatively high test-retest reliability has been reported for the assessment of fibre type composition (ICC of 0.88, 0.82, 0.56 for type I, Ila and IIX fibres) and fibre-type specific area (ICC of 0.74-0.82) (Simoneau et al., 1986). Moreover, a 4-7% variation in fibre type composition has been reported between duplicate biopsy samplings, while the variation in fibre type distribution within a single biopsy was small (2-3%) when 200 fibres were analysed (Blomstrand & Ekblom, 1982). However, it should be recognized, that greater variations (12-19%) in fibre type composition and area have been reported with duplicate biopsy sampling procedures in the human VL muscle (Halkjaer-Kristensen & Ingemann-Hansen, 1981). Yet, despite the needle biopsy sampling technique in the human VL muscle may show substantial within-subject variability (Lexell & Taylor, 1989; Lexell & Taylor, 1991), this remains the only known method for the evaluating of cellular morphology and phenotype composition in human skeletal muscle in vivo.

5.8.2 Myogenic stem cells and myonuclei

In Study VIII myogenic stem cells, also referred to as satellite cells (SCs), were identified by using antibody staining of monoclonal cells located between the basal lamina and the sarcolemma, to mark SCs expressing Pax-7 (Kadi et al., 2005; Mack-
eye et al., 2009). In contrast to the membrane localization of membrane-bound neural cell adhesion molecule (N-CAM/CD56), the paired-box transcription factor Pax-7 is confined to the satellite cell nucleus (Mackey et al., 2009), resulting in a lower density of Pax-7+ compared to the number of CD56+ cells typically observed in resting human skeletal muscle (Mackey et al., 2009; Mikkelsen et al., 2009), although fairly similar SC numbers also have been reported (Lindstrom et al., 2010).

5.8.3 Gene expression analyses
In Study IV, VII and VIII total RNA was isolated (Kadi et al., 2004b) in order to study the molecular regulation in muscle size during disuse and subsequent re-training. Total RNA (500 ng) was converted into cDNA in 20 ml using the Omniscript reverse transcriptase (Qiagen, CA, USA) according to the manufacturer's protocol. The mRNA expression of FoxO1, FoxO3, FoxO4, PGC-1a, PGC-1β, IL-6, MGF, IGF-1Ea, GAPDH and RPLP0 were analysed by quantitative real-time RT-PCR. The amplification was monitored real-time using the real-time PCR analysis (MX3000P; Stratagene, CA, USA). The threshold cycle (Ct) values were related to a standard curve made with the cloned PCR products and specificity ensured by melting curves analysis and the quantities were normalized to RPLP0. Furthermore, TaqMan based quantitative real-time RT-PCR of MuRF-1, Atrogin-1, NF-kB, Bax, BCL2L1, p53, TNF-α, ATG4B, GABARAPL1, and RPLP0 mRNA were performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using ABI TaqMan Low Density Arrays (Applied Biosystems). Each sample was run in triplicates with 4 samples per card. Raw data were extracted and analysed using the SDS 2.1 software (Applied Biosystems), while qBasePlus (Biogazelle) was used to quality-check Ct-values, assess triplicates, exclude runs for difference among triplicates >0.5 Ct and finally to normalize data to RPLP0 using the 2-ΔΔCt method (Livak & Schmittgen, 2001).

5.8.4 Protein quantification
In Study VII and IX mRNA expression data were supplemented by protein quantification using Western Blotting analysis. From each muscle biopsy 150 cryosections (10 µm) were homogenized in a micro vial containing 1 silicium carbide crystal, 5 steel beads (2.3 mm) and 250 µl ice-cold homogenization buffer (Complete, Roche, Basel, Switzerland), using a Fast-Prep-24 (MP Biomedicals, Solon, OH, USA) homogenizer. Laemmli buffer was added and protein concentrations were determined with the EZQ Protein Quantitation Kit according to the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). After heating samples were separated by SDS-PAGE and gels were blotted (Trans-blot cell, Bio-Rad, 400 mA, 2 h) to polyvinylidene difluoride membranes (Amersham Hybond LFP, GE Healthcare, Buckinghamshire, UK). Total and phosphorylated protein pairs were detected simultaneously on the same membrane. Band intensities were quantified using densitometry analysis (ImageJ; National Institutes of Health, Bethesda, MD, USA).

6. RESULTS AND DISCUSSION
6.1 EFFECTS OF SKELETAL MUSCLE DISUSE
Skeletal muscle mass and muscle strength are both known to decline in response to disuse, and the effects of inactivity on human skeletal muscle have been studied in a variety of different modes including bed-rest, unilateral lower limb suspension, immobilisation as well as actual and simulated microgravity (Appell, 1990; Booth & Gollnick, 1983; Hvid et al., 2010) in order to gain knowledge about how muscle disuse affects the human locomotor system. Yet, the effect of ageing on human skeletal muscle disuse has not previously been given much attention, although it seems important to enhance our understanding of disuse-atrophy in bed-ridden patients and to shed light on its contribution to sarcopenia in older individuals.

Based on such recent experiments, we here present and discuss data from three different human muscle disuse interventions to focus on the effects of ageing on skeletal muscle disuse-atrophy. At the outset, our population of elderly patients with hip-osteoarthritis were examined in order to enhance our knowledge about the effects of chronic muscle disuse on aging skeletal muscle (Study V). Further, in order to investigate potential age-related differences to human skeletal muscle disuse, we studied two well-defined periods of immobilisation in able-bodied elderly individuals, compared to young individuals with a comparable activity level (Study VI-VIII).

6.1.1 The impact of chronic disuse on aged human skeletal muscle
Chronic joint pain is a surprisingly frequent condition, which is estimated to affect more than 33% of individuals above the age of 45 years of age (Felson et al., 2000). Furthermore, osteoarthritis (OA) has been shown to be the most common cause of inactivity and long-term disability in persons above the age of 65 years (Felson et al., 2000). The impact of osteoarthritis on disability is therefore substantial and the risk of disability due to osteoarthritis is greater than any other medical condition in elderly persons (Guccione et al., 1990). Moreover, the presence of concurrent chronic conditions further increases the likelihood of subsequent disability (Felson et al., 2000). Based on this knowledge, we studied a mixed population of men and women with osteoarthritis of the hip on the waiting list for a hip-replacement operation, to get deeper insights into how long-term (months to years) inactivity affects muscle function in elderly individuals (Study V).

6.1.1.a Skeletal muscle size and architecture
Decreased loading in terms of immobilisation, bed-rest or spinal-cord injury is known to induce marked muscle atrophy in younger human individuals (de Boer et al., 2007a; Hespel et al., 2001; Hortobagyi et al., 2000; Jones et al., 2004; LeBlanc et al., 1988; Wall et al., 2014). Notably, the relative loss in muscle mass over time is not linear but tends to plateau over time, with the highest atrophy rate observed within the first 7-10 days (de Boer et al., 2007a; Narici & de Boer, 2011; Suëtta et al., 2012a). Even though chronic disuse due to joint pain may not be directly comparable to a standardised period of immobilisation, patients with chronic hip osteoarthritis in Study V showed
a ~10% reduced anatomical quadriceps muscle cross sectional area (ACSA) on the affected side compared to the unaffected side (Study V). Additional contributing factors to the decrement in muscle strength with ageing comprise changes in structural components, such as increased intramuscular fat and connective tissue (Lexell et al., 1988). Therefore, in addition to ACSA, lean tissue cross-sectional area (LCSA) as well as inter- and intramuscular fat CSAs were measured using known CT density limits for fat and lean tissue to discriminate between contractile and non-contractile tissue within the muscle compartment area in Study V (Sipila & Suominen, 1995). Both men and women showed an 8-10 % reduced lean quadriceps muscle CSA (LCSA) on the affected compared to the unaffected side (Study V), in line with findings by Rasch et al that observed marked atrophy in all muscles around the hip and knee in a similar group of patients (Rasch et al., 2007). These results were further underlined by our findings of a reduced myofibre area (type I and type IIA fibres) on the affected compared to the non-affected side (Study III).

In addition to a reduction in muscle size, changes in muscle architecture also contribute to the decrease in muscle force production with aging and/or disuse (Kawakami et al., 2000; Narici & Cerretelli, 1998). Consequently, in sarcopenia as well as skeletal muscle disuse-atrophy, it has been demonstrated that muscle fibre fascicles have a reduced pennation angle compared to healthy young individuals (Morse et al., 2005a; Narici et al., 2003). In agreement with these findings, muscle fibre pennation angles on the osteoarthritic side in Study III were significantly smaller (-15%) compared to the healthy side, indicating that chronic disuse leads to significant changes not only in muscle size but also muscle architecture.

6.1.1.b Neuromuscular function

Maximal contractile muscle strength and rapid force characteristics (RFD) are known to decrease with aging as well as in response to disuse (Aagaard et al., 2010a; Vandervoort, 2002a). Notably, isokinetic strength has been demonstrated to be an important predictor of pain and disability in patients with gonarthrosis (Madsen et al., 1995). Somewhat surprisingly, maximal isometric quadriceps strength (MVC) of the unaffected leg was similar to that of healthy age-matched subjects in our cohort of patients with unilateral hip-osteoarthritis, (Esmarck et al., 2001; Lange et al., 2002; Sueta et al., 2006). In contrast, MVC on the affected side was markedly reduced (~20 %), along with a decline in muscle quality reflected by decreased specific strength (MVC moment/CSA), underlining the severe consequences of chronic pain/disuse on mechanical muscle function (Study V). Notably, the clinical consequences of muscle strength asymmetry in the lower limbs are significant since a close relation to postural balance problems, decreased walking speed, as well as increased risk of falling has been shown (Portegijs et al., 2005; Portegijs et al., 2006).

In general, women demonstrate lower muscle mass and maximal muscle strength than men throughout the adult life span, and therefore the risk of frailty is increased with ageing in women in particular (de Rekeneire N. et al., 2003). Accordingly, women showed markedly lower MVC on both sides (~40%) compared to male subjects (Study V). Notably, no difference between genders was detected when maximal muscle strength was normalised to lean cross sectional area (MVC/LCSA) (Study V), in agreement with earlier investigations (Lindle et al., 1997; Tracy et al., 1999; Vandervoort & McComas, 1986). Importantly, however, specific strength on the affected side was reduced compared to the unaffected side in both genders of about 12-14%, in line with earlier results demonstrating a lower specific strength in sedentary elderly subjects compared to young individuals, whereas elderly subjects with a long-term history of strength or endurance training typically show similar specific strength compared to young individuals (Klitgaard et al., 1990). Furthermore, immobilisation leads to decreased specific force capacity in single muscle fibres of the quadriceps muscle after immobilisation in both young and old individuals (D’Antona et al., 2003; Hvid et al., 2011), indicating a deterioration in cellular muscle quality with skeletal muscle disuse.

In parallel with the decrease in maximal contractile muscle strength, the ability to develop force rapidly (i.e. contractile RFD) is substantially reduced in healthy elderly compared to young individuals of both genders (Clarkson et al., 1981; Vandervoort & McComas, 1986; Thelen et al., 1996; Izquierdo et al., 1999), although not a universal finding (Thelen et al., 1996; Clarkson et al., 1981). However, we found absolute contractile RFD significantly to be reduced on the affected side compared to the unaffected side in both men and women (Study V). Notably, the affected side remained reduced when RFD was normalized to CSA in both genders, supporting the finding of a decrease in muscle quality with prolonged disuse.

Along with changes in mechanical muscle function, marked reductions in maximal EMG signal amplitude were observed during MVC testing on the affected side compared to the unaffected side in both genders (Study V), in agreement with earlier findings in young individuals after 4 weeks of unloading (Berg et al., 1991a), suggesting that the decreases in contractile RFD on the affected limb at least partly was explained by changes in neuromuscular activation. In further support hereof, using interpolated twitch analysis we observed a significant muscle activation deficit on the affected compared to the non-affected side (Study V), in line with that observed by Stevens et al. after 7 weeks of cast immobilization in young subjects (Stevens et al., 2004).

In summary, the present data demonstrate that chronic muscle disuse in the elderly is associated with marked quantitative as well as qualitative neuromuscular impairments. More specifically, chronic muscle disuse leads to decreases in muscle strength, muscle size (ACSA, LCSA, and myofibre area of type I and IIA fibres), accompanied by impairments in muscle architecture (muscle fibre pennation angle), contractile properties (rapid muscle force characteristics) and neuromuscular activation (maximal EMG amplitudes). Furthermore, large side-to-side deficits were observed for specific strength (MVC/LCSA) and normalised RFD (RFD/CSA), indicating that a major part of the observed changes with disuse are explained by decreases in muscle quality.

6.1.2 The effect of ageing on skeletal muscle disuse

The plasticity in skeletal muscle mass homeostasis in response
to decreased activity is fairly well described in young adults (Adams et al., 2003;Appell, 1990;Berg et al., 1991b). On the other hand, almost no attention has been given to the combined effects of ageing and skeletal muscle disuse, although muscle contractile function is known to be a crucial factor to maintain an independent lifestyle with ageing. Notably, the deterioration of mechanical muscle function with ageing seems to be a result of changes in both quantitative and qualitative factors (Aagaard et al., 2010b;Doherty, 2003;Vandervoort, 2002b). Moreover, in addition to changes in intrinsic factors, the level of physical activity is known to modify the age-related loss in muscle size and function (Klittgaard et al., 1990;Rantanen et al., 2000;Aagaard et al., 2007;Pearson et al., 2002). Hence, the fact that that our young and older able-bodied participants reported comparable levels of activity in the two present immobilisation experiments (Study VI-IX) lead us to believe that the observed differences between young and old prior to the intervention were mainly attributable to the effect of aging per se.

6.1.2.a Changes in muscle size and muscle architecture
Muscle disuse in terms of immobilisation and bed-rest is known to induce significant reductions in anatomical muscle cross sectional area, muscle fibre area and muscle fibre pennation angle in young individuals (de Boer et al., 2008;Hespel et al., 2001;Jones et al., 2004;Narici & Cerretelli, 1998). Data from older human individuals are limited, but in the animal model the majority of studies, find a higher magnitude of muscle atrophy in young compared to old animals (Alway et al., 2001;Brown & Hassler, 1996;Carlson et al., 2002;Pistilli et al., 2007) although not all agree (Deschenes et al., 2001;Si et al., 2006). In agreement with earlier findings in young subjects, we found significant reductions in muscle size (anatomical muscle cross sectional area and quadriceps muscle volume) in young individuals after 2 weeks of immobilisation (de Boer et al., 2007b;Hespel et al., 2001;Jones et al., 2004, Study VI), and recently also demonstrated after 5 days of immobility (Wall et al., 2014). However, in agreement with the majority of various animal models (Alway et al., 2001;Brown & Hassler, 1996;Carlson et al., 2002;Pistilli et al., 2007), the atrophy response was significantly smaller in old compared to young individuals (Study VI).

In contrast, a larger muscle atrophy response has been reported in old compared to young adults, after 14 days of immobilisation of the human adductor pollicis muscle (Urso et al., 2006). Part of the explanation may be due to the different muscle groups investigated, as smaller muscle groups have been suggested to respond differently to changes in loading (Gandevia et al., 1990). Yet, a significantly larger muscle loss has also been observed after 10 days bed-rest in able-bodied older individuals (Kortebein et al., 2007b). In general, immobilisation and bed-rest induce similar atrophy responses of the lower limbs (Adams et al., 2003;Convertino et al., 1997) which makes the difference between the marked muscle loss (950 g lean lower limb mass) shown by Kortebein et al. (Kortebein et al., 2007b) and the more moderate atrophy response observed in our study (Study VI) somewhat puzzling. The difference may, however, be explained by the observation of a negative nitrogen balance before the intervention with a further decline during bed rest in the bed-ridden participants (Kortebein et al., 2007b), since a negative nitrogen balance is known to aggravate the magnitude of skeletal muscle loss with immobilisation (Paddon-Jones et al., 2006).

In addition, changes in muscle size were assessed by histological analyses of muscle fibre cross sectional area after 4 and 14 days of immobility. Prior to the immobilisation interventions, myofibre CSA of type II fibres were smaller (~25-30%) in old compared to young muscle in both intervention studies, whereas no difference was observed in type I fibre CSA. Notably, decreases in mean myofibre area of approximately 10% were detected in both age groups, despite the brief period of muscle disuse (4 days) (Study VII). However, no difference was observed between the decline in type 1 and type 2 fibres at the 4 day time-point (Study IX), despite that the decline in type 2 fibres was significantly larger than in type 1 fibres in both young and old following 14 days of immobilisation (Hvid et al., 2010). Yet, in line with our whole muscle assessments, the decrease in muscle fibre CSA following 14 days of immobilisation was larger in young compared to older individuals (Hvid et al., 2010), Study VII) and consequently elderly individuals demonstrated less overall muscle loss with disuse than their young counterparts after 14 days of immobility (Study VI & VII).

Along with the reduction in muscle size, marked changes in muscle architecture were observed following 14 days of immobilisation (Study VI). Accordingly, more marked decreases in muscle fibre pennation angles of the VL fascicles were observed in young subjects compared to aged individuals (Study VI), underpinning the importance of muscle architecture to explain part of the discrepancy between the average relative decrease in muscle strength, which was about twice as large compared with the average relative decrease in muscle mass (Aagaard et al., 2001;Narici, 1999;Rutherford & Jones, 1992).

Collectively our findings in Study VI, VII and IX, are in line with previous findings obtained using various animal models, indicating that skeletal muscle disuse leads to larger loss of muscle mass (quadriceps volume, anatomical CSA, muscle fibre CSA and muscle fibre pennation angles) in young compared to older individuals, at least with more prolonged immobilisation (14 days). Interestingly, however, the muscle atrophy response observed within the first 4 days of immobilisation did not seem to be affected by age, since similar reductions in myofibre area were observed in young and old muscle.

6.1.2.b Changes in neuromuscular function
It is evident, that ageing as well as muscle disuse bring about negative effects on the neuromuscular system, and although there are indications of differential effects of muscle disuse in young and old animals, data from human individuals are still limited.

In Study VI and IX, various parameters of mechanical muscle function (dynamic & isometric knee extensor muscle strength, specific force (MVC/CSA) and contractile rate of force development (RFD)) were assessed prior to immobilisation. In agreement with previous data (Aagaard et al., 2001;de Boer et al., 2007b;Deschenes et al., 2008;Hespel et al., 2001;Hortobagyi et al., 2000;Jones et al., 2004) marked reductions
in contractile capacity were demonstrated following 4 days as well as 14 days of lower limb disuse, independently of age (Study VI & IX). Notably, the magnitude and time-course of changes were similar to earlier findings in young (Bamman et al., 1998; Berg & Tesch, 1996; de Boer et al., 2007b; Labarque et al., 2002) as well as old individuals (Deschenes et al., 2008; Kortebein et al., 2008). Of notice, maximal leg extension power and rapid muscle force capacity (RFD) have been shown to decline to a greater relative extent than maximal muscle strength with aging (Klass et al., 2008; Pearson et al., 2002; Skelton et al., 1994) and more importantly, has been advocated to be of greater importance than maximal muscle strength for the observed decline in functional status and the ability to counteract a fall (Portegijs et al., 2006; Skelton et al., 1999; Skelton et al., 2002). Notably, our data demonstrated rapid force capacity (RFD, impulse) was affected to a greater extent in older individuals compared to young after 4 days and 14 days of disuse, especially during the very initial phase of muscle contraction (0–50 ms) (Study IX, Hvid et al., 2010). In support hereof, maximal dynamic muscle strength during fast contractions (120°/s) has been shown to decline to a greater extent in old compared to young individuals following 7 days of immobilisation (Deschenes et al., 2008).

In addition to mechanical muscle strength parameters, the magnitude of central activation (neuromuscular activation) (Merton, 1954; Strojnik & Komi, 1998) and resting twitch characteristics were assessed in the present line of experiments. Indicating changes in intrinsic (“qualitative”) mechanical muscle function with ageing, older subjects demonstrated lower resting twitch peak torque and resting twitch RFD prior to intervention compared to young individuals, followed by comparable decreases in activation as a result of muscle disuse (Study VI). In contrast, both young and old adults showed similar levels of central activation prior to immobilisation, in line with previous findings (Hakkinen et al., 1995; Kent-Braun & Ng, 1999; Roos et al., 1999) and further supporting the observation of comparable habitual activity levels between our two age-groups. However, following immobilisation (14 days) older individuals experienced a decline (Study VI), whereas young subjects in agreement with previous findings remained unaffected (de Boer et al., 2007a). Collectively, these observa-
tions suggest that disuse may enhance the age-related gap in neuromuscular function, observed with natural ageing.

Taken together, our neuromuscular data demonstrate that young and aged men experience similar declines in muscle contractile function following a brief period of muscle unloading at isometric and slow velocities of muscle contraction, whereas at faster contraction speeds as well as in terms of rapid force characteristics (RFD and Impulse), older individuals may experience more marked declines than young. Moreover, the present findings points toward an age-related difference in the susceptibility of central activation parameters to short-term disuse.

6.2.1.c Molecular regulation of muscle disuse-atrophy

Although, important insights have been made concerning the molecular and genetic bases of skeletal muscle atrophy and ageing in cell culture and animal models, only little is known about the underlying molecular mechanisms of skeletal muscle atrophy with ageing and disuse in humans. Certain controversy exists in the literature regarding whether muscle atrophy in human skeletal muscle is regulated primarily by increases in myofibrillar protein degradation or a decrease in protein synthesis (Figure 2). In support of the latter, solid evidence in the murine model, has pointed at protein degradation as the driving factor for skeletal muscle atrophy, with the ubiquitin-dependent proteolytic system being rapidly activated (Gomes et al., 2001; Bodine et al., 2001a; Lecker et al., 2004) in relation to unloading and various disease states (Bodine et al., 2001a; Lecker et al., 2004; Sacheck et al., 2007). In contrast, data from human in vivo studies have been less consistent (de Boer et al., 2007b; Jones et al., 2004; Chen et al., 2007; Abadi et al., 2009; Leger et al., 2006). Our data revealed a 1-2 fold up-regulation in MuRF-1 and Atrogin-1 mRNA expression in both young and old muscle during the initial days of immobility (~2-4 days), supporting a role for the ubiquitin-proteasome pathway in the initiation of human skeletal muscle atrophy. However, the fact that we observed more modest changes compared to previous animal reports may reflect that more drastic and hence more systemic wasting models were used in these animal studies (Lecker et al., 2004; Gomes et al., 2001; Bodine et al., 2001a) compared to human immobilisation models. Notably, the present data revealed that the expression levels of both Atrogin-1 and MuRF-1 returned to basal levels after 14 days of immobility, indicating that in human skeletal muscle the ubiquitin-proteasome pathway may not be important to maintain a more chronic atrophy response but rather plays a role in the very initial atrophy response (~days). In support of this notion, a bi-phasic time-course has previously been shown to exist for the mRNA expression profiles of selected atrogenes in the rodent model (Sacheck et al., 2007), which may explain that early transcriptional changes have been overlooked in previous human studies, which mainly have studied later time points of disuse.

A coordinated regulation of the ubiquitin-proteasome and the autophagy-lysosome pathways has been shown to exist in the murine model (Mammucari et al., 2007; Sandri, 2008; Zhao et al., 2007), but somewhat surprisingly we did not observe any change in the mRNA expression profiles of ATG4, GABARAPL or FoxO3 (Study VII). However, we did see a trend towards an increase in LC3B II/I protein ratio selectively in young muscle after 1 and 4 days of immobility, indicating that the autophagic process (lipidation) was initiated at least in the young myofibres and thus, crosstalk between the ubiquitin-proteasome and the autophagy-lysosome pathways may also exist in the human model. The limited activation of the autophagy pathway could indicate that cross-talk between ubiquitin-proteasome and autophagy-lysosome pathways mainly occurs in more systemic atrophy models, although species-specific differences between the rodent and human model have been suggested to exist as well (Welle et al., 2001).

In addition to being a central regulator of muscle protein synthesis and muscle hypertrophy the IGF-1/Akt signalling pathway has been proposed to be a potent suppressor of myofibrillar proteolysis and atrophy related ubiquitin ligases, respectively (Bodine et al., 2001b). Importantly, our finding of an age-specific decrease in P-Akt protein content indicates that immobility leads to a rapid (2-4 days) as well as more sustained (14 days) decrease in myofibrillar protein synthesis exclusively in young muscle, which at least in part explains the observation of a larger muscle loss in young compared old individuals (Study VII). In support of this notion, a diminished phosphorylation of Akt pathway components has been reported following 48h of immobility in young human subjects (Abadi et al., 2009). In contrast, the present data point toward an up regulation of the muscle specific IGF1-pathway exclusively in old subjects (Study IV), which in combination with a lack of change in the Akt pathway may explain the attenuated atrophy response in old muscle. However, our current knowledge regarding the age-related differences in the regulation of this pathway remain highly limited, and more studies clearly are needed to uncover the mechanisms underlying the apparent age-specific influence on disuse induced muscle loss that was observed in the present line of experiments.

Further, a marked down-regulation in genes involved in mitochondrial metabolism was observed (Study VII), consistent with recent human gene array studies (Abadi et al., 2009; Chen et al., 2007). A rapid decrease in PGC-1α mRNA and PGC-1B mRNA gene expression was observed in young but not old muscle in the most initial phase of immobilisation (1 day), where the response in old muscle was slightly slower (Study VII), although reaching statistical significance within the first 4 days of immobilisation (Study VII). However, there was no difference between age groups at the 14 days’ time-point, in accordance with recent data from Gram et al. (Gram et al., 2014). These findings support the hypothesis that down-regulation in PGC-1α and PGC-1B are important determinants for the initiation of human skeletal muscle atrophy, as also observed in rodents (Sandri et al., 2006), although no indications of FoxO3-dependant transcriptional changes were noted in the present study in contrast to previous animal data obtained using systemic muscle wasting (Sandri et al., 2004; Sandri et al., 2006). It can be speculated, that one reason for the slower and/or attenuated atrophy response to immobility in aged compared to young human muscle could be a consequence of the general decrease in oxidative metabolism observed with aging. However, recent findings from Gram et al elegantly have demonstrated that 14 days of inactivity and subsequent...
re-training alter mitochondrial biogenesis to a similar extend in young and elderly males (Gram et al., 2014).

Another topic of debate has been the role of the apoptotic pathway in human skeletal muscle atrophy and sarcopenia. Using animal models there are a significant amount of data indicating an important role for apoptosis in the development of muscle atrophy observed with aging (Dirks & Leeuwenburgh, 2002;Marzetti et al., 2008;Marzetti et al., 2009;Phillips & Leeuwenburgh, 2005;Pistilli et al., 2006;Siu et al., 2005), whereas human data have been more inconsistent (Malmgren et al., 2001;Strasser et al., 2000;Whitman et al., 2009). In essence, our data showed a marked and rapid increase in the expression of apoptotic markers with immobilisation, with indications of a more pronounced response in old muscle cells (Study VII). Notably, despite that a general (i.e. non-specific) increase in TUNEL-positive nuclei was observed primarily in muscle biopsies from old individuals after immobilisation, specific myocellular TUNEL-positive myonuclei did not appear to increase in neither young nor old adults, in contrast to previous findings in the murine model (Dupont-Versteegden et al., 2006). Thus, in the present experiments an up-regulated number of TUNEL-positive nuclei mainly were localized in the interstitiel space, indicating that intrinsic myofibre apoptosis may not play a key role for the mediation of human disuse-muscle atrophy.

In summary, our data point toward a number of intracellular signalling pathways for both muscle atrophy and hypertrophy being activated in the very initial phase of immobility, in turn leading to a rapid initial atrophy response in both young and aged muscle, followed by a decaying atrophy response at later time-points. Notably, our data showed a parallel activation of the ubiquitin-proteasome pathway along with the IGF/Akt indicating that proteolyses may be an important component in the initiation of human disuse atrophy in both young and old muscle, whereas the myocellular regulation in protein synthesis and mitochondrial function seems more age-dependent. Although fundamental mechanistic questions still remain to be answered, our data indicate that the orchestrating of human skeletal muscle atrophy is age-dependent, with a number of cellular signalling pathways being modified independently of each other.

6.2 RE-GROWTH OF HUMAN SKELETAL MUSCLE

Human skeletal muscle is a highly plastic tissue, which is reflected in its rapid ability to adapt to changes in loading intensity, at least in young individuals (Hvid et al., 2010;Wall et al., 2014). In contrast, the ability of skeletal muscle to repair and re-growth is known to diminish with aging (Brooks & Faulkner, 1990;Chakravarthy et al., 2000;Grounds, 1998;Zarzhevsky et al., 2001). Yet, the mechanisms responsible for the diminished ability of aged skeletal muscle to re-growth are largely unknown and the cellular and molecular mechanisms that contribute to the recovery in muscle mass after reduced mechanical loading are just beginning to become unravelled. However, as muscle atrophy not only compromises physical functioning but also is associated with increased frailty and mortality, it seems important to expand our understanding of the mechanisms involved in muscle re-growth to develop methods to maintain or improve muscle mass during or following periods of disuse. Thus, in order to expand our knowledge regarding the ability of aged human skeletal muscle to recover from longer or shorter periods of disuse, we investigated the capacity for muscle re-growth and restoration of mechanical muscle function in the three groups of patients/participants described above who underwent skeletal muscle disuse of various lengths (hip-replacement patients, as well as two groups of healthy elderly and young individuals, respectively).

### Table 3. Overview of skeletal muscle signalling responses during immobilisation in young and old individuals

<table>
<thead>
<tr>
<th>Measured variable from signalling pathways</th>
<th>Young</th>
<th>Old</th>
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<tr>
<td><strong>Protein degradation</strong></td>
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<tr>
<td>Atrogen-1</td>
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<td>MALRF-1</td>
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<tr>
<td><strong>Apoptosis</strong></td>
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6.2.1 Effects of re-training in elderly post-operative patients

An infinite number of medical and surgical illness states lead to the development of hospital associated deconditioning (Kortebein, 2009). Several common etiologic factors contribute to this effect, including the specific medical or surgical illness necessitating hospitalisation, the adverse effects of treatment (including surgical interventions), bed rest inactivity, as well as the detrimental effects of aging per see (Appell, 1986;Hill & Hill, 1998;Kortebein et al., 2007b;Vandervoort, 2002b).

Although much effort is done to minimize surgical intervention, effects of major surgery are still associated with an increased risk of morbidity, convalescence and disability (Kehlet, 2006;Kamel et al., 2003;Covinsky et al., 2003;Foss et al., 2006). Major surgery is furthermore known to elicit a cata- bolic stress response that leads to a reduced protein synthesis and a reduction of lean tissue mass (Convertino et al., 1997;Kehlet & Wilmore, 2002). Consequently, a significant number of elderly patients experience a decline in functional performance after surgery, and more importantly a large proportion of these patients do not regain their functional level without specific intervention programs (Magaziner et al., 1990;Reardon et al., 2001a;Sashika et al., 1996;Shih et al., 1994;Sicard-Rosenbaum et al., 2002;Trudelle-Jackson et al., 2002;Visser et al., 2000;Walsh et al., 1998). To minimize de-
conditioning and enhance recovery the concept of “fast-track surgery” has been introduced as a coordinated effort to combine uni-modal evidence-based principles of care into a multi-modal effort, which has evolved as a valid concept to improve post-operative outcome (Kehlet, 2006;Swanson et al., 1998). Yet, loss of muscle mass is not completely counteracted by the implementation of “fast-track regimes” (Kehlet & Wilmore, 2002) and growing evidence indicates that rehabilitation programmes have to be highly specific and of sufficient intensity to counteract decreases in muscle strength and muscle mass in postoperative elderly patients (Hauer et al., 2002;Sashika et al., 1996; Study I & II. Trudelle-Jackson & Smith, 2004). Notably, the use of resistance training is known to improve muscle strength and functional performance both when initiated in the early post-operative phase (weeks to months) (Hauer et al., 2002) as well as in the late post-operative phase (months to years) (Trudelle-Jackson & Smith, 2004;Sashika et al., 1996;Sherrington & Lord, 1997). Yet, knowing that the detrimental effects on muscle tissue properties are most dramatic during the first weeks of immobilisation (Appell, 1990; Convertino et al., 1997, Study VII) it seems rational to initiate specific training intervention as soon as possible after surgery. We therefore set out to investigate the effect of various rehabilitation regimes initiated in the very early postoperative phase (1-2 days post-surgery) in elderly individuals undergoing elective hip-replacement surgery (Study I & II). Based on the findings from earlier studies (Sashika et al., 1996;Sherrington & Lord, 1997;Trudelle-Jackson & Smith, 2004) voluntary resistance training was compared to peripheral muscle stimulation (NMES) and conventional rehabilitation activities in order to evaluate the neuromuscular adaptations elicited by these three different exercise modalities (Study I-IV).

6.2.1.a Skeletal muscle size and architecture Despite resistance training is known to induce marked increases in anatomical muscle cross sectional area and muscle fibre area, in aged (Hakkinen et al., 1996;Hakkinen et al., 1998a; Morse et al., 2005b;Roth et al., 2001;Tracy et al., 1999;Welle et al., 1996) as well as in very old individuals (Fiatarone et al., 1990;Kryger & Andersen, 2007) the use of resistance training is still not widely used to rehabilitate elderly patients following major surgery. In fact, only few studies have tried to apply intensive strengthening exercises in the acute post-operative phase (Study I, II) (Husby et al., 2009;Jakobsen et al., 2012). In the light hereof, one of the most important findings of Study I and II was the feasibility of applying resistance training and electrical stimulation in the acute post-operative phase (1 day after surgery). Moreover, there was marked differences between the three different rehabilitation regimes investigated (resistance training, electrical muscle stimulation and functional exercises) on quadriceps muscle size and quadriceps muscle architecture. Despite successful surgical outcome and the use of early mobilisation strategies during hospitalisation, we observed a further decrease in CSA at five as well as twelve weeks post-surgery in the patients who received the conventional rehabilitation program based on functional exercises with no external loads applied. In contrast, 1 hour/day of neuromuscular electrical stimulation (NMES) of the quadriceps muscle, nearly counteracted a decline in muscle size (Study I & II), in accordance with previous results from young patients (Arvidsson et al., 1986;Gibson et al., 1988), and recently also in intensive care unit patients (Dirks et al., 2015). Amplifying this effect, resistive exercises not only prevented the surgery associated muscle atrophy at five weeks, but further increased CSA after twelve weeks. As a result, a significant difference in treatment outcome was observed between the resistance-training group and the conventional rehabilitation group after 12 weeks of rehabilitation. Further, muscle fibre area increased by 32% following 12 weeks of resistance training, and in line with previous studies in young (Aagaard et al., 2001) and old individuals (Kryger & Andersen, 2007) more marked gains were observed for the type Ia and Ix) fibres compared to the type I fibres (Study III). Consequently, side-to-side deficits in anatomical CSA and muscle fibre CSA (type 1 and type 2a) were fully eliminated after 12 weeks of resistance training, while still persistent following NMES and conventional rehabilitation (Study III).

In addition to the muscular changes relating to muscle fibre size, ageing also leads to marked alterations in muscle architecture that potentially contribute to the loss in muscle strength (Narici et al., 2003). A reduction in muscle fibre pennation angle in old compared to young individuals has previously been observed, suggesting that a significant part of the decrease in muscle function with aging may be related to changes in muscle architecture (Narici et al., 2003). Both in sarcopenia and disuse atrophy, muscle fibre fascicles seem to have a reduced pennation angle compared to healthy young individuals, likely due to decreased amounts of contractile tissue (Morse et al., 2005a;Narici & Cerretelli, 1998). In agreement with these findings, muscle fibre pennation angles on the osteoarthritic side were substantially reduced compared to the unaffected side (Study III). However, after 12 weeks of RT there was a significant increase in VL muscle fibre pennation angle, comparable to the changes typically reported following resistance training in able-bodied young and old individuals after comparable periods of resistance training (Aagaard et al., 2001;Morse et al., 2006). In contrast, no improvements in muscle fibre pennation angle were observed for patients subjected to electrical stimulation or functional exercises.

6.2.1.b Neuromuscular function and functional capacity Alongside the above morphological adaptations, marked changes in mechanical muscle strength parameters and functional capacity were observed after 12 weeks of rehabilitation, however, the observed changes were highly dependent on the type of rehabilitation regime (Study I & II). Notably, marked increases was observed maximal muscle strength (dynamic and isometric) in response to 12 weeks of resistance training, despite patients being rather frail during the initial 4-6 weeks after surgery. Comparable gains in maximal muscle strength have been demonstrated following resistance training in healthy elderly individuals (Hakkinen et al., 1998b;Lexell et al., 1995; Reeves et al., 2005), as well as in frail elderly (Beyer et al., 2007; Fiatarone et al., 1990;Harridge et al., 1998;Harridge et al., 1999).

In contrast, no gains in maximal muscle strength parame-
ters could be observed with electrical stimulation (Study II), which is in agreement with earlier findings in young patients after ACL-reconstruction and conventional rehabilitation (Arvidsson et al., 1986), while also lacking to be demonstrated in studies evaluating the effect of physiotherapy exercises (with no external loads) after hip-surgery (Sicard-Rosenbaum et al., 2002;Trudelle-Jackson et al., 2002). Consequently, the muscle strength asymmetry observed in all patients prior to the operation was fully reversed following twelve weeks of resistance training, while not affected by electrical stimulation or conventional rehabilitation (Study I). This finding is potentially of high importance since asymmetry in lower limb muscle strength is related to fall prevalence in elderly adults (Skelton et al., 2002;Portegijs et al., 2006). Moreover, the elimination of muscle asymmetry is noteworthy, since the non-operated side was equally strong compared to that of able-bodied age-matched individuals (Esmarck et al., 2001), resulting in a normalisation of neuromuscular performance after only 12 weeks of resistance training in patients subjected to many years of chronic disuse and subsequent hip-replacement surgery. Of note, is also the fact that no training related complications were observed in any of the three intervention groups, despite all three training regimes were commenced in the very early post-operative phase (1-2 days after surgery).

The ability to develop force rapidly (i.e., demonstrating high contractile RFD) is an important performance characteristic, especially in older people, contributing to several tasks of daily life such as climbing stairs, walking, and attempting to avoid a fall (Bassey et al., 1992;Fleming et al., 1991). However, significant increases in RFD and elevated neuromuscular (EMG) activity have been demonstrated in healthy elderly individuals following 3-6 months of resistance training with special focus on increasing muscle power (Hakkinnen et al., 1985;Hakkinnen et al., 2001). In order to avoid postoperative injuries while still ensuring a sufficiently high loading intensity to induce measurable gains in muscle size (de Vos et al., 2005;Fiatarone et al., 1990) and neuromuscular performance characteristics, as previously demonstrated in young individuals (Aagaard et al., 2002) we used a progressively adjusted exercise program known to be effective of inducing adaptive muscular changes in able-bodied elderly individuals (Esmarck et al., 2001). To our best knowledge, potential changes in RFD characteristics have not previously been evaluated after NMES or conventional rehabilitation.

In accordance with earlier findings in young individuals (Aagaard et al., 2002;Hakkinnen et al., 1998b;Kraemer et al., 2001), resistance training lead to marked increases in rapid force production, in the very initial phase (30–50 ms) as well as the later part (100–200 ms) of the isometric force-time curve, with similar gains in contractile impulse. Notably, the increase in RFD was still present after normalising for muscle size (RFD/CSA), indicating qualitative changes in muscle contraction characteristics may have occurred, such as increased maximal motor unit firing frequency (Van et al., 1998) and/or changes in myosin heavy chain isoform composition toward an increase in type II fibre area percentage (Aagaard et al., 2001). The importance of these positive adaptations in rapid muscle force characteristics are underlined by the fact, that a positive correlation was observed between the increase in maximal gait speed and the increase in absolute RFD following 12 weeks of resistance training, which was even stronger when related to normalized RFD (RFD/CSA) in the very initial phase of muscle contraction (0–30 ms). In contrast, no relationship could be observed between the change in maximal walking speed and changes in maximal muscle strength characteristics and/or muscle size (Study II).

Apart from gains in muscle size and neuromuscular characteristics, resistance training and NMES produced marked increases in functional performance parameters (walking speed, chair rise performance and stair climbing), although most present following resistance training (Study I). The enhancement in horizontal walking speed was particularly noticeable, since maximal and habitual walking speed are powerful predictors of future disability and dependency (Guralnik et al., 1995;Sonn & Asberg, 1991). Moreover, it is thoughtful that despite all resistance training exercise were performed unilaterally the marked changes in unilateral muscle strength and muscle size were translated (nearly 1:1) into increases in functional tasks that comprise two-legged coordinated movement. This finding underlines the importance of specifically focusing on reducing observable deficits (muscle size and strength) between sides before both legs are trained simultaneously. Another puzzling finding was the observed increase in functional performance with NMES that, although being more modest than the changes observed with resistance training, clearly were superior to those achieved by conventional rehabilitation (Study I). However, despite there was no measurable gains in neither neuromuscular function nor muscle size with NMES, a majority of the decline observed in the assessed parameters at 5 weeks post-operatively with conventional rehabilitation was largely prevented with NMES (Study I), underlining the importance of focused intervention to counteract the decline in muscle mass and function during hospitalisation.

In summary, the marked increases in neuromuscular performance observed with resistance training were translated into significant gains in activities of daily life (ADL) function, manifested by increased walking speed, enhanced ability to rise from a chair and improved stair climbing performance (Study I-II). In contrast, despite producing no increases in neuromuscular performance characteristics (dynamic and isometric MVC, RFD, EMG and contractile impulse) NMES led to moderate albeit statistically significant gains in functional performance, while conventional rehabilitation did not result in any increases in neuromuscular or functional performance (Study I-II).

6.2.1.c The effect of post-operative re-training on the IGF-1 pathway

The IGF-I pathway is known to be an important stimulator of anabolic signalling and one of the key factors responsible for increasing rate of protein synthesis in skeletal muscle (Adams, 1998). However, based on the findings that senescent muscle in the animal model demonstrates an attenuated recovery response after immobilisation and injury (Brooks & Faulkner, 1990;Chakravarthy et al., 2000;Grounds, 1998;Zarzhevsky et al., 2001), it has been hypothesized that sarcopenia may in part be due to a failure to generate IGF-I isoforms necessary to
initiate the remodelling of muscle by stimulating satellite cell activation and proliferation (Goldspink & Harridge, 2004). In order to describe the potential interaction between changes in muscle morphology and IGF-I splice variants in elderly frail patients, changes in muscle fibre area and mRNA expression levels of IGF-IeA and the mechano sensitive IGF isoform IGF-Iec (MGF) were assessed in our group of hip replacement patients prior to surgery and subsequent to our three intervention regimes (Study IV). In human exercise studies, the expression of IGF-I mRNA has been found to increase acutely after a single bout of resistance exercise (Bamman et al., 2001), although no total agreement exists (Hameed et al., 2003;Psilander et al., 2003). However, more consistent increases in IGF-I have been demonstrated at both the mRNA level (Hameed et al., 2004;Kvorning et al., 2007;Petrella et al., 2006) and the protein level (Singh et al., 1999) following prolonged periods of resistance training.

The activation of myogenic stem cells (satellite cells) and their donation of new nuclei to the exercised myofibres seem required for hypertrophied fibres to maintain an optimal DNA to protein ratio (Kadi et al., 2000). Since MGF is suggested to play an important role in the activation of satellite cells (Yang & Goldspink, 2002), the possibility exists that IGF isoforms are involved in the promotion of muscle growth and repair during the process of reloading subsequent to periods of disuse. Known to be involved in muscle repair (Goldspink & Yang, 2001) it does not seem surprising that MGF mRNA levels were elevated in all three intervention groups compared to the non-operated-side at 48 h post-surgery (Study IV). However, in contrast to NMES and conventional rehabilitation, MGF mRNA expression levels did not decrease in the RT group, which could support the hypothesis that MGF could be involved in both muscle repair (1-2 days post-surgery) as well as in the robust hypertrophy response observed with resistance training (5 weeks and 12 weeks). Moreover, increases in absolute levels of IGF-IeA mRNA and MGF mRNA were only observed in response to resistance training intervention, with no changes detected following NMES or conventional rehabilitation (Study IV).

Importantly, albeit the response may be attenuated in old human muscle as well as in old muscles of various animals, older muscles appear capable of up-regulating the expression of both IGF-IeA and MGF mRNAs in response to a period of prolonged resistance training even after surgery and/or immobilisation.

Collectively, Study I-IV, demonstrates that elderly patients who undergo elective hip-replacement surgery achieve significant muscular, neuronal and functional benefits from intensive physical training initiated in the very early the postoperative phase. Specifically, resistance training more effectively increased muscle morphology (size and architecture), neuromuscular characteristics (muscle strength, RFD, impulse, EMG, central activation) and functional performance (walking speed, chair rise and stair climbing) compared to NMES or conventional rehabilitation.

6.2.2 The effect of aging on skeletal muscle re-growth

It is well known that the ability to produce muscle re-growth after injury and immobilisation is impaired in animal senescent muscle (Chakravarthy et al., 2000;Grounds, 1998;Zarzhevsky et al., 2001). In human individuals however, it still remains largely unknown, which factors and mechanisms promote or hinder the recovery of muscle mass following short or longer periods of disuse. Based on the findings of an impaired ability for muscle re-growth in animal models and our findings of an age-specific regulation and time-course of skeletal muscle disuse-atrophy, we hypothesized that a similar age-specific regulation might exist in response to human skeletal muscle recovery after disuse atrophy. In the light of a steadily increasing aging population leading to an increasing number of persons recovering from shorter or longer periods of muscle disuse, it seems rational to expand our current understanding of the mechanisms involved in human muscle re-growth in order to facilitate the development of approaches to maintain and regain muscle mass during periods of skeletal muscle disuse. We therefore assessed the effect of recovery following 4 days and 14 days of immobilisation, respectively. To optimize the conditions for complete muscle recovery, supervised resistance training was applied in both age groups. Following the 4 days disuse intervention participants were admitted to a 7 days recovery regime (Study IX), whereas participants exposed to 14 days disuse intervention were retrained for 4 weeks (Study VIII).

6.2.2.a Skeletal muscle size and muscle architecture

Reloading of disuse-induced skeletal muscle atrophy, by means of resistive types of exercise, is known to fully restore muscle mass through hypertrophy in young human individuals (Berg et al., 1991b; Boesen et al., 2013;Hespel et al., 2001;Hortobagyi et al., 2000;Jones et al., 2004), however, only few data exists on the changes in skeletal muscle size following brief periods (less than a week) of unloading followed by active exercise-based recovery (Study IX). Thus, our somewhat limited assessments of changes in muscle size and architecture following 7 days of recovery subsequent to 4 days of disuse (Study IX) reflected that we did not expect major changes following such a brief intervention period. Yet, 4 days of unloading induced substantial atrophy in both type I and type II myofibres of young and old individuals alike (Study VII & IX), which was reversed to predisuse values in both age-groups following 7 days of recovery, except for type I myofibre area in old individuals that remained suppressed compared to pre-levels (Study IX).

In comparison, despite a larger atrophy response following 14 days of immobilisation young individuals demonstrated a greater increase in quadriceps muscle size (Qvol, ACSA and PCSA) in response to 4 weeks of retraining leading to a full restoration in muscle mass. In contrast, despite a smaller atrophy response, old individuals did not fully recover quadriceps muscle size after retraining. A similar pattern was observed for the changes in muscle architecture, with a more modest decrease in muscle fibre pennation angle seen in old compared to young with no full reversal in old participants despite 4 weeks of intensive re-training (Study VI). This finding was further supported by our analyses of muscle fibre cross sectional area which showed robust increases in type I and II myofibre area in young subjects during the recovery phase, whereas aged individuals showed no changes in neither type I nor type II myofibre area, leading to an overall difference in the recovery response to re-
training between young and old (Study VIII). Notably however, apart from reflecting a significant age-related difference to reloading, the observed differences between young and old adults may also reflect that a limited period of re-training was used in the present experiments, as substantially longer intervention periods have demonstrated significant gains in quadriceps size (Boesen et al., 2014, Study I-II), muscle architecture (Study III) and muscle fibre area (Study III) in older individuals recovering from muscle disuse and/or surgery.

Altogether, the present data indicate that despite intensive rehabilitation efforts aging is accompanied by an impaired ability to recover from short-term disuse muscle atrophy, and consequently old individuals may need a longer time to recover from periods of disuse compared to young individuals.

6.2.2.b Neuromuscular function
Parameters of mechanical muscle output (contractile capacity) and neuromuscular function are both strong predictors of general functional capacity, quality of life, and risk of mortality in aged individuals (Buchman et al., 2007; Newman et al., 2006; Wyszomierski et al., 2009), which underlines the importance of gaining more insight to the time-course and potential age-related differences in the recovery of neuromuscular function in human individuals following periods of disuse. In young adults, mechanical muscle function (isometric and dynamic MVC) has been demonstrated to be fully reversed following 3-6 weeks of resistance training subsequent to 2 weeks of unloading/immobilisation (Boesen et al., 2013; Hespel et al., 2001; Jones et al., 2004; Labarque et al., 2002) in agreement with the present findings (Study VI, Hvid et al., 2010). Moreover, it is noteworthy that decreases in dynamic muscle strength observed following 4 weeks of unloading in young individuals were fully reversed after 7 weeks, despite no training intervention (Berg et al., 1991b). However, almost no previous studies have focused on disuse lasting less than 1 week, and the subsequent recovery phase. Based on the present experiments, 7 days recovery (subsequent to 4 days immobilisation) appeared effective of restoring knee extensor mechanical muscle function in young subjects, while in contrast maximal muscle strength characteristics remained suppressed in our old subjects (Study IX). Similar trends were observed for rapid force capacity (RFD, impulse) at the very initial phase of contraction (0–50 ms) that tended to remain reduced relative to baseline levels after 4 weeks of re-training subsequent to 14 days of disuse (Hvid et al., 2010). The observed impairment in restoring lower limb mechanical muscle function following short-term disuse in old adults may in part reside from alterations in qualitative muscle factors, as contractile rate of force development tended to remain reduced in old individuals following 7 days of recovery (Study IX), as well as following 4 weeks of re-training (Hvid et al., 2010), whereas maximal isometric strength capacity recovered to a more full extent. Yet, our older individuals were able to reverse the deficit in central activation with 4 weeks of resistance training (following 14 days of immobilization), whereas young individuals reached values above the baseline activation level (Study VI).

In summary, the findings of the present data suggest that the magnitude and time-course of changes in mechanical muscle function during the recovery phase following short-term disuse are compromised in old compared to young individuals.

6.2.2.c Myogenic stem cells
The regulation in muscle growth and maintenance of muscle mass is known to be influenced by a unique population of muscle resident stem cells referred to as myogenic progenitor cells or satellite cells (SCs) (Mauro, 1961). Satellite cells represent a heterogeneous population of adult muscle stem cells that are normally quiescent and were identified more than 50 years ago as nuclei located in a niche between the sarcolemma and the basement membrane of the muscle fibre, and known to play a key role in the maintenance, growth and repair of myofibres (Heslop et al., 2001; Mauro, 1961; Moss & Leblond, 1970; Moss & Leblond, 1971).

In humans, the pool of SCs seems to be maintained into the sixth-to-seventh decade of life (Petrella et al., 2006; Roth et al., 2000), with a decline in content and activation capabilities with progressive ageing (Kadi et al., 2004a; Verdijk et al., 2007) leading to a reduced muscle regeneration capacity in response to myofibre injury and disuse (Carlson et al., 2001; Carlson & Conboy, 2007). However, despite the group of aged individuals in the present experiments our old participants (~67 years) demonstrated impaired SC proliferation capacity compared to young individuals (~24 years), indicating that the SC response to re-loading and exercise might be attenuated. Interestingly, age-related differences in SC proliferative capacity were detectable in the acute (+ 3 days) as well as the prolonged (+ 4 weeks) phase of re-training, in line with reports by Dreyer et al. (Dreyer et al., 2006) who reported a greater increase in SC content in young compared to aged human skeletal muscle within 24h following 92 eccentric contractions (Dreyer et al., 2006).

The importance of SC number in relation to muscle size has previously been reported by Petrella et al (Petrella et al., 2008) that found a positive association between SC number at baseline and gains in muscle fibre area after 16 weeks of resistance training in young and older human individuals (Petrella et al., 2008), suggesting that the individual myogenic potential may at least partially be pre-determined by the availability of satellite cells prior to training. Expanding those observations, we observed for the first time in human individuals a positive relationship exists between SC number and mean fibre area (MFA) following disuse-atrophy (2 weeks) as well as moderate-to-strong associations the magnitude of muscle hypertrophy and gains in SC number in response to exercise-based reloading (4 weeks) (Study VIII).

As aging is associated with a preferential reduction in muscle fibre type II size it may be speculated that SC content would decrease more in type II fibres compared to type I fibres. In young human individuals SC content is similar between type I and II muscle fibres (Kadi et al., 2006; Verdijk et al., 2007). In contrast, age-related type II muscle fibre atrophy is accompanied by a type II muscle fibre specific reduction in SC content (Verdijk et al., 2007; Verney et al., 2008). Interestingly, in the present line of experiments positive associations were observed between the number of Pax7+ cells and the size of both type I and II fibres, respectively. In further support of the
importance of SC proliferation for muscle re-growth, a relationship was observed between the relative change in MFA and total number of Pax7+ cells, as well as the relative change in fibre type I area and the change in number of type I associated Pax7+ cells subsequent to 4 weeks of re-training (Study VIII). However, although muscle regenerative capacity appears to decline at a more advanced age reflected by a decline in SC number and/or activation (Gallegly et al., 2004; Renault et al., 2002; Shefer et al., 2010), a reduced SC pool does not abolish the myogenic potential for adaptive muscle hypertrophy if the intervention period is sufficiently long (~12 weeks), even at old age (Mackey et al., 2007; Shefer et al., 2010).

In summary, our data demonstrated substantial age-specific differences in the re-growth capacity of human skeletal muscle following immobility-induced muscle atrophy. Specifically, aged individuals showed a reduced responsiveness to the re-loading exercise paradigm, reflected by reduced gains in myofibre area that were accompanied by an attenuated capacity for SC proliferation.

6.2.2.d Molecular regulation of skeletal muscle re-growth
The reduced capacity of aging skeletal muscle to recover after disuse indicates that molecular signalling pathways regulating muscle hypertrophy/re-growth are altered at increasing age and/or that negative regulators of muscle mass become progressively more active with aging. We therefore set out to profile a range of positive and negative growth factors associated with local skeletal muscle milieu that are known to stimulate the proliferation of SCs (Gopinath & Rando, 2008). Among those factors, Insulin-like growth factor (IGF-1Ea) and mecha-no-growth factor (MGF) mRNA expression levels and protein content have been shown to correlate with the increase in whole muscle DNA content in response to compensatory muscle overloading (Adams & Haddad, 1996). Further, MyoD and myogenin mRNA expression levels were also assessed in the present experiments since these factors are part of the family of myogenic regulatory factors (Myf5, MyoD, Mrf4 and myogenin) that play a key factor in the myogenic specification and differentiation of SCs in mature skeletal muscle (Goldspink, 1998; Olson et al., 1991). MyoD is primarily related to SC activation and proliferation, whereas myogenin reflects the phase of terminal myoblast differentiation (Kuang et al., 2007; Olson et al., 1991). Among the multiple growth factors associated with the local skeletal muscle milieu that stimulate the proliferation of SCs, hepatocyte-growth factor (HGF) is considered one of the most important parameters (Gopinath & Rando, 2008). Together with its trans-membrane receptor (c-met) HGF is a vital link in the cascade of signalling events that lead to activation of skeletal muscle SCs when myofibres are exposed to strain or injury (Wozniak & Anderson, 2007). Furthermore, a number of studies have identified various fibroblast growth factors (FGFs) and their receptors (FGFRs) to be key regulators of both senescence and self-renewal capacity in a variety of stem cell types (Sheehan & Allen, 1999). Among those, fibroblast growth factor 2 (FGF2) and its receptor FGFR1 are known to stimulate SC proliferation (Allen & Boxhorn, 1989; Mezzogiorno et al., 1993). In addition to these factors, we also assessed the expression levels of myostatin mRNA, a member of the transforming growth factor-β family, which exerts a strong negative regulation on skeletal muscle mass (McPherron et al., 1997) partly by inducing a sustained satellite cell quiescence (Joulia et al., 2003; McCroskery et al., 2003).

Together, our experiments showed age-independent differences in the time-course of IGF-1Ea and MGF regulation, respectively, with an acute and sustained up-regulation of MGF mRNA expression in response re-training, whereas IGF-1Ea expression was up-regulated only in the later phase of re-loading (Study VIII). Notably, changes in myofibre area were positively related to the corresponding changes in IGF-1Ea and MGF expression levels after 4 weeks of resistance training, strongly suggesting an essential role of these IGF isoforms for the induction of human muscle hypertrophy with training/re-loading. Moreover, MyoD and myogenin demonstrated markedly up-regulated expression levels with immobilisation in both age groups, whereas the subsequent recovery phase led to acute and sustained decreases in both MyoD and myogenin mRNA in young as well as aged skeletal muscle. While elevated mRNA expression levels of myogenic regulatory factors have been reported following prolonged (months) resistance exercise in both young and older adults (Kim et al., 2005; Kosek et al., 2006; Raue et al., 2006), only a modest up-regulation in myogenin expression was observed following 4 weeks of resistance training in the present experiments (Study VIII), which may indicate that short-term (days-weeks) re-training after disuse-atrophy generate a different molecular signalling stimuli compared to that evoked by more prolonged exercise intervention. Moreover, marked increases in HGF mRNA expression were observed in response to immobilisation as well as early and sustained re-training (+3d and +4wks) in line with the overall increase in SCs at these time-points (Study VIII). However, despite the age-dependent differences in SC proliferation no age-specific difference was found in the expression profiles of HGF. These seemingly conflicting observations may be due to a somewhat low number of subjects examined and/or could be caused by the relative large variation seen especially in the elderly subjects. Further, there was no significant change in the expression levels of FGF2 at any time-points, indicating that FGF may be of minor importance for SC proliferation in human skeletal muscle irrespectively of age.

Notably, we observed an age-specific influence on the pattern of myostatin regulation, with a larger increase seen in response to immobilisation followed by a smaller reduction with re-loading in our aged individuals (Study VIII). In turn, these observations may partly explain the impaired ability of SC proliferation and myofibre re-growth observed with re-training in aged skeletal muscle (Study VIII). In line with these findings, myostatin mRNA expression has previously been shown to increase following disuse in young individuals, where the elevation in myostatin expression was related to the magnitude of myofibre atrophy (Raidon et al., 2001b). Notably, our data revealed that the change in myostatin expression within the first days of re-loading (+3d) was negatively related to the change in Pax7+ cells, underlining the negative effect of myostatin on SC proliferation (Joulia et al., 2003; McCroskery et al., 2003).

Collectively, our data demonstrate that important age-
specific differences exist for the capacity of myofibrillar re-growth of human skeletal muscle following immobility-induced muscle atrophy. Specifically, aged individuals seemed to respond less sensitively to the re-loading program, as reflected by significantly smaller gains in myofibre area, in parallel with a smaller increase in SC number despite that no age-related differences were observed in local growth factors known to promote skeletal muscle hypertrophy and satellite cell proliferation (IGF-Ea, MGF, MyoD, myogenin, HGF). This attenuated responsiveness of old adults to the re-loading stimulus may partly be explained by a reduced sensitivity to the above growth factors since basal MRF mRNA expression appears to be generally up-regulated in senescent muscle (Hameed et al., 2003; Kim et al., 2005; Kosek et al., 2006; Raue et al., 2006). In addition, the present experiments also indicate that myostatin may play an important role for the impaired re-growth response of aged human skeletal muscle, as the regulation in myostatin mRNA expression was influenced by age, with old adults demonstrating greater increases with immobilisation followed by a less degree of down-regulation in response to subsequent re-loading. Notably, an association between the down-regulation in myostatin mRNA expression and SC proliferation was observed in the acute phase of re-loading, indicating a strong influence of myostatin signalling on the myogenic potential of human skeletal muscle in vivo.

7. MAJOR CONCLUSIONS
In summary, chronic muscle disuse in the elderly was associated with marked quantitative as well as qualitative neuromuscular impairments. More specifically, decreases were ob-
erved in muscle strength, quadriceps muscle size and myofibre area, muscle architecture, contractile properties and neuromuscular activation. Furthermore, substantial side-to-side differences in specific strength (MVC/LCSA) and normalised rapid muscle force capacity (RFD/CSA) were observed, indicating that a significant part of the observed changes in mechanical muscle function with disuse were explained by impairments in muscle quality.

Importantly, within the first 4 days of immobility the observed atrophy responses did not seem affected by age, as manifested by comparable reductions in myofibre area in young and old individuals. However, in line with previous observations using various animal models, we observed a larger loss in muscle mass in young compared to older individuals after more prolonged immobilisation (14 days). Conversely, old individuals were more negatively affected with respect to neural function and rapid force characteristics than their young counterparts.

Moreover, we showed that the initiation and regulation of human skeletal muscle atrophy with short-term disuse is age-dependent. Based on the present experiments it can be concluded that a multitude of signalling pathways related to both muscle atrophy and protein synthesis are activated in the initial phase of disuse, which in turn lead to a rapid initial atrophy response (~1-4 days) in both young and old individuals followed by a gradually attenuated atrophy response at later time-points (~2 weeks). Notably, during the first 1-2 days of immobility a parallel activation of the ubiquitin-proteasome pathway and the IGF1/Akt pathway seem to occur along with a deactivation of PGC-1α and PGC-1β, suggesting that cellular proteolysis plays an important role in the initiation of human disuse atrophy in both young and old muscle, whereas the concurrent regulation in protein synthesis signalling and proteolysis inhibition appears to affect young adults more pronouncedly compared to older adults.

Gaining a better understanding of the ability of human skeletal muscle to recover from disuse-induced atrophy has important implications for the development and implementation of effective countermeasures against physical frailty in the increasing population of elderly. Importantly therefore, the present experiments demonstrate that resistance training is highly effective of increasing maximal muscle strength and neuromuscular function in elderly post-operative patients. Importantly, these increases in mechanical muscle function were accompanied by gains in muscle size, architecture and in the expression of IGF-1 mRNA splice variants, resembling that typically seen in young healthy individuals when exposed to resistance training. In contrast, these positive adaptations could not be achieved with the use of neuromuscular electrical stimulation or conventional rehabilitation efforts alone. Collectively, these findings strongly underline the importance of implementing resistive exercises in future rehabilitation programs for elderly individuals.

In addition, comparing young and old able-bodied individuals, we observed that the magnitude and time-course of changes in mechanical muscle function during the recovery phase following short-term disuse were compromised in old compared to young individuals. Likewise, aged individuals demonstrated an impaired response to re-loading reflected by attenuated gains in myofibre area, in parallel with smaller increases in satellite cell number despite no age-related differences were observed in factors known to promote skeletal muscle hypertrophy and SC proliferation (IGF-Ea, MGF, MyoD, myogenin, HGF). Moreover, an age-specific regulation in myostatin mRNA expression was observed, characterized by an amplified increase in aging skeletal muscle with immobilisation that was followed by less down-regulation during the subsequent phase of re-loading. In combination with an association observed between the changes in myostatin expression and satellite cell proliferation in the acute phase of re-loading, these data indicates that myostatin play an important role in the impaired ability of aged human skeletal muscle to recover from immobility-induced muscle atrophy.

8. PERSPECTIVES

The present line of experiments revealed that the adaptive plasticity in skeletal muscle mass and central nervous system function associated with unloading and subsequent re-mobilisation, differ between young and older individuals (Study VI-IX). Notably, this influence of aging on muscle mass homeostasis is well documented in various animal models (Brooks & Faulkner, 1990;Chakravarthy et al., 2000;Grounds, 1998;Zarzhevsky et al., 2001). However, in human research there has been a tendency to overlook the importance of investigating the very early phase of disuse/unloading (1-5 days) where the atrophy response is most strongly manifested and important information regarding the regulation of human disuse atrophy has therefore been left unnoticed. Yet, our findings (Study VII) as well as others (Tesch et al., 2008; de Boer et al., 2007b;Abadi et al., 2009) show evidence of an early rise in atrogenes during human disuse with time-course patterns similar to what have previously been demonstrated in the murine model (Sachse et al., 2007). Collectively, these findings indicate that the regulation of human muscle disuse is more complex and not merely driven by a decrease in myofibrillar protein synthesis, as previously suggested (Glover et al., 2008;Rennie et al., 2010).

Despite, many links still remain to be elucidated in the puzzle of human muscle plasticity, the observation that the initiation and regulation of human skeletal muscle atrophy is age dependant may be important for the identification of biomarkers and future therapeutic intervention paradigms, which can be used to counteract human skeletal muscle atrophy in relation to aging and disuse.

Moreover, our finding that aging is accompanied by an impaired ability to recover from disuse muscle atrophy despite intensive re-training efforts, and, consequently, need a longer time to recover from periods of disuse may also explain the somewhat disappointing results from shorter rehabilitation studies (Jakobsen et al., 2014).

Importantly, however, the findings from study I-IV clearly demonstrate, in line with previous data (Fiatarone et al., 1990;Kryger & Andersen, 2007) that elderly skeletal muscles respond very well to prolonged intensive resistance training. Consequently, this intervention modality should be more clearly recognized as one of the key tools in the rehabilitation of elderly individuals, including very old and frail patients. The
findings from Study I-IV showed that resistance training can be successfully initiated during a hospital stay, including the acute post-operative phase and in the initial days after discharge in order to counteract the decline in muscle function and loss of muscle mass normally associated with hospitalisation in elderly patients (Kortebein, 2009). Additionally, the observation from Study II & IV that rapid muscle force capacity (RFD) and neuromuscular function remain trainable in elderly recovering from surgery has important implications for the design of future rehabilitation programs, especially when considering the importance of rapid muscle force capacity on postural balance control, maximal walking speed and other tasks of daily living (Aagaard et al., 2010).

9. SUMMARY
In order to study the influence of disuse and aging on skeletal muscle homeostasis, different human models were employed. Effects of chronic disuse were investigated in elderly patients suffering from uni-lateral hip-osteoarthritis, whereas the effect of short-term disuse (4 and 14 days of unilateral lower limb immobilisation) was assessed in healthy young and old individuals.

In summary, chronic muscle disuse in the elderly was associated with marked quantitative as well as qualitative neuromuscular impairments. More specifically, decreased were observed in muscle strength, quadriceps muscle size and myofibre area, muscle architecture, contractile properties and neuromuscular activation. Furthermore, substantial side-to-side differences in specific strength (MVC/LCSA) and normalised rapid muscle force capacity (RFD/CSA) were observed, indicating that a significant part of the observed changes in mechanical muscle function with disuse were explained by impairments in muscle quality.

Importantly, within the first 4 days of immobility the observed atrophy responses did not seem affected by age, as manifested by comparable reductions in myofibre area in young and old individuals. However, in line with previous observations using various animal models, we observed a larger loss in muscle mass in young compared to older individuals after more prolonged immobilisation (14 days). Conversely, old individuals were more negatively affected with respect to neural function and rapid force characteristics than their young counterparts.

Moreover, we showed that the initiation and regulation of human skeletal muscle atrophy with short-term disuse is age-dependent. Based on the present experiments it can be concluded that a multitude of signalling pathways related to both muscle atrophy and protein synthesis are activated in the initial phase of disuse, which in turn lead to a rapid initial atrophy response (~1-4 days) in both young and old individuals followed by a gradually attenuated atrophy response at later time-points (~2 weeks). Notably, during the first 1-2 days of immobility a parallel activation of the ubiquitin-proteasome pathway and the IGF-1/Akt pathway seem to occur along with a deactivation of PGC-1α and PGC-1β, suggesting that cellular proteolysis plays an important role in the initiation of human disuse atrophy in both young and old muscle, whereas the concurrent regulation in protein synthesis signalling and proteolysis inhibition appears to affect young adults more pronouncedly compared to older adults.

Gaining a better understanding of the ability of human skeletal muscle to recover from disuse-induced atrophy has important implications for the development and implementation of effective countermeasures against physical frailty in the increasing population of elderly. Importantly therefore, the present experiments demonstrate that resistance training is highly effective of increasing maximal muscle strength and neuromuscular function in elderly post-operative patients. Importantly, these increases in mechanical muscle function were accompanied by gains in muscle size, architecture and in the expression of IGF-I mRNA splice variants, resembling that typically seen in young healthy individuals when exposed to resistance training. In contrast, these positive adaptations could not be achieved with the use of neuromuscular electrical stimulation or conventional rehabilitation efforts alone. Collectively, these findings strongly underline the importance of implementing resistive exercises in future rehabilitation programs for elderly individuals.

In addition, comparing young and old able-bodied individuals, we observed that the magnitude and time-course of changes in mechanical muscle function during the recovery phase following short-term disuse were compromised in old compared to young individuals. Likewise, aged individuals demonstrated an impaired response to re-loading reflected by attenuated gains in myofibre area, in parallel with smaller increases in satellite cell number despite no age-related differences were observed in factors known to promote skeletal muscle hypertrophy and myogenic stem cell proliferation (IGF-Ea, MGF, MyoD, myogenin, HGF). Moreover, an age-specific regulation in myostatin mRNA expression was observed, characterized by an amplified increase in aging skeletal muscle with immobilisation that was followed by less down-regulation during the subsequent phase of re-loading. In combination with an association observed between the changes in myostatin expression and satellite cell proliferation in the acute phase of re-loading, these data indicates that myostatin play an important role in the impaired ability of aged human skeletal muscle to recover from immobility-induced muscle atrophy.

10. DANISH SUMMARY
For at få en bedre forståelse af den human skeletmuskels evne til at komme sig efter længere tids inaktivitet og et ope-rativt indgreb, undersøgte vi effekten af henholdsvis neu-romuskulær elektrisk stimulation (NMES) eller styrketræning som supplement til den konventionelle rehabilitering efter total høftealloplastik. Vores resultater viste med stor tyde-lighed, at styrketræning er en effektiv måde at øge muskel-styrken og muskelfunktionen hos ældre postoperative patien-ter. Endvidere blev fremgangen i muskelfunktion ledsaget af en betydelig øgning i muskelstørrelse og muskelarkektur. I modsætning hertil blev disse positive ændringer ikke opnået med NMES eller konventionel rehabilitering, hvilket er med til at understrege betydningen af at implementere styrke-træning i fremtidige rehabiliteringsprogrammer til ældre per-soner.

For at undersøge hvilken betydning alder har for effekten af inaktivitet på skeletmuskaturene, supplerede vi ovenstå-ende studier med to interventionstudier, hvor en gruppe af raske aktive unge og ældre personer fik det ene ben immobiliseret (med gips eller Don-Joy skinner) i henholdsvis 14 og 4 dage. Efter blot 4 dages immobilisering observerede vi et signifikant fald i muskelfiberarealet hos både unge og ældre, uden at dette atrofi-respons var påvirket af alder. I modsæt-ning hertil, observerede vi et større tab af muskelmasse hos unge sammenlignet med ældre personer efter 14 dages im-mobilisering, hvorimod den centrale muskelaktivering og ev-nen til at udvikle kraft hurtigt (RFD) var mere udtalt hos de ældre personer.

Samlet set viste de to immobiliseringsstudier endvidere at initieringen og reguleringen af den humane skeletmuskelaftrofi er aldersrelateret, og at en række signaleringsveje relateret til både muskelatrofi og proteinsyntese aktiveres indenfor de første dage af en immobiliseringsperiode. I lighed med tidligere observationer i forskellige dyremo-deller blev det endvidere vist, at det tager længere tid for æl-dre personer at genvinde deres muskelstyrke og muskelfunktion efter en immobiliseringsperiode sammenholdt med yngre personer. Parallelt hermed observeredes en nedsat evne til at genvinde muskelmassen og øge antallet af myogene stamcel-ler (satelliteceller) i forbindelse med re-træning hos de ældre forsøgspersonaeren, på trods af at der ikke blev fundet aldersrela-terede forskelle i genexpressionen af faktorer der vides at have betydning for muskelvækst (IGF-Ea, MGF, MyoD, myoge-nin, HGF). Derimod var reguleringen af myostatin, der vides at hæmme aktiveringen af de myogene stamcellers aldersbetin-get, og der observeredes således en større øgning i myostatin mRNA i relation til immobiliserings og en mindre nedregulering som reaktion på re-træning i den ældre skeletmuskulatur. Disse fund indikerer samlet set, at myostatin spiller en vigtig rolle for den nedsatte evne til at genvinde et muskelsttab efter en kortere eller længerevarende immobiliseringsperiode, der ses hos ældre personer.

11. REFERENCES


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Resistance Training in the Early Postoperative Phase Reduces Hospitalization and Leads to Muscle Hypertrophy in Elderly Hip Surgery Patients—A Controlled, Randomized Study

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OBJECTIVES: To better understand how immobilization and surgery affect muscle size and function in the elderly and to identify effective training regimes.

DESIGN: A prospective randomized, controlled study.

SETTING: Bispebjerg University Hospital, Copenhagen, Denmark.

PARTICIPANTS: Thirty-six patients (aged 60–86) scheduled for unilateral hip replacement due to primary hip osteoarthrosis.

INTERVENTION: Patients were randomized to standard home-based rehabilitation (1 h/d × 12 weeks), unilateral neuromuscular electrical stimulation of the operated side (1 h/d × 12 weeks), or unilateral resistance training of the operated side (3/wk × 12 weeks).

MEASUREMENTS: Hospital length of stay (LOS), quadriceps muscle cross-sectional area (CSA), isokinetic muscle strength, and functional performance. Patients were tested presurgery and 5 and 12 weeks postsurgery.

RESULTS: Mean ± standard error LOS was shorter for the resistance training group (10.0 ± 2.4 days, P < .05) than for the standard rehabilitation group (16.0 ± 7.2 days). Resistance training, but not electrical stimulation or standard rehabilitation, resulted in increased CSA (12%, P < .05) and muscle strength (22–28%, P < .05). Functional muscle performance increased after resistance training (30%, P < .001) and electrical stimulation (15%, P < .05) but not after standard rehabilitation.

conventional home-based rehabilitation program in a group of elderly patients scheduled for elective hip-replacement surgery.

METHODS

Study Design

The study was designed as a prospective randomized, controlled study and included patients scheduled for primary unilateral hip replacement at Bispebjerg University Hospital, Copenhagen, Denmark. The patient group was chosen in part because it permitted familiarization and baseline tests before the operation. These tests were conducted approximately 2 and 1 week preoperatively, and patients were subsequently randomly allocated to one of the following groups: home-based standard rehabilitation (SR), SR plus unilateral lower-limb resistance training (RT), or SR plus unilateral NMES. The randomization procedure was performed with the aid of a computer program (Minimize version 2.1, C. V. Jensen, Rigshospitalet; Copenhagen, Denmark), and patients were stratified by age and sex. The RT and electrical stimulation (ES) groups performed the additional training or received ES on the operated leg, so the nonoperated side could serve as a within-subject control. Patients were retested 5 weeks and 12 weeks postsurgery. Measurement outcomes included hospital length of stay (LOS), functional performance, muscle cross-sectional area (CSA), and maximal quadriceps strength. The ethics committee of Copenhagen approved the study in accordance with the Declaration of Helsinki, and written informed consent was obtained.

Study Population

Medical records of 168 patients on a waiting list for a hip replacement operation were reviewed to identify eligible subjects from May 2000 to May 2002. Eligibility criteria included age of 60 and older and unilateral primary hip replacement due to primary hip osteoarthrosis in patients without cardiopulmonary, neurological, or cognitive problems. To avoid differences in comorbidity between groups, only patients with an American Society of Anesthesiologists (ASA) score of I to II (I = no comorbidity, II = comorbidity but no systemic affection) were included. Sixty-eight persons that met the inclusion criteria were contacted, of whom 36 gave written informed consent to participate. The most common reasons for refusal to participate in the study were the time-consuming test sessions before the operation and the randomization of the postoperative intervention. Excluded patients did not significantly differ from included patients with respect to age, sex, comorbidity, or degree of hip osteoarthrosis.

LOS in the Hospital

LOS in the hospital was defined as the day of admission to the day of discharge. Patients were considered ready for discharge when they were independent in activities of daily living and able to walk minor indoor distances (<10 m) with crutches. Personnel in the orthopedic department, including those involved in discharge planning, were blinded to the training intervention.

Functional Performance

Three functional parameters that have been shown to correlate with physical disability, dependence, and the risk for falling were measured. Maximal gait speed over a 10-meter course was measured to the nearest 0.1 seconds, and stair-climbing performance was measured as the time to ascend 10 steps (height 20 cm). Both tests were initiated from a standing position and stopped when both feet were at the predefined finish position. The ability to rise from a chair (sit-to-stand test; five repetitions) was measured to the nearest 0.1 seconds.

Strength Assessment

Muscle strength was measured as the maximal voluntary isokinetic knee extension moment (peak moment, Nm) during concentric quadriceps contraction at slow (60°/s) and fast (180°/s) knee joint angular velocities. All moment values were corrected for the effect of gravity of the lower limb, as has been described in detail elsewhere. Measurements were performed on both thighs and were preceded by a familiarization trial conducted on a separate day. The nonaffected side was tested first to increase the subject’s comfort with the procedure. Strict care was taken to ensure identical test protocols for all subjects, which included standardized verbal encouragement and visual feedback provided by a real-time display of the force output. Successive trials were performed until peak moment could not be improved any further, which typically included seven to nine attempts at each velocity. Although pain will clearly influence any measure of muscle strength, it is noteworthy that pain has not been addressed in previous training studies on patients. In the present study, the subjects were requested to indicate pain level during the test on a standard visual analog scale. The strength data were discarded if the visual analog scale score exceeded three. Peak moment of the operated side divided by that of the nonoperated side was defined as the quadriceps strength index.

Muscle CSA

Computed tomography (Picker 5000, Picker, Inc., Cleveland, OH) with an image matrix of 512 by 512 pixels was used to obtain muscle CSA of the quadriceps femoris muscle. Slice thickness was 8 mm, and scanning time was 5 seconds. The scan of the quadriceps muscle was obtained at the midpoint between the great trochanter and lateral joint line of the knee. An experienced radiologist who was blinded to intervention and time evaluated the scans three times. The mean value was recorded as the result. The CSA was calculated using Picker VOXEL-Q CT/MR Software Package for real-time analysis. The coefficient of variation between two consecutive measurements was less than 2%.

INTERVENTION

Home-Based Standard Rehabilitation

All three groups were provided the same standard rehabilitation procedure for hip-replacement patients at Bispebjerg Hospital. The standard rehabilitation program consisted of 15 exercises divided in two parts. The first part consisted of six bed exercises: ankle dorsiflexion and plantarflexion and isometric exercises for the glutei, pelvic, and thigh muscles. The second part consisted of knee extensions in a seated position and hip abduction, knee flexion, step training, and calf stretching while standing. No additional weights or resistance bands were used. During the hospital stay, a trained physical therapist who was blinded to the intervention
trained all patients in transfer situations and ambulation, as well as the above exercises. Patients received a pamphlet with the 15 exercises to be continued at home. The SR group was instructed to perform the exercises twice a day and attend weekly control sessions in the physical therapy department, during which an experienced physical therapist guided them through all the exercises to ensure they were performed correctly. Identical instructions were given to the two other treatment groups.

Neuromuscular Electrical Stimulation
The group that received additional unilateral NMES began the stimulation program on the affected leg 1 day after the operation. Patients were carefully instructed in the use of the stimulator and the placement of the electrodes. The stimulator was a pocket-sized battery-operated unit (Elpha 2000, Biofina, Denmark) that delivered a constant biphasic current (0–60 × mA). After careful preparation of the skin, two electrodes (Bio-Flex, 50 × 89 × mm, Biofina A/S; Odense, Denmark) were placed over the quadriceps muscle 5 cm below the inguinal ligament and 5 cm above the patella. The pulse rate was 40 Hz, with a pulse width of 250 microseconds, and each stimulation lasted for 10 seconds, followed by 20 seconds of rest. The amplitude increased and decreased gradually during the first and last 2 seconds. The intensity of the stimulation was adjusted according to patient tolerance. The total stimulation time was 1 h/d for 12 weeks, and all patients registered total stimulation time and intensity. After discharge from the hospital, the stimulator was used at home, and weekly controls were conducted.

Resistance Training
Resistance training was unilateral progressive training for the quadriceps muscle of the operated leg. During hospitalization, the patients performed daily knee extension exercises (3 × 10 repetitions) in a seated position with sandbags strapped to the ankle. Training was performed using adjustable leg-press and knee-extension machines (TechnoGym International, Gambettola, Italy) three times per week as soon as possible (~Day 7). After a 10-minute warm-up on a stationary bicycle, seated knee extensions and leg presses were performed in a supine position (~90° of hip flexion to avoid hip luxation). A trained physical therapist carefully supervised all training sessions. Training intensity was progressively increased in intensity from 20-repetition maximum (RM) (~50% of 1RM) the first week to 15RM (~65% of 1RM) during Weeks 2 to 4 to 12RM (~70% of 1RM) during Weeks 5 to 6 and finally to 8RM (~80% of 1RM) the last 6 weeks. Progressive increases and fairly low intensity during the first 6 weeks were used to avoid training-associated injuries. During each training session, patients performed three to five sets of 10 repetitions during Weeks 1 to 6 and three to five sets of eight repetitions during Weeks 6 to 12. The training load was carefully adjusted weekly to ensure that all patients trained at the appropriate intensity. The load was measured using multiple-RM testing based on goal repetitions.

Statistical Analysis
Nonparametric statistics were used for the analysis, because not all data were normally distributed. Friedman tests with Wilcoxon post hoc tests were used to evaluate the effect of intervention over time. The Kruskal-Wallis tests with Mann-Whitney U post hoc tests were used to analyze for intergroup differences. Data are presented as mean values ± standard error. $P < .05$ was considered significant.

RESULTS
Thirty of the 36 patients completed the study. Two from the SR group withdrew immediately because of dissatisfaction with the randomization outcome. Two became ill (1ES; 1 RT) for reasons unrelated to the study, and two withdrew because of personal problems (1SR; 1ET) (Figure 1). There were no differences between the three groups at the start of the study with respect to anthropometric data (Table 1), comorbidity, muscle strength, or functional performance parameters (Table 2). There were no training-related complications observed in any of the three groups.

LOS differed significantly between the treatment groups (Figure 2A); mean LOS was 37% shorter for the RT group (10 ± 2.4 days, range 8–14) than for the SR group (16 ± 7.2 days, range 9–35) ($P < .05$). This difference remained when the patients with the longest LOS in the SR group were excluded ($P < .05$). A tendency ($P = .07$) toward a shorter LOS was seen for the ES group (12 ± 2.8 days, range 8–16) than for the SR group.

All three functional skills improved in the RT and ES groups after 3 months of training (Table 2). RT increased maximal gait speed 30% ($P < .001$), and ES increased maximal gait speed 19% ($P < .05$). Stair-climbing performance improved 28% ($P < .005$) in RT and 21% ($P < .001$) in ES. The sit-to-stand test improved 30% ($P < .001$) in RT and 21% ($P < .001$) in ES. No improvements were seen in SR from baseline values to 12 weeks after surgery. RT and ES improved to a greater extent than SR in the sit-to-stand test from 0 to 12 weeks (RT > SR, $P = .002$ and ES > SR, $P = .03$), whereas no between-group difference could be observed for maximal gait speed ($P = .11$ to .20) or stair-climbing performance ($P = .33$ to .55).

Quadriceps muscle CSA in the SR group decreased 13% ($P < .05$) on the operated side 5 weeks postsurgery and remained 9% below baseline values ($P < .05$) 12 weeks postsurgery (Figure 2B). In the RT group, CSA of the operated leg was unaltered 5 weeks postsurgery and increased 12% over
baseline (P < .05) 12 weeks postsurgery. In the ES group, CSA decreased 4% on the operated side 5 weeks postsurgery (P < .05) and increased 7% from 5 to 12 weeks postsurgery (P < .05). The nonoperated side was unaffected in all three groups. A significant between-group difference was observed from baseline to 5 weeks and from baseline to 12 weeks. Changes in muscle CSA for RT were greater than for ES (5 weeks P = .04, 12 weeks P < .0001) and SR (5 weeks P = .002, 12 weeks P < .001).

Peak torque did not differ between the groups at baseline. Peak torque was greater on the nonoperated than the operated side in all three groups at baseline (P < .05) (Table 3). Peak torque increased on the operated side in RT 28% at 60°/s (P < .001) and 22% at 180°/s (P < .05) 12 weeks postsurgery. Muscle strength was unchanged on the operated side in the SR group and the ES group (Table 3), and no change in peak torque was observed in the nonoperated leg in any of the groups. Consequently, the quadriceps strength index (operated leg/nonoperated leg) rose from 78% to 100% at 60°/s and from 80% to 99% at 180°/s in the RT group. In contrast, the corresponding quadriceps strength index remained at 73% and 75% (60°/s and 180°/s, respectively) in the SR group 12 weeks after surgery and at 87% and 83% in the ES group. The improvement in peak torque at 60°/s was significantly greater in the RT group than in the SR group from baseline to 5 weeks (P = .02) and than in the SR group (P = .001) and the ES group (P = .001) from baseline to 12 weeks. The RT group improved significantly more than the SR group from baseline to 12 weeks (P = .033) at 180°/s but not than the ES group. There was no difference in the change in peak torque between the ES group and the SR group at any time.

**DISCUSSION**

The present study investigated whether elderly patients who undergo hip-replacement surgery could benefit from additional training in the early postoperative phase and more specifically what training modality would be the most effective. The data indicate that RT is an effective and safe way to increase muscle mass, maximal muscle strength, and functional performance. In addition, RT reduced the hospitalization period more than a conventional rehabilitation regimen or NMES. Furthermore, it was shown that, although the conventional rehabilitation program included early mobilization, quadriceps CSA on the operated side decreased 9%, whereas the RT program augmented CSA 12% 12 weeks after surgery.

Previous studies have demonstrated that major surgery elicits a catabolic stress response that leads to general weight loss and a reduction of lean tissue mass, but the combined effect of surgery and physical training on muscle CSA in elderly patients has not yet been investigated. Maximal muscle strength is directly related to muscle mass and CSA.
and reduced lower-limb muscle strength has been correlated with functional impairments.\textsuperscript{19,30} Therefore, attempts to ameliorate hospitalization-associated muscle atrophy in the elderly is of the utmost importance. In the present study, body weight and muscle CSA on the nonoperated side remained unchanged 5 and 12 weeks after surgery. It is possible that the present sample, which consisted of relatively healthy patients, experienced minor surgery-related catabolism and that the loss of muscle mass represented disuse atrophy, which is possible to prevent, in part, with physical training. The data may suggest that weight loss was regained after 5 weeks or that the perioperative interventions effectively prevented weight loss, but this was contrasted by the decline in muscle CSA on the operated side in the SR group at 5 (\(-13\%\)) and 12 weeks (\(-9\%\)) postsurgery, which may reflect that the conventional rehabilitation program produced insufficient muscle contractile activation. The decline in CSA in the ES group (\(-4\%\)) was less than in the SR group (\(-13\%\)) 5 weeks after surgery, and there was no decrease in the ES group 12 weeks after surgery, which is in accordance with studies in young patients.\textsuperscript{18,19} The increase in CSA from 5 to 12 weeks in the ES group without any accompanying increase in isokinetic strength is in accordance with studies that demonstrate that NMES may influence muscle mass but not voluntary strength.\textsuperscript{18,19} In addition, it cannot be excluded that the knee position during the ES or during isokinetic testing may have contributed to the apparent disparity in CSA and isokinetic muscle strength. Notably, RT not only prevented postsurgery muscle atrophy, but also augmented CSA after 12 weeks, which represented a substantial numerical difference (\(-21\%\)) relative to the observed atrophy in the SR group. The relative gain in muscle strength in the RT group was comparable with that observed in other studies using isokinetic strength assessment in healthy elderly after RT intervention\textsuperscript{11,12} and was likely a function of the augmented muscle mass, but because RT has consistently been shown to induce neural adaptations in healthy elderly individuals\textsuperscript{33,34} and most recently also in patients recovering from hip replacement surgery (unpublished data), it is also plausible that a portion of the observed strength gain can be attributed to changes in neural function.\textsuperscript{33}

Studies of the effects of physical intervention introduced several months to years after hip replacement surgery show that, despite a successful operation and uncomplicated conventional postoperative rehabilitation, a significant deficit in muscle strength persists in the operated limb relative to the nonoperated side.\textsuperscript{5,7} In contrast, the present data demonstrate that the preoperative side-to-side difference in muscle strength was eliminated after 12 weeks of unilateral RT. This elimination of the strength deficit was not accomplished in the two other groups that used conventional rehabilitation or NMES.

An important clinical finding in the present study was the marked increase in functional performance in the RT (30\%) and ES groups (15–20\%). Because RT and ES were performed unilaterally, there is no intuitive link between the changes in unilateral muscle strength/CSA and the increases in function that requires two-legged coordinated movement, but because the increase in strength/CSA only occurred on the weak and trained side, it is plausible that the unilateral increase in strength/CSA may have helped to reduce any deficit between legs and thereby have improved function. The amelioration in walking speed with RT and NMES in the present study was particularly noticeable, because previous randomized, controlled studies on hip-fracture patients that have demonstrated a reduced LOS have included combinations of interventions.\textsuperscript{12,37} It should be mentioned that, in the present study, LOS represents a sum of the acute surgical

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<th>Table 3. Changes in Isokinetic Muscle Strength at Baseline and 5 and 12 Weeks Postsurgery</th>
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<tr>
<td><strong>Peak Torque</strong></td>
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Note: Results from the isokinetic quadriceps strength measurements (60°/s and 180°/s) and quadriceps cross-sectional area on the operated and the nonoperated leg from all three intervention groups at three time points.

\( ^{1}\)Peak torque was greater on the nonoperated than operated side in all 3 groups at baseline (\(P<0.05\)).

\( ^{2}\)P < 0.05 refers to intergroup differences, resistance training being significantly different from standard rehabilitation and electrical stimulation.

Nm = Newton meter.
LOS and the rehabilitative LOS, and thus any specific effect on the separate components cannot be ascertained. Furthermore, the present study included a limited number of patients, and it can therefore not be excluded that a few of the parameters have not been studied with a sufficiently high power. However, the randomization and lack of difference at baseline, as well as the blinding of the department staff, strengthens the findings and supports the notion that specific muscle training is an effective intervention tool to attain improved function that also permits earlier hospital discharge.

In summary, the present study demonstrates that elderly patients who undergo hip-replacement surgery can, without any side effects, benefit from intensive physical training that is initiated early in the postoperative phase. Moreover, RT more effectively increased muscle mass, maximal muscle strength, and functional performance and decreased LOS than ES or conventional rehabilitation.

Figure 2. Length of stay in hospital and change in muscle cross-sectional area (CSA). A. Difference in hospitalization length between the three intervention groups. B. Quadriceps muscle cross-sectional area of the operated leg in all three intervention groups at three points. Data are presented as means ± standard error (SE). *P < .05 significantly different from baseline (Pre); †P < .05 significantly different from 5 weeks after the operation (5w); ‡P < .05 resistance training (RT) significantly different from standard rehabilitation (SR).

ES = electrical stimulation; 12w = 12 weeks after the operation.

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Training-induced changes in muscle CSA, muscle strength, EMG, and rate of force development in elderly subjects after long-term unilateral disuse

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Suetta, Charlotte, Per Aagaard, Anna Rosted, Ane K. Jakobsen, Benn Duus, Michael Kjaer, and S. Peter Magnusson. Training-induced changes in muscle CSA, muscle strength, EMG, and rate of force development in elderly subjects after long-term unilateral disuse. J Appl Physiol 97: 1954–1961, 2004. —The ability to develop muscle force rapidly may be a very important factor to prevent a fall and to perform other tasks of daily life. However, information is still lacking on the range of training-induced neuromuscular adaptations in elderly humans recovering from a period of disuse. Therefore, the present study examined the effect of three types of training regimes after unilateral prolonged disuse and subsequent hip-replacement surgery on maximal muscle strength, rapid muscle force [rate of force development (RFD)], muscle activation, and muscle size. Thirty-six subjects (60–86 yr) were randomized to a 12-wk rehabilitation program consisting of either (1) strength training (3 times/wk for 12 wk), (2) electrical muscle stimulation (1 h/day for 12 wk), or (3) standard rehabilitation (1 h/day for 12 wk). The nonoperated side did not receive any intervention and thereby served as a within-subject control. Thirty subjects completed the trial. In the strength-training group, significant changes were observed in maximal isometric muscle strength (24%, P < 0.01), contractile RFD (26–45%, P < 0.05), and contractile impulse (27–32%, P < 0.05). No significant changes were seen in the two other training groups or in the nontrained legs of all three groups. Mean electromyogram signal amplitude of vastus lateralis was larger in the strength-training group than in the standard-rehabilitation group at 5 and 12 wk (P < 0.05). In contrast to traditional physiotherapy and electrical stimulation, strength training increased muscle mass, maximal isometric strength, RFD, and muscle activation in elderly men and women recovering from long-term muscle disuse and subsequent hip surgery. The improvement in both muscle mass and neural function is likely to have important functional implications for elderly individuals.

The debilitating effects of disuse on maximal muscle strength (4, 40), muscle mass (13, 21, 35), and muscle activation (44) are well documented and occur already during the first week of immobilization. Most of the current knowledge concerning the effect of immobilization on skeletal muscle is based on studies in healthy young individuals during bed rest (35), limb unloading (9), and spaceflight (13, 36) or in young patients recovering from anterior cruciate ligament reconstruction (5, 19, 55). However, because elderly persons in particular are prone to periods of immobilization and disuse either due to joint pain or hospitalization (11), it appears paramount to gain a better understanding of how immobilization affects muscle size and neural function in this population, as well as to identify training regimes that ensure an effective rehabilitation.

It is well known that, with increasing age, muscle strength and muscle power decrease for both sexes, especially beyond the sixth decade (10, 22, 42, 48, 53, 56). Interestingly, it has been shown that the ability to develop high muscle power declines more rapidly and relates more to functional performance than to maximal muscle strength (7, 48, 53). In daily life, however, many types of movements, such as preventing a fall, are characterized by a limited time to develop force (0–200 ms), which is considerably less time than it takes to achieve maximal contraction force (~400–600 ms) (2, 51). Consequently, during such time-restricted contraction conditions (<200 ms), the ability to develop a rapid rise in muscle force [i.e., a high rate of force development (RFD) = force/time] may become more important than maximal muscle force and power. Despite this, limited information is available on training-induced neuromuscular adaptations in elderly subjects after a period of immobilization.

Previously, RFD has been investigated in young, healthy individuals (2, 46, 51) and has been demonstrated to increase in response to heavy strength training (2, 46, 51) and combined strength/power training (26), whereas low strength training seems to have no effects (46). In elderly individuals, RFD is reduced compared with young individuals of both genders (12, 30, 33, 50, 54); however, combined power/strength training has been shown to induce marked increases in RFD and muscle activation [electromyogram (EMG) amplitude] in healthy, elderly individuals of both genders (24, 25, 27). The effect of strength training on RFD has not previously been investigated in elderly subjects who recover from a period of immobilization.

Another method to restore muscular function after immobilization is by the application of percutaneous neuromuscular electrical stimulation (NMES), which has been used primarily in young patients rehabilitating from anterior cruciate ligament reconstruction (5, 19), whereas studies on elderly subjects are scarce (41, 43). None of the aforementioned studies have evaluated the effect of NMES on muscle activation and rapid muscle strength properties. Because muscle contraction induced by NMES partly bypasses the central nervous system (CNS), it could be hypothesized that training involving volitional strength exercise more effectively improves neuromuscular function and RFD. On the other hand, NMES might be a more tolerable training modality for frail, elderly individuals.
Therefore, the purpose of the present study was to compare the effect of additional unilateral strength training or electrical muscle stimulation with conventional physiotherapy on neuromuscular adaptation in a group of elderly individuals rehabilitating from unilateral, long-term disuse and immobilization. Measurements were focused on the type and magnitude of neuromuscular adaptation in the very early phase of muscle contraction (0–200 ms), with respect to RFD and muscle activation. Furthermore, the study evaluated the effect of strength training vs. electrical muscle stimulation to compare the neuromuscular adaptation to training involving either the functioning nervous system or training using peripheral muscle stimulation, which bypasses effector motor output of the CNS.

**METHODS**

**Subjects.** The study was designed as a prospective randomized controlled study and included 36 elderly individuals with long-term unilateral disuse due to osteoarthritis of the hip. Subjects were scheduled for primary unilateral hip-replacement operation at Bispebjerg University Hospital, Copenhagen, Denmark. Eligibility criteria included age over 60 yr and radiological and clinical primary hip osteoarthritis. All subjects were carefully examined by a blinded physician to exclude subjects with cardiopulmonary, neurological, or cognitive problems. Also, lower limb problems other than the hip osteoarthritis and/or pain during testing or training measured on a visual analog scale (>3) were exclusion criteria.

Subjects were randomly allocated to one of the following groups after baseline tests: 1) standard rehabilitation (SR), 2) SR plus unilateral strength training (ST), or 3) SR plus unilateral NMES (ES). The ST and ES groups only performed the additional training on the operated leg so that the nonoperated side could serve as a within-subject control; the SR group served as a control group.

Subjects were retested 5 and 12 wk after surgery. Measurement outcomes were quadriceps muscle cross-sectional area (CSA), maximal voluntary isometric quadriceps strength, rapid muscle force defined as the contractile RFD (change in force/change in time), normalized RFD, and contractile impulse (force dtime). EMG recordings were obtained in vastus lateralis (VL), vastus medialis (VM), and rectus femoris during maximal isometric quadriceps contraction (2) to evaluate the change in muscle activation induced by the different training regimes. The ethics committee of Copenhagen approved the study in accordance with the Helsinki Declaration, and written, informed consent was obtained from all participants.

**Maximal isometric muscle strength and RFD.** Muscle strength was measured as the maximal voluntary isometric knee extension torque exerted in an isokinetic dynamometer (KinCom; Kinetic Communicator, Chattecx, Chattanooga, TN). The reliability and validity of this dynamometer have been verified in detail elsewhere (14). Subjects were seated 10° reclined and firmly strapped at the hip and thigh. The axis of rotation of the dynamometer lever arm was visually aligned to the axis of the lateral femur condyle of the subject, and the lower leg was attached to the lever arm of the dynamometer just above the medial malleolus. Individual setting of the seat, backrest, dynamometer head, and lever arm length was registered, so identical positioning was secured at all time points. To correct for the effect of gravity, the passive mass of the lower leg was measured by the dynamometer at a knee joint angle of 45° (3). Subjects were carefully instructed to contract as fast and hard as possible. Visual feedback was provided to the subjects as a real-time display of the dynamometer force output on a computer screen (34). After careful warm-up with several dynamic and submaximal isometric contractions, subjects performed three maximal isometric contractions at a knee joint angle of 60° (0° = full knee extension). The trial with the highest maximal voluntary contraction was selected for further analyses (2). All measurements were performed on both thighs and were preceded by a familiarization trial that was conducted on a separate day. The nonaffected side was tested first to increase subjects’ comfort with the procedure, as described in detail elsewhere (2). Contractile RFD was derived as the average slope of the initial phase of the force-time curve (change in force/change in time) at 30, 50, 100, and 200 ms relative to the onset of contraction. The onset of contraction was defined as the instant where force increased 3.5 N·m above the rising baseline level, corresponding to 2% of the peak moment. Contractile impulse was determined as the area under the force-time curve (force·dt) in the same time intervals. By incorporating the aspect of contraction time, contractile impulse provide important information to rapid muscle strength characteristics, although this parameter is only rarely reported (2, 6). Normalized RFD was calculated as the slope of the force-time curve normalized relative to CSA. The measurement procedure has been described in more detail elsewhere (2).

**EMG recordings.** After careful preparation of the skin by shaving and cleaning with alcohol, pairs of surface electrodes (Medicostet Q-10-A, 20-mm interelectrode distance) were placed over the belly of VL, VM, and rectus femoris. All electrode positions were carefully measured in each subject to ensure identical recording sites throughout all tests. The EMG electrodes were connected directly to small custom-built electromyograph amplifiers with a frequency response of 10–10,000 Hz and common mode rejection ratio exceeding 100 dB. EMG and dynamometer strain gauge signals were synchronously sampled at a 1,000-Hz analog-to-digital conversion rate using an external analog-to-digital converter (dt 2801-A, Data Translation, Marlboro, MA). During later offline analysis, EMG signals were digitally high-pass filtered with a fourth-order, zero-lag Butterworth filter with a 5-Hz cutoff frequency, followed by a moving root mean square filter with a time constant of 50 ms. To reflect neural adaptations in the early phase of contraction, integrated EMG of the root mean square-filtered signal was calculated in time intervals of 0–30, 50, 100, and 200 ms relative to the onset of EMG integration, which was initiated 70 ms before force onset to account for electromechanical delay (2). To yield mean average voltage (MAV), integrated EMG was divided by integration time (MAV = integrated EMG/time integration).

**Muscle CSA.** CSA of the quadriceps femoris muscle was obtained by computed tomography (Picker 5000) with an image matrix of 512 × 512 pixels, slice thickness of 8 mm, and scanning time of 5 s. The scans of the quadriceps muscle were obtained at the midpoint between the great trochanter and lateral joint line of the knee. Each scan was blinded, CSA was measured three times by a radiologist, and the mean value was recorded as the result. The coefficient of variation between two consecutive measurements was <2%.

**Strength training.** Strength training was performed as unilateral progressive training of the leg muscles with the focus on the quadriceps muscle of the affected limb. During hospitalization, patients performed daily unilateral knee extension exercises (3 × 10 repetitions) in a sitting position with sandbags strapped to the ankle of the operated leg. As soon as possible (approximately day 7), training was performed in adjustable leg-press and knee-extension machines (Technogym International) three times per week. After a 10-min warm-up on a stationary bicycle, sitting knee-extension and leg-press exercises in a supine position were performed. Training intensity was decreased from 20 to 12 repetition maximum (RM; 3–5 sets × 10 repetitions) from weeks 0 to 6 to avoid injuries and was thereafter maintained at 8 RM (3–5 sets × 8 repetitions). The training load was adjusted on a weekly basis, and in the final 6–8 wk, when the subjects were familiar with the training, they were supervised to perform the exercises as rapidly as possible in the concentric phase and keep a slow speed in the eccentric phase (8).

**NMES.** The ES group received the stimulation program on the affected side the first day after surgery. Subjects were carefully instructed in the use of the stimulator and placement of the electrodes. The stimulator was a pocket-size, battery-operated device (Elkha...
2000, Biofina) that delivered a constant biphasic current (0–60 mA).
After careful preparation of the skin, two electrodes (Bio-Flex, 50 × 89 mm) were placed over the quadriceps muscle 5 cm below the inguinal ligament and 5 cm above the patella. The pulse rate was 40 Hz with a pulse width of 250 μs, and each stimulation lasted for 10 s followed by 20 s of rest. The amplitude increased and decreased gradually the first and last 2 s. The intensity of the stimulation was adjusted according to individual subject tolerance. The stimulation regime was applied for 1 h per day on the affected leg for 12 wk. All subjects registered daily the total stimulation time and intensity. After discharge from hospital, the stimulator was used at home, and weekly controls were conducted in the physiotherapy department to ensure the stimulation was performed correctly.

ST. The SR group, as well as the two other subject groups, were provided the same rehabilitation procedure for hip-replacement patients at Bispebjerg Hospital. The rehabilitation program consisted of a home-based training program that included 15 physiotherapy exercises aimed at improving function, range of motion, and muscle strength around the hip. No external loads or rubber bands were used in the program. During the hospital stay, all subjects were trained in all 15 exercises by an experienced physiotherapist, who was blinded to the intervention. All subjects received a pamphlet with the 15 exercises to be continued at home. The SR group who served as a control group was instructed to perform the exercises twice a day and to come to weekly controls in the physiotherapy department.

Statistical analysis. Nonparametric statistics were used for the analyses, since not all data met the criterion of normality. To evaluate the effect of intervention over time, a Friedman test was used with post hoc Wilcoxon test. Any between-group differences were analysed with Kruskall-Wallis tests and subsequently the Mann-Whitney U-test. Data are presented as means ± SE. A P value of <0.05 was considered significant.

RESULTS

Thirty of the 36 subjects completed the study. Two subjects from the SR group withdrew immediately because of dissatisfaction with the randomization outcome, two subjects became ill for reasons unrelated to the study, and two withdrew because of personal problems. There was no difference between the three groups with respect to anthropometric data (Table 1), maximal isometric muscle strength, or muscle CSA (mCSA; Table 2) at the inclusion of the study. No training-related complications were seen in any of the three groups.

Maximal isometric strength. Maximal isometric quadriceps strength increased by 24% in the ST group on the operated side 12 wk postsurgery compared with baseline ($P < 0.05$), whereas there was no increase in quadriceps strength in the two other training groups when 12-wk values were compared with baseline (Table 2). In the SR group, there was a decrease in peak torque from presurgery to 5 wk postsurgery (22%, $P < 0.05$), with a subsequent increase from 5 to 12 wk (27%, $P < 0.05$). A similar decrease in peak torque at 5 wk postsurgery was not observed in the two other groups. There was no change on the nonoperated side in any of the three groups (Table 2).

Although there was no difference in peak torque between groups at inclusion time, a significant difference in the relative change between the ST group and the SR group was observed at 5 ($P < 0.005$) and 12 wk ($P < 0.001$), and between the ST group and the ES group at 12 wk ($P < 0.005$). There were no statistically significant differences in the change in peak torque between the ES group and the SR group at any time point.

Quadriceps CSA. Quadriceps mCSA in the SR group decreased 13% on the operated side at 5 wk ($P < 0.05$) and remained 9% below baseline values at 12 wk ($P < 0.05$, Table 2). In the ST group, mCSA of the operated leg was unchanged at 5 wk and increased 12% compared with baseline at 12 wk ($P < 0.05$). In ES, there was a 4% decrease in CSA on the operated side from baseline to 5 wk ($P < 0.05$) and a 7% increase from 5 to 12 wk ($P < 0.05$). There was no change on the nonoperated side in any of the three groups (Table 2).

There were no differences in CSA between groups at inclusion but a significant difference in the relative change between the ST group and SR group after 12 wk of training ($P < 0.05$).

Contractile RFD and impulse. In the ST group, a steeper slope of the moment-time curve of the affected leg was observed after 12 wk of strength training (Fig. 2). Specifically, contractile RFD increased for peak RFD (21%, $P < 0.005$), and at time intervals of 0–30 ms (45%, $P < 0.05$), 0–50 ms (31%, $P < 0.05$), 0–100 ms (26%, $P < 0.05$), and 0–200 ms (30%, $P < 0.005$) of the affected side at the end of the 12-wk training period (Fig. 3A). When RFD was normalized to mCSA (RFD/CSA), there was a 25% increase (from 11.27 to 14.12 N·m·s⁻¹·cm⁻², $P < 0.05$) in the very initial part of the contraction phase (0–30 ms). In contrast, RFD/CSA remained unchanged in the intervals of 0–50 ms (15.28 vs. 17.33 N·m·s⁻¹·cm⁻²), 0–100 ms (12.84 vs. 14.20 N·m·s⁻¹·cm⁻²), and 0–200 ms (8.96 vs. 10.22 N·m·s⁻¹·cm⁻²). RFD did not change in the two other training groups from preexercise to 12 wk (Fig. 3, B and C) or in the nonaffected side in any of the three groups (data not shown). Contractile impulse increased in the time intervals of 0–30 ms (32%, $P < 0.05$), 0–50 ms (32%, $P < 0.05$), 0–100 ms (28%, $P < 0.05$), and 0–200 ms (27%, $P < 0.005$) on the trained leg in the ST group (Fig. 4A). There were no change in impulse in the ES or SR group on the affected side (Fig. 4, B and C), and there were no changes on the nonaffected side in any of the three groups (data not shown).

Quadriceps muscle EMG. In the ST group, MAV increased significantly for VL on the affected leg in the time intervals of 0–30 ms (36%, $P < 0.05$), 0–50 ms (40%, $P < 0.05$), 0–100 ms (38%, $P < 0.05$), and 0–200 ms (41%, $P < 0.05$), and for VM at 0–200 ms (21%, $P < 0.05$) from 5 to 12 wk of training (Fig. 5). In contrast, there were significant decreases in VL of the affected leg in the SR group from pretraining to 5 wk of training in the time intervals of 0–100 ms (45%, $P < 0.05$) and 0–200 ms (43%, $P < 0.05$) and a strong trend toward a decrease was observed in VM (0–200 ms) from pretraining to 5 wk of training (43%, $P = 0.06$) (Table 3). A subsequent increase in MAV (0–200 ms) was observed in VL (57%, $P < 0.05$) and VM (37%, $P < 0.05$) from 5 to 12 wks. In the ES group, there was no change in MAV on the affected side as
well as on the nonaffected side in any of the three groups (Table 3). At the inclusion time, there was no difference in MAV between the three training groups; however, the above-mentioned changes led to significant differences between VL MAV (0–200 ms) in the ST and SR group at 5 wk (ST > SR, P < 0.05) and at 12 wk (ST > SR, P < 0.05), and a difference between ES and SR was observed at 5 wk (ES > SR, P < 0.05) (Fig. 5). For VM MAV (0–200 ms), there was only a tendency toward a difference between ST and SR (ST > SR, P = 0.09), whereas there were significant differences for the rectus femoris muscle at 5 wk (ST > SR, P < 0.05) and at 12 wk (ST > SR, P < 0.05) in the time intervals of 0–100 and 0–200 ms.

**DISCUSSION**

The present study examined specific neural adaptations to training, involving the CNS and peripheral muscle stimulation partly bypassing the CNS, by comparing the effects of 12 wk of strength training to electrical muscle stimulation (NMES) and conventional physiotherapy after unilateral hip replacement surgery.

For the first time, it was demonstrated that strength training is an effective way to increase muscle mass, muscle activation, and rapid muscle force characteristics (RFD) in elderly individuals rehabilitating after long-term disuse and surgery-related hospitalization. Importantly, the data show that strength training resulted in marked increases both in RFD and in contractile impulse in the time intervals of 0–30, 0–50, 0–100, and 0–200 ms, and in normalized RFD in the initial phase of muscle contraction (0–30 and 0–50 ms). In contrast, NMES and conventional rehabilitation did not produce such increases in these outcome measures.

Previous studies have demonstrated positive effects of strength training on mCSA and muscle strength in healthy elderly individuals (18, 37) and in very old subjects (16, 31). However, the use of strength training is seldomly used in elderly subjects rehabilitating from surgery, and the number of studies in this area are scarce. Only a few previous studies have investigated the effect of resistive exercises after hip surgery (32, 38, 45, 47) and reported significant increases in muscle strength, but none of these studies have reported results on muscle size or neuromuscular adaptations with training. In contrast to the above-mentioned studies, the baseline measures of the present study were obtained before the time of surgery, which enabled us to evaluate the effect of limb immobilization.

### Table 2. Isokinetic muscle strength and quadriceps muscle CSA

<table>
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<th>ST</th>
<th>ES</th>
<th>SR</th>
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<tr>
<td><strong>Quadriiceps CSA, m²</strong></td>
<td></td>
<td></td>
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<tr>
<td>Pre</td>
<td>4,964 ± 451</td>
<td>5,479 ± 415</td>
<td>5,765 ± 569</td>
</tr>
<tr>
<td>5 wk</td>
<td>5,215 ± 409</td>
<td>5,624 ± 1457</td>
<td>5,624 ± 525</td>
</tr>
<tr>
<td>12 wk</td>
<td>5,530 ± 465*</td>
<td>5,736 ± 440</td>
<td>5,825 ± 483</td>
</tr>
<tr>
<td><strong>Isometric strength, N·m</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>122.9 ± 17.2</td>
<td>156.3 ± 20.9</td>
<td>157.2 ± 19.1</td>
</tr>
<tr>
<td>5 wk</td>
<td>130.8 ± 17.2*</td>
<td>152.0 ± 20.9</td>
<td>130.8 ± 15.8</td>
</tr>
<tr>
<td>12 wk</td>
<td>151.9 ± 17.8*</td>
<td>157.8 ± 19.1</td>
<td>147.8 ± 16.3</td>
</tr>
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</table>

Values are means ± SE. Maximal isometric quadriceps strength measurements (60°) and quadriceps cross-sectional area (CSA) from the operated side (Op-leg) and the nonoperated side (Con-leg) in the 3 training groups (ST, ES, SR) at 3 time points (baseline (Pre), at 5 wk (5 wk) and at 12 wk (12 wk) of training). *P < 0.05, 12 wk significantly different from baseline. †P < 0.05, baseline significantly different from 5 wk. ‡P < 0.05, 12 wk significantly different from 5 wk. §P < 0.05, ST significantly different from SR (ST > SR). *P < 0.05, ST significantly different from ES (ST > ES).

### Table 3. EMG signal amplitudes

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<th>ST</th>
<th>ES</th>
<th>SR</th>
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<tr>
<td><strong>0–30 ms, µV</strong></td>
<td></td>
<td></td>
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<tr>
<td>Pre</td>
<td>72.8 ± 11.3</td>
<td>73.4 ± 19.6</td>
<td>76.1 ± 20.5</td>
</tr>
<tr>
<td>5 wk</td>
<td>47.8 ± 5.8</td>
<td>67.7 ± 16.2</td>
<td>47.1 ± 11.1</td>
</tr>
<tr>
<td>12 wk</td>
<td>65.3 ± 9.1*</td>
<td>68.0 ± 13.1</td>
<td>50.5 ± 7.2</td>
</tr>
<tr>
<td><strong>0–100 ms, µV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>109.0 ± 26.0</td>
<td>98.8 ± 27.8</td>
<td>95.7 ± 19.0</td>
</tr>
<tr>
<td>5 wk</td>
<td>63.1 ± 5.4</td>
<td>85.6 ± 17.9</td>
<td>55.4 ± 11.1</td>
</tr>
<tr>
<td>12 wk</td>
<td>88.3 ± 11.0*</td>
<td>88.3 ± 16.8</td>
<td>70.1 ± 10.0</td>
</tr>
<tr>
<td><strong>0–200 ms, µV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>127.4 ± 31.8</td>
<td>117.8 ± 34.3</td>
<td>123.2 ± 23.0</td>
</tr>
<tr>
<td>5 wk</td>
<td>91.6 ± 7.3</td>
<td>107.2 ± 19.7</td>
<td>68.2 ± 13.3†</td>
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due to surgery 5 and 12 wk after the operation. Surprisingly, although the affected limb clearly had disuse-associated muscle atrophy, a further decline in muscle size (Table 2) was observed with conventional rehabilitation (SR group, 13%) 5 wk postsurgery, which was fully prevented with strength training and partly with electrical stimulation. Albeit not statistically significant, it appears that the muscle loss was less pronounced in the ES group (−4%) compared with the SR group (−13%) 5 wk after surgery.

Fig. 1. Computer tomography images taken from the midthigh region of a female subject in the strength training (ST) group before and after training. The 2 images are shown at the same scale. Quadriceps muscle cross-sectional area of the operated side (op-leg) increased 32% in this subject; the nonoperated side (con-leg) did not change from pretraining to posttraining.

Fig. 2. Average moment-time curves obtained for the ST group (n = 11) before (pre; solid line) and after 12 wk (post; dashed line) of training. Onset of contraction is denoted by a solid circle, and vertical lines indicate time intervals of 30, 50, 100, and 200 ms relative to the onset of contraction. Posttraining peak isometric torque increased significantly from 122.9 ± 17.2 to 151.9 ± 17.8 N·m in parallel with a steeper slope of the moment-time curve. The increase in slope was reflected by a significant increase in contractile rate of force development both in the initial (30 and 50 ms) and later (100 and 200 ms) phases of rising force.

Fig. 3. Contractile rate of force development (RFD) at baseline and 5 and 12 wk posttraining in the trained leg of all 3 intervention groups [ST, electrical stimulation (ES), and standard rehabilitation (SR)]. Contractile RFD was derived as the average slope of the initial phase of the force-time curve (change in force/change in time) at 30, 50, 100, and 200 ms relative to the onset of contraction. In addition, peak RFD was determined in the time interval of 0–200 ms. Values are means ± SE. *Significant difference between 12-wk and baseline values (P < 0.05).
Moreover, there was no decrease in mCSA in the ES group 12 wk after surgery, in accordance with earlier studies in young anterior cruciate ligament patients (5, 19). However, it was not possible to detect any overall statistical difference in treatment outcome between these two intervention groups (SR and ES groups). In contrast, strength training not only prevented the postsurgery muscle atrophy at 5 wk but also augmented mCSA after 12 wk (12%), which resulted in a significant difference in treatment outcome between the ST group and the two other groups (Table 2).

With respect to isometric strength, similar changes were observed (Table 2) with a 22% decrease in the SR group 5 wk postsurgery, which was prevented in the ES and ST groups. Furthermore, maximal muscle strength increased 24% in the ST group from baseline to after 12 wk of training in contrast to the ES and SR groups. The observed improvements in the ST group corresponds to previous findings in elderly individuals.

Fig. 4. Contractile impulse at baseline and 5 and 12 wk posttraining in the trained leg of all 3 intervention groups (ST, ES, and SR). Contractile impulse, defined as the area covered by the moment-time curve (\(J_{\text{moment dt}}\)), was calculated in time intervals of 0–30, 50, 100, and 200 ms relative to the onset of contraction. In addition, peak RFD was determined in the time interval of 0–200 ms. Values are means \(\pm\) SE. *Significant difference between 12-wk and baseline values (\(P < 0.05\)).

Fig. 5. Electromyogram (EMG) signal amplitudes at baseline (0), 5 wk (5), and 12 wk (12) posttraining in the trained leg of all 3 intervention groups (ST, ES, and SR). Data presented were calculated as the mean integrated EMG divided by the integration time (200 ms) relative to onset of EMG integration for vastus lateralis (VL), vastus medialis (VM), and rectus femoris (RF). Values are means \(\pm\) SE. MAV, mean average voltage. #Significant difference between 12-wk and 5-wk values (\(P < 0.05\)). §Significant difference between 5-wk and baseline values (\(P < 0.05\)). *ST significantly different from SR (\(P < 0.05\)).
after prolonged strength training intervention regardless of age and gender (18, 20, 49).

Although the loading intensity of the three different training regimes was not directly comparable in the present study, the fact that neither electrical stimulation nor conventional rehabilitation induced increases in maximal muscle strength or muscle mass may reflect inadequate muscle activation with these training modalities and emphasizes the importance of loading intensity in rehabilitation programs.

The ability to develop force rapidly (i.e., contractile RFD) is an important performance characteristic, especially in older people, contributing to several tasks of daily life such as climbing stairs, walking, and attempting to avoid a fall (7, 17). At the same time, reduced muscle strength in older people, e.g., after a period of immobilization or disuse, may be associated with muscle atrophy, a lowered ability to produce force rapidly, and thereby an increased risk of falling (17). In healthy elderly individuals, it has been demonstrated that RFD is reduced compared with young individuals of both genders (12, 33, 50, 54), although when RFD is normalized to maximal voluntary contraction the results are conflicting (12, 50). Likewise, in healthy elderly individuals, Hakkinen and coworkers demonstrated significant increases in RFD and elevated EMG as a result of combined power/strength training performed for 12 wk (23), 21 wk (29), and 6 mo (24), whereas no effects on RFD could be demonstrated with a 10-wk mixed-methods training program in healthy elderly men (28). The strength training program used in the present study was designed as a progressively adjusted program. The aim was to avoid postoperative injuries while still ensuring a sufficiently high loading intensity (~8 RM) to induce adaptive changes in muscle size (15, 18) and muscle activation, as previously demonstrated in young individuals (2, 14, 46). In accordance with these studies, the present strength training regime resulted in marked increases in rapid force production, both in the very initial phase (30–50 ms, 31–45%) as well as the later part (100–200 ms, 26–30%) of the isometric force-time curve (Fig. 2). Similar changes occurred with respect to contractile impulse, which was determined as the integrated area under the force-time curve, both in the initial phase of contraction (30–50 ms, 32%) and in the later part (100–200 ms, 27%) (Fig. 4). Importantly, the present data are the first to demonstrate a 25% increase in the very initial phase of muscle contraction (30 ms) for RFD normalized to mCSA. This increase indicates that qualitative changes may have occurred in muscle contraction characteristics, such as increased maximal motor unit firing frequency (52) or changes in myosin heavy chain isoform composition toward an increased type II dominance (1). The importance of these rapid muscle force characteristics is stressed by the fact that a positive correlation was observed between RFD (0–30–50 ms) and maximal walking speed (data not presented) at baseline ($r = 0.51–0.55$, $P = 0.005$) but not between walking speed and maximal isometric muscle strength. Correspondingly, after 12 wk of strength training, maximal gait speed increased by 30% ($P < 0.001$) and correlated to the increase in absolute RFD ($r = 0.79$, $P = 0.004$) and normalized RFD ($r = 0.86$, $P = 0.001$) in the very initial phase of muscle contraction (0–30 ms). In contrast, the change in maximal walking speed did not correlate to the adaptive change in maximal muscle strength or mCSA.

The fact that marked increases (36–41%) were observed in EMG signal amplitude, especially for VL, during the early (30–50 ms) and later (100–200 ms) phase of rising muscle force indicates that the increases in RFD and impulse at least partly was explained by adaptive changes in neural function. The lack of increased EMG with strength training in the early phase of training was somewhat surprising comparing with results from earlier studies (23, 39); however, it should be noted that the observed adaptations from preexercise to 5 wk of exercise reflect not only the training intervention but also the immobilization period due to surgery. Thus marked decreases in muscle activation (43–45%) were observed in the SR group from preexercise to 5 wk of exercise (Fig. 5), which seemed to be prevented in both the ST the ES groups.

In summary, the present study demonstrates that strength training is an effective way to induce marked increases in maximal muscle strength and enhanced rapid muscle force characteristics in elderly subjects after long-term unilateral limb disuse compared with rehabilitation regimes using electrical muscle stimulation or conventional physiotherapy. Furthermore, the gains in maximal muscle strength and rapid muscle force characteristics were accompanied by significant increases in EMG amplitudes and increased mCSA of the quadriceps muscles. Although the relative contribution to the observed changes in rapid muscle strength from neural vs. morphological adaptations could not be determined in the present study, the results underline the importance of training both aspects in elderly individuals. Furthermore, the observation that rapid muscle force capacity of the neuromuscular system remains trainable in elderly patients recovering from prolonged limb disuse may have important implications for future rehabilitation programs, especially when the importance of rapid muscle force capacity on postural balance, maximal walking speed, and other tasks of daily life actions are considered.

REFERENCES


Resistance training induces qualitative changes in muscle morphology, muscle architecture, and muscle function in elderly postoperative patients

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Although the negative effects of bed rest on muscle strength and muscle mass are well established, it still remains a challenge to identify effective methods to restore physical capacity of elderly patients recovering from hospitalization. The present study compared different training regimes with respect to muscle strength, muscle fiber size, muscle architecture, and stair walking power in elderly postoperative patients. Thirty-six patients (60–86 yr) scheduled for unilateral hip replacement surgery due to hip osteoarthritis were randomized to either 1) resistance training (RT: 3/wk × 12 wk), 2) electrical stimulation (ES: 1 h/day × 12 wk), or 3) standard rehabilitation (SR: 1 h/day × 12 wk). All measurements were performed at baseline, at 5 wk and 12 wk postsurgery. After 12 wk of resistance training, maximal dynamic muscle strength increased by 30% at 60°/s (P < 0.05) and by 29% at 180°/s (P < 0.05); muscle fiber area increased for type I (+17%, P < 0.05), type IIa (+37%, P < 0.05), and type IIx muscle fibers (+51%, P < 0.05); and muscle fiber pennation angle increased by 22% and muscle thickness increased by 15% (P < 0.05). Furthermore, stair walking power increased by 35% (P < 0.05) and was related to the increase in type II fiber area (r = 0.729, P < 0.05). In contrast, there was no increase in any measurement outcomes with electrical stimulation and standard rehabilitation.

The present study is the first to demonstrate the effectiveness of resistance training to induce beneficial qualitative changes in muscle fiber morphology and muscle architecture in elderly postoperative patients. In contrast, rehabilitation regimes based on functional exercises and neuromuscular electrical stimulation had no effect. The present data emphasize the importance of resistance training in future rehabilitation programs for elderly individuals.

aging; muscle power; exercise

Sarcopenia has long been recognized as a major cause of loss in muscle strength with old age. In fact, aging and disuse are two of the main conditions leading to skeletal muscle atrophy in humans. In both conditions, the loss of muscle mass leads to a decrease in muscle force production, and recent evidence suggests that a significant additional contribution might come from changes in muscle architecture (38, 39). However, only a few studies have examined changes in muscle architecture and muscle fiber morphology in response to aging and physical training, and although the results suggest that a significant plasticity exists, there is generally a lack of data as to what extent different conditioning stimuli may affect muscle architecture in elderly individuals recovering from hospitalization.

The loss of muscle mass with aging accelerates from the sixth decade onward, partly owing to a decreased number of muscle fibers and also as the result of general muscle fiber atrophy (32, 33). Although several cross-sectional studies indicate that type II fibers are more vulnerable to the aging process than type I fibers (4, 27, 29, 32), some find a more marked type I atrophy (13). In essence, the loss of muscle mass with aging is profound and has been estimated to decrease ∼30% during the life span (30, 33). Considering these morphological changes, it is not surprising that maximal muscle strength is reduced as a result of aging by ∼1.5% per year from the sixth decade (45).

In addition to the muscular changes pertaining to muscle fiber area, aging also leads to marked alterations in muscle architecture that potentially contribute to the loss of muscle strength (39). A reduction of 10–13% in muscle fiber pennation angle in old compared with young individuals has been demonstrated by Narici et al. (39), suggesting that a significant part of the decrease in muscle function with aging may be related to changes in muscle architecture. However, there is a paucity of data describing architectural adaptations to different types of loading, although understanding the impact of different training stimuli on muscle architecture in the elderly seems important to determine effective intervention programs to improve muscle function after chronic disuse and/or illness.

The most commonly used rehabilitation regimes for elderly individuals are based on functional types of exercises without external loading, although it has been demonstrated that this type of intervention cannot prevent further muscle atrophy (40) or restore muscle strength and functional performance in elderly postoperative patients (43, 44). Percutaneous neuromuscular electrical stimulation is another method used to restore muscular function after immobilization, although primarily investigated in young individuals (5, 16). During the last decades, resistance training has emerged as an effective method to induce muscle hypertrophy and increase muscle strength and functional performance in frail elderly (12, 21) and in patients with chronic diseases (10, 23, 26). Furthermore there is increasing evidence that resistance training used in the late postoperative phase is an effective method to restore muscle function in elderly patients (22, 34, 42). Despite this, resistance training is still rarely used in the rehabilitation of elderly patients and especially in the elderly who have been hospitalized.
Previously, we have studied the neuromuscular and functional changes, before and after unilateral resistance training, neuromuscular electrical stimulation, and a standard rehabilitation program in elderly patients recovering from hip replacement surgery (46, 47). These results indicated that resistance training is more effective to restore muscle mass, contractile rate of force development, and functional performance than rehabilitation regimes based on functional exercises and electrical stimulation (46, 47). However, although muscle architecture has been shown to be an important factor for muscle function in young individuals (1), no studies have previously investigated the changes in muscle fiber morphology and muscle architecture with different types of intervention in the elderly.

To describe more closely the potential interaction between muscle morphology, muscle architecture, and contractile capacity, the aim of the present study was therefore, in the same group of patients, to examine the relationship between muscle fiber area and muscle fiber pennation angle of the vastus lateralis (VL) muscle before and after the three intervention regimes: resistance training, electrical stimulation, and standard rehabilitation. It was hypothesized that resistance training would be more effective to increase muscle fiber pennation angle and muscle fiber area than standard rehabilitation and electrical stimulation and furthermore that these changes might be related to the potential gains in maximal voluntary contraction capacity and stair-climbing power. The present study is the first in which measurements of muscle fiber pennation angle, muscle fiber area, maximal dynamic strength, and stair-climbing power were combined to examine the specific adaptations to different training regimes in elderly individuals.

METHODS

Subjects and study design. Thirty-six elderly individuals, 18 women (age range 60–86 yr) and 18 men (age range 60–79 yr) volunteered to participate in the study. The subjects were scheduled for unilateral hip replacement surgery at Bispebjerg University Hospital, Copenhagen, Denmark due to hip osteoarthritis. Before the operation all subjects were randomized to one of three groups: 1) unilateral resistance training (RT; n = 13), 2) unilateral electrical stimulation of the quadriceps muscle (ES; n = 11), 3) standard rehabilitation (SR; n = 12). Randomization was performed by a computer program (Minimize version 2.1), and patients were stratified by age and sex. All three training regimes have been described in detail elsewhere (47). In brief, RT consisted of a 12-wk (3/wk) unilateral progressive training program (weeks 1–2: 3 × 10 (20 RM, where RM is repetition maximum); weeks 3–4: 3 × 12 (15 RM); weeks 5–6: 4 × 10 (12 RM); weeks 7–8: 5 × 8 (8 RM); weeks 9–10: 4 × 8 (8 RM); weeks 11–12: 3 × 8 (8 RM)) with focus on knee extension and leg press exercises. The ES group performed neuromuscular electrical stimulation of the quadriceps muscle of the operated limb 1 h/day (40 Hz). The SR group performed a rehabilitation program consisting of functional exercises with focus on improving mobility and strength without external loading. Measurements were performed 1 wk (±2–3 days) before the operation, 5 and 12 wk postsurgery, and the muscle biopsies were taken 2 days after surgery. The local Ethics Committee approved the conditions of the study, the experimental procedures were performed in accordance with the Declaration of Helsinki, and written informed consent was obtained. Measurements were performed bilaterally and were preceded by a familiarization trial conducted on a separate day. The nonaffected side was tested first to increase the subject’s comfort with the procedure. Strict care was taken to ensure identical test protocols for all subjects, which included standardized verbal encouragement and visual feedback provided by a real-time display of the force output (25). Successive trials were performed until peak moment could not be improved any further, which typically included seven to nine attempts at each velocity (2).

Muscle biopsy sampling and analyses. Bilateral muscle samples were obtained from the middle portion of the VL utilizing the percutaneous needle biopsy technique of Bergström (7) by the same investigator. Following intervention, efforts were made to extract tissue from the same depth and location (within ~1–2 cm). After being dissected of all visible blood, adipose, and connective tissue, the muscle samples were oriented in embedding medium (Tissue-Tek) frozen in isopentane cooled with liquid nitrogen and stored at −80°C. Subsequently, serial transverse sections (10 μm) were cut in a cryotome at −20°C and stained for myofibrillar ATPase at pH 9.4 after both alkaline (pH 10.3) and acid (pH 4.3 and 4.6) preincubations (9). All samples of each individual person were stained in the same batch to avoid interassay variation. Muscle fiber-type and cross-sectional area (CSA) analyses were conducted in a blinded fashion and an average of 397 ± 22 fibers were analyzed in each biopsy. On the basis of the ATPase staining pattern, muscle fibers were characterized as type I, I/IIa, IIa, IIx, and Ix (3). Because of a low number of type I/IIa and IIx fibers in some individuals, the individual analyses were collapsed into three fiber types, type I, type IIa, and type Ix, before the final statistical analyses were performed (3). The reduction in fiber type was based on the following equations: type I = I + I/IIa; type IIa = I/IIa + IIa + Ix; and type Ix = IIa + Ix. For the determination of muscle fiber size, only truly horizontally fibers were used; thus a restricted number of fibers (minimum of 50 fibers) were included for this analysis. A videoclip consisting of a microscope (Olympus BX 50) and color video camera (Sanyo high-resolution charge-coupled device) in combination with Tima Image-analysis System (Scanbeam Denmark) were used to calculate the mean fiber area values of each fiber type.

Muscle fiber pennation angle and muscle thickness. Sagittal ultrasound (UL) images of the quadriceps femoris muscle were recorded with a Siemens real-time scanner with a 7.5-MHz linear array transducer. Images were obtained in the seated position (90° flexion in the hip and knee joint) at 50% of femur length over the midbelly of the VL (1). To ensure the same scan position, traces were drawn on acetate paper, which was aligned relative to skin marks and anatomic landmarks. VL fiber pennation angle was measured as the angle between VL muscle fiber fascicles and the deep aponeurosis of the insertion (1) (Fig. 1). VL muscle thickness was obtained with the UL-transducer in the same position and measured as the distance between the deep and superficial aponeurosis of the VL muscle. Two images from each limb were obtained from each subject. Each image was evaluated three times and the mean value was recorded. The coefficient of variation between two consecutive measurements was <5%.

Stair walking power. Maximum stair walking power per kilogram body mass (W/kg) was calculated as the distance of vertical displacement of the body center mass times g (9.81 m/s²), i.e., the change in potential energy, divided by the fastest time of stair ascent. Each subject performed three trials, and the stairs consisted of 10 steps each with a height of 16.5 cm for a total vertical displacement of 1.65 m. Statistical analysis. Nonparametric statistics were used for the analyses, since not all data were normally distributed. To evaluate the effect of intervention over time, a Friedman test was used with post hoc Wilcoxon’s test. Any between-group differences were analyzed with Kruskal-Wallis tests and subsequent Mann-Whitney U-test. Spearman’s Rho was used for the correlation analysis on a limited
of RT this asymmetry was eliminated, in contrast to SR and ES. Muscle fiber CSA in the control leg did not change in any of the intervention groups ($P > 0.05$). At baseline, there was no difference between groups; however, the delta changes in type I muscle fiber CSA observed with 12 wk of RT and ES were greater than after 12 wk of SR ($RT > SR$ and $ES > SR$, $P < 0.05$), and the increases in type Ila muscle fiber CSA with 12 wk of RTs were greater than after 12 wk of ES and SR ($RT > ES$ and $RT > SR$, $P < 0.05$).

Changes in muscle fiber pennation angles and muscle thickness. Muscle architecture was altered following 12 wk of RT training as reflected by a 22% increase in VL muscle fiber pennation angle ($7.2 \pm 0.5 to 8.6 \pm 0.6^\circ$, $P < 0.05$), which was contrasted by a 11% decrease following SR ($7.6 \pm 0.3 to 6.7 \pm 0.2^\circ$, $P < 0.05$). No change was observed with ES (Fig. 3). At baseline, there was no difference between groups; however, the delta changes with RT were greater than those observed with SR and ES both at 5 wk ($RT > ES$, $P < 0.05$ and $RT > SR$, $P < 0.05$) and at 12 wk ($RT > ES$ and $RT > SR$, $P < 0.05$). Muscle thickness of the VL muscle increased by 14.8% after 12 wk of RT ($15.3 \pm 1.3 to 17.5 \pm 1.6$ mm, $P < 0.05$), whereas there was no increase with ES or SR (Fig. 4). Moreover, the delta changes with 12 wk of RT was greater than those observed with SR ($RT > SR$, $P < 0.05$).

Stair walking power. Maximum stair walking power (W/kg) increased after 12 wk of RT ($2.6 \pm 0.4$ W/kg to $3.5 \pm 0.4$ W/kg, $P < 0.05$) and 12 wk of ES ($2.6 \pm 0.3$ W/kg to $3.4 \pm 0.4$ W/kg, $P < 0.05$) but not after 12 wk of SR ($2.2 \pm 0.3$ W/kg to $2.5 \pm 0.2$ W/kg). Furthermore, the delta changes with RT were greater than those observed with SR at 12 wk ($RT > SR$, $P < 0.05$), but there was no difference between ES and SR ($P = 0.495$).

Correlation analyses. The relative change in VL muscle fiber pennation angle was related to the individual change in dynamic torque at both contraction velocities (60°/s: $r = 0.619$, 180°/s: $r = 0.530$, $P < 0.05$), to the change in total mean fiber area ($r = 0.429$, $P < 0.05$), and to the change in VL muscle thickness ($r = 0.479$, $P < 0.05$). The initial delta change in type II muscle fiber area after 12 wk of RT was related to the delta change in stair walking power ($r = 0.729$, $P < 0.05$). Furthermore, the increase in muscle fiber pennation angle after 12 wk of RT was strongly related to the increase in muscle thickness ($r = 0.733$, $P < 0.05$).

DISCUSSION

Although immobilization and hospitalization are more frequent in old age and lead to an increased risk of disability (24),

Table 1. Anthropometric data

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<th>RT</th>
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<tr>
<td>$n$</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>ns</td>
</tr>
<tr>
<td>Age, yr</td>
<td>71 (61–86)</td>
<td>69 (60–75)</td>
<td>69 (62–78)</td>
<td>ns</td>
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<tr>
<td>Sex</td>
<td>6 W/4 M</td>
<td>5 W/5 M</td>
<td>4 W/4 M</td>
<td>ns</td>
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<tr>
<td>Body weight, kg</td>
<td>76.7±5.7</td>
<td>79.9±4.6</td>
<td>86.8±6.0</td>
<td>ns</td>
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<tr>
<td>Height, cm</td>
<td>167.7±2.6</td>
<td>168.3±3.1</td>
<td>170.5±2.4</td>
<td>ns</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.9±1.7</td>
<td>28.0±1.0</td>
<td>29.4±1.6</td>
<td>ns</td>
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Values are means ± SE (range); $n$, no. of subjects. No significant differences were observed between groups at inclusion time (ns). RT, resistance training; ES, electrical stimulation; SR, standard rehabilitation; W, women; M, men; BMI, body mass index.
the effects of different types of loading on qualitative changes in muscle architecture and muscle morphology has not previously been investigated in elderly postoperative patients. The present study is the first to simultaneously measure maximal contractile muscle function, muscle fiber morphology, muscle architecture, and functional performance with different training modalities (resistance training, electrical stimulation, or standard rehabilitation) in elderly individuals recovering from hip surgery. The main finding was that 12 wk of resistance training led to substantial increases in maximal contractile muscle strength that were accompanied by gains in both type I and II single muscle fiber CSA and gains in VL muscle fiber pennation angle and stair walking power. Notably, in contrast to resistance training, no changes occurred in these parameters following the most commonly employed types of training to elderly patients, i.e., a rehabilitation program based on functional exercises (standard rehabilitation) or neuromuscular electrical stimulation.

Although the subjects in the present study were rather frail, especially the first 4–6 wk after surgery, maximal dynamic muscle strength increased by ~30% (Table 2) in response to 12 wk of resistance training. Similar gains in muscle strength have been demonstrated following resistance training in healthy elderly individuals (18, 31, 41) and recently in frail elderly (8, 27). In contrast, electrical stimulation maintained the preoperative level of muscle strength, which is in line with previous findings in young patients after anterior cruciate ligament reconstruction (5, 16). Notably, the standard rehabilitation regime did not result in any increases in maximal dynamic muscle strength, which is in accordance with studies that have evaluated the effect of physiotherapy exercises after hip surgery (44, 48). Importantly, although all three regimes were commenced already 1–2 days after surgery, there were no training-related complications in any of the groups.

Although average muscle fiber area increased by 32% following 12 wk of RT, more pronounced gains in fiber CSA were seen for the type IIa (+37%) and IIx (+51%) fibers compared with that of the type I fibers (+17%), indicating a more marked hypertrophy of the type II fibers. This is in agreement with previous studies in young (1) and old individuals (27), although not consistently shown (17, 31). Furthermore, the pronounced increase in type II fiber CSA seen with RT compensated for the preoperative difference in type I and II fiber CSA (II < 1), which disappeared following RT. In contrast, muscle fiber area remained unaffected by ES or SR. Importantly, the present data demonstrates that changes in type II fiber CSA with RT translated into an improved stair walking power, which is an important functional adaptation (6, 28). Interestingly, the delta changes in type I muscle fiber CSA were significantly larger after both RT and ES than after SR (RT > SR and ES > SR, P > 0.05), whereas RT was the most effective intervention to induce changes in type III muscle fiber CSA (RT > ES and RT > SR, P > 0.05). These findings are in line with previous studies indicating that ES mainly leads to hypertrophy of type I fibers (15, 20) whereas RT effectively induce hypertrophy of type II fibers (1, 27). Furthermore, the present data demonstrate that the preoperative side-to-side difference in muscle fiber CSA (type I and type IIa) was eliminated after 12 wk of RT, in contrast to ES and SR.

In both sarcopenia and disuse atrophy, muscle fiber fascicles seem to have a reduced pennation angle compared with healthy young individuals, likely because of decreased amounts of contractile tissue (35, 37). In agreement with these findings, muscle fiber pennation angles on the osteoarthritic side were significantly smaller compared with the healthy side (control leg) in the present study. However, the muscle tissue of old individuals also shows a remarkable plasticity with resistance training. After 12 wk of RT there was a 22% increase in VL muscle fiber pennation angle (Fig. 3), which was comparable to that seen in both young and old individuals after a period of resistance training (1, 36). That is contrasted by the lack of change in muscle fiber pennation angle for the elderly individuals subjected to ES training or SR. Interestingly, there was a positive relationship between the training-induced change in VL muscle fiber pennation angle and the corresponding changes in dynamic torque at slow-to-fast contraction velocities (60°/s: r = 0.619, 180°/s: r = 0.530, P < 0.05), emphasizing the importance of muscle architecture for the contractile function of the muscle. Moreover, the individual delta changes in muscle fiber pennation angle was positive related to the

### Table 2. Changes in dynamic muscle strength normalized to body weight

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<tr>
<td>Peak torque (N·m·kg⁻¹)</td>
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<tr>
<td>Pre, 60°/s</td>
<td>1.31±0.15</td>
<td>1.64±0.11</td>
<td>1.28±0.12</td>
<td>1.48±0.10</td>
<td>1.22±0.11</td>
<td>1.70±0.13</td>
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<tr>
<td>5 wk</td>
<td>1.34±0.14†</td>
<td>1.67±0.12</td>
<td>1.16±0.13</td>
<td>1.42±0.10</td>
<td>1.08±0.08</td>
<td>1.53±0.12</td>
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<tr>
<td>12 wk</td>
<td>1.64±0.15†</td>
<td>1.64±0.12</td>
<td>1.25±0.13</td>
<td>1.42±0.11</td>
<td>1.16±0.10</td>
<td>1.66±0.13</td>
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<td>Peak torque (N·m·kg⁻¹)</td>
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<tr>
<td>Pre, 180°/s</td>
<td>0.88±0.10</td>
<td>1.08±0.09</td>
<td>0.84±0.09</td>
<td>0.98±0.08</td>
<td>0.96±0.07</td>
<td>1.14±0.09</td>
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<tr>
<td>5 wk</td>
<td>0.89±0.08</td>
<td>1.09±0.09</td>
<td>0.83±0.10</td>
<td>0.98±0.08</td>
<td>0.85±0.06</td>
<td>1.15±0.10</td>
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<tr>
<td>12 wk</td>
<td>1.07±0.09†</td>
<td>1.08±0.09</td>
<td>0.88±0.10</td>
<td>0.97±0.08</td>
<td>0.93±0.07</td>
<td>1.16±0.09</td>
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</table>

Isokinetic quadriceps strength measurements normalized to body weight. Data are presented from both sides, the operated side (OP) and the control side (CO), from all three intervention groups at baseline (Pre), 5 wk after the operation (5 wk), and 12 wk after the operation (12 wk). The 3 intervention groups are RT, ES, and SR. Values are means ± SE. *P < 0.05 significantly different from baseline. †P < 0.05 refers to intergroup differences, RT being significantly different from SR and ES.
changes in VL muscle thickness (r = 0.479, P < 0.05), which was further emphasized looking separately at the RT group (r = 0.733, P < 0.05). Notably, there was no relation between the changes in muscle thickness and the anatomic CSA (ACSA) measured by computed tomography scan (P > 0.05), yet, the changes in ACSA was positively related to the delta changes in muscle fiber pennation angle (r = 0.600, P < 0.05).

In line with previous data in young individuals a positive relationship was observed between the changes in ACSA and the individual changes in type I muscle fiber CSA.
In the present study, we found a mismatch between the increase in muscle fiber CSA (+32%) and the gain in muscle thickness (+14%) or as previously reported in ACSA (+12%) after 12 wk of resistance training (47). Similar findings of a mismatch between the gains in ACSA and muscle fiber CSA after a period of resistance training has previously been found in both young (1) and old individuals (21, 44, 19). The observed increase in muscle fiber pennation angle (+22%) in the present study indicates the importance of muscle architecture to explain for some of this mismatch, since a steeper muscle fiber pennation angle allows for a larger physiological fiber area for a given muscle volume and it should therefore be recognized that ACSA may not be a very representative measure of changes in the physiologica Cla (1).

In conclusion, the present study demonstrated that resistance training offers an effective way of increasing maximal muscle strength in elderly postoperative patients. Importantly, the increase in muscle fiber size was accompanied by gains in muscle fiber size and pennation angle that resemble that typically seen in young healthy individuals. In contrast, these positive adaptations were not achieved by daily neuromuscular resistive exercises in future rehabilitation programs for elderly (SR). Thus the present data emphasize the importance of using positive adaptations to increase in muscle function was accompanied by gains in strength in elderly postoperative patients. Importantly, the present study indicates the importance of muscle architecture to explain for some of this mismatch, since a steeper muscle fiber pennation angle allows for a larger physiological fiber area for a given muscle volume and it should therefore be recognized that ACSA may not be a very representative measure of changes in the physiological CSA (1).

ACKNOWLEDGMENTS

We greatly acknowledge the patients who volunteered to participate in this study. The study was supported by the IMK Foundation, The Danish Rheumatology Association and the Maraes McKinley Moller Foundation.

REFERENCES


Coordinated increase in skeletal muscle fiber area and expression of IGF-I with resistance exercise in elderly post-operative patients

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A B S T R A C T

Hypertrophy of developing skeletal muscle involves stimulation by insulin-like growth factor-I (IGF-I), however, the role of IGF-I in adult muscle is less clarified. In the present study, the mRNA splice variants of IGF-I (IGF-Iea and MGF) and the changes in muscle fiber cross sectional area after 12 weeks of training were studied in elderly post-operative patients. About 28 subjects, 14 men and 14 women (age 69, range 60–86 years) were randomized to unilateral resistance training (RT: 3/week), electrical stimulation (ES: 1 h/day) or functional exercises (FE: 1 h/day). The non-operated-side served as a within subject control. Muscle biopsies were obtained from the vastus lateralis of both limbs at +2d post-operative (baseline), at 5 weeks and 12 weeks post-surgery to analyze for changes in type 1 and type 2 muscle fiber area. Changes in expression levels of IGF-I mRNA isoforms were determined using real-time RT-PCR, normalized to the ribosomal protein large protein 0 (RPLP0) mRNA and presented relative to the control-side. At baseline there was no difference between the three groups in muscle fiber area or resting levels of IGF-Iea and MGF. RT resulted in a significant increase in muscle fiber area of type 1 (+17%, p < 0.05) and type 2 (+36%, p < 0.05) parallel to an increase in the expression of IGF-Iea and MGF, in contrast to ES and FE. The present study demonstrates that resistance training initiated in the acute post-operative phase is highly effective in increasing mean fiber area and in addition induces marked increases in the expression of IGF-I splice variants, supporting the idea that IGF-I is involved in regulating muscle hypertrophy.

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1. Introduction

The consequences of the age-related loss of muscle mass (sarcopenia) are potentially grave and have been related to loss of muscle function and an increased risk of disability and mortality [1,2]. Although several conditions have been linked to this syndrome, the underlying mechanisms and the potential reversibility is still far from clear.

A part from the mechanical properties, the muscle tissue has another important role, in that it represents the largest protein store in the body which is required in catabolic situations such as critical illness or surgery [3]. Importantly, there are indications, that the muscle tissue of old animals demonstrates an attenuated response to recover after immobilization and injury [4,5]. Thus, it has been suggested that sarcopenia may in part be due to failure to generate an isoform of IGF-I that is necessary to initiate the remodeling of muscle by stimulating satellite cell activation and proliferation [6]. This knowledge becomes very important considering the vast amount of elderly individuals who undergo a period of critical illness or surgery and it therefore seems paramount to gain knowledge about which type of muscle overload is the most effective to restore muscle mass in these elderly individuals.

The GH-IGF-I axis is regarded to play a key role in the regulation of postnatal muscle growth and development [7] and there is good evidence that IGF-I is an important factor in the hypertrophic adaptation of muscle to resistance exercise especially in developing muscles of animals [8,9]. Two isoforms of the IGF-I mRNA are expressed by skeletal muscle cells when subjected to mechanical stimulation, although the two splice variants seems to be affected in different ways by mechanical loading [10,11]. One of them, IGF-Iea, is similar to the isoforms produced by the liver and is thought to have an endocrine function being expressed both in resting and working muscle [10]. Interestingly, it has been shown that muscles from transgenic mice that over-express this isoform have a marked muscle hypertrophy and further, in old mice signs of protection against development of sarcopenia was demonstrated.
The expression of IGF-I mRNA has been more consistent at both the mRNA level [19] and the protein level [20]. However, the number of studies evaluating the effect of prolonged resistance training on IGF-I mRNA expression and protein levels are sparse, especially in elderly individuals [19,20]. This is contrasted by the fact that elderly individuals are more often hospitalized and it therefore seems paramount to gain more knowledge about the cellular responses to different types of exercises, in order to provide optimal rehabilitation regimes to elderly individuals. Previously, we have studied the neuromuscular and functional adaptations to three different training regimes based on functional exercises, electrical muscle stimulation and resistance training in elderly post-operative individuals and these results indicated that resistance training is more effective to restore muscle function and muscle mass than rehabilitation regimes based on functional exercises or muscle stimulation [21,22]. However, to our knowledge, no study has previously investigated the expression level of IGF-Ia and MGF in combination with changes in muscle fiber CSA as a result of different types of muscle over-loading in old individuals.

The aim of the present study was therefore, in order to describe the potential interaction between changes in muscle morphology and IGF-I splice variants in elderly frail patients, to examine changes in muscle fiber area and expression levels of IGF-Ia and MGF mRNA in the same group of patients before and after the three intervention regimes. We put out the hypothesis that potential increases in the expression of IGF-I splice variants would be related to changes in muscle morphology and that resistance training would be a more potent stimulus to increase the expression levels of IGF-Ia and MGF mRNA than exercise regimes based on functional exercises or electrical muscle stimulation in post-operative elderly individuals.

2. Methods
2.1. Subjects and study design

Subjects were scheduled for unilateral hip-replacement surgery at Bispebjerg University Hospital, Copenhagen, Denmark due to hip osteoarthritis. The study was part of a larger study and a subpopulation of these subjects, 14 women (W: age 70, range 60–85 years) and 14 men (M: age 69, range 60–79 years) volunteered to undergo the muscle biopsy procedure. Before the operation subjects were randomized (stratified for age and gender) to one of three groups: (1) unilateral resistance training (RT, n = 10), (2) unilateral electrical stimulation of the quadriceps muscle (ES, n = 10), (3) functional exercises (FE, n = 8). All three training regimes have been described in detail elsewhere [21,23]. In brief, resistance training (RT) consisted of a 12 week (3/week) unilateral progressive training-program (week 1–2: 3 × 10 [20RM], week 3–4: 3 × 12 [15RM], week 5–6: 4 × 10 [12RM], week 7–8: 5 × 8 [8RM], week 9–10: 4 × 8 [8RM], week 11–12: 3 × 8 [8RM]) with focus on knee-extension and leg-press exercises. The ES group was subjected to neuromuscular electrical stimulation (NMES) of the quadriceps muscle of the operated limb 1 h/day (40 Hz). The aim was stimulate as much of the muscle as possible by placing the electrodes with as much distance as possible (from right below the groin to 2 cm above patella). The FE group performed a rehabilitation program consisting of functional exercises with focus on improving mobility and strength without external loading. Muscle biopsies were obtained from vastus lateralis of both legs, in order for the non-operated-side to serve as an internal control. Biopsies were obtained at three time points, at 48 h post-surgery (baseline), at 5 weeks and 12 weeks post-surgery (48 h after the last training bout). The 48 h post-surgery time-point was chosen in order to have a “baseline” measure from the beginning of the training intervention. Biopsies were analyzed for changes in muscle fiber area of types 1 and 2 fibers, and expression levels of IGF-Ia and MGF mRNA. The local Ethics Committee approved the conditions of the study and the experimental procedures were performed in accordance with the Declaration of Helsinki.

2.2. Muscle biopsy sampling and analyses

Bilateral muscle samples were obtained from the middle portion of M. vastus lateralis utilizing the percutaneous needle biopsy technique of Bergström [24] by a single investigator. Following intervention, efforts were made to extract tissue from the same depth and location (within ~1–2 cm). After dissecting the muscle samples of all visible blood, adipose and connective tissue, the muscle samples were oriented in embedding medium (Tissue Tec) frozen in isopentane cooled with liquid nitrogen and stored at −80°C. Subsequently serial transverse sections (10 μm) were cut in a cryotome at −20°C and stained for myofibrillar ATPase at pH 9.4 after both alkaline (pH 10.3) and acid (pH 4.3 and 4.6) preincubations [25]. All samples of each individual person were stained in the same batch to avoid interassay variation. Based on the ATPase staining pattern muscle fibers were characterized as types I and II and an average of 397 ± 22 fibers were analyzed in each biopsy. For the determination of muscle fiber size only truly horizontally fibers were used, with a minimum of 50 fibers included for the analysis. A videoscope consisting of a microscope (Olympus BX 50) and color video camera (Sanyo high resolution CCD) in combination with Tema Image-analyses System (Scanbeam, Denmark) were used to calculate the mean fiber area of the muscle fibers.

2.3. RNA purification

Total RNA was isolated from muscle biopsies by phenol extraction (TriReagent; Molecular Research Center, OH, USA) as previously described [26]. Intact RNA was confirmed by denaturing agarose gel electrophoresis.

2.4. Real-time RT-PCR

The mRNA expression of IGF-Ia, MGF, and GADPH was analyzed by real-time RT-PCR. Total RNA (500 ng) was converted into cDNA in 20 μl using the OmniScript reverse transcriptase (Qiagen, CA, USA) according to the manufacturer’s protocol. For each target mRNA, 0.25 μl cDNA was amplified in a 25 μl SYBR Green PCR reaction containing 1× Quantitect SYBR Green Master Mix (Qiagen) and 100 nm of each primer (Table 1). The amplification was monitored real-time using the MX3000P real-time PCR machine (Stratagene, CA, USA). The threshold cycle (Ct) values were related to a standard curve made with the cloned PCR products and...
specificity ensured by melting curves analysis. The quantities were normalized to the RPLP0 mRNA \cite{27}. To test the stability of the "housekeeping" gene (RPLP0), another "housekeeping" gene GAPDH was measured and normalized to RPLP0 (Fig. 1). Data are presented in absolute levels normalized to RPLP0 (Figs. 2 and 3) as well as in fold changes relative to the CO-side (Figs. 4 and 5).

2.5. Statistics

All data from the mRNA analyses were log-transformed before statistical analyses and are presented as geometric means ± back-transformed SEM. A two-way ANOVA on intervention × time was performed using SAS procedure mixed with autoregressive modeling. Non-parametric statistics were used for the analyses of changes in muscle fiber CSA, since not all of these data were normally distributed. To evaluate the effect of intervention over time a Friedman test was used with post hoc Wilcoxon test. Any between-group differences were analyzed with Kruskal–Wallis tests and subsequent Mann–Whitney U test. Data are presented as mean values ± SEM. A p-value of less than 0.05 was considered significant.

3. Results

3.1. Normalization of mRNA data

To validate the use of RPLP0 as internal reference for the mRNA, data were normalized to GADPH, which proved to be constitutively expressed. There were no difference in GADPH resting levels between the three groups and there were no changes with any of the interventions (Fig. 1).

3.2. IGF-I\textsubscript{Ea}

At baseline, there was no difference in the resting levels of IGF-I\textsubscript{Ea} between the three groups or between the two sides (CO vs. OP) expressed in absolute values (Fig. 2) or when OP was expressed relative to CO (Fig. 3). RT resulted in a significant increase in absolute levels of IGF-I\textsubscript{Ea} after 5 weeks (5w) and 12 weeks (12w) (Fig. 2) and relative to the CO-side after 12 weeks (Fig. 3). In contrast, 12 weeks of ES and

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
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<tr>
<td>IGF-I\textsubscript{Ea}</td>
<td>GACATGCCCAAGACCCAGAAGGA</td>
<td>CGGTGGCATGTCACTCTTCACTC</td>
</tr>
<tr>
<td>MGF</td>
<td>GCCCCCATCTCCACAAACAAGAACAC</td>
<td>CGGTGGCATGTCACTCTTCACTC</td>
</tr>
<tr>
<td>RPLP0</td>
<td>GAAACTCTCGATCTTTCTCTCTCTC</td>
<td>CAGAGAATGTTCCTACCGCCCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCTCCTGCACCACCAACTGCTT</td>
<td>GAGGGGCCATCCACAGTCTTCTC</td>
</tr>
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</table>

IGF-I\textsubscript{Ea}, insulin-like growth factor-I isoform Ea; MGF, mechano-growth-factor. RPLP0, large ribosomal protein P0; GAPDH.

Fig. 1. GAPDH mRNA expression was normalized to RPLP0 mRNA expression to validate the use of RPLP0 as internal reference for the mRNA. There were no difference in GADPH resting levels between the three groups and there were no changes with any of the interventions.

Fig. 2. Insulin-like growth factor-I isoform Ea (IGF-I\textsubscript{Ea}) mRNA normalized to RPLP0 mRNA and presented in absolute values. At baseline, there was no difference in the resting levels of IGF-I\textsubscript{Ea} between the three interventions (functional exercises, FE; electrical simulation, ES; and resistance training, RT) or between the two sides (CO vs. OP). RT resulted in a significant increase in absolute levels of IGF-I\textsubscript{Ea} after 5 weeks (5w) and 12 weeks (12w). * denotes significantly different from baseline, # denotes RT-OP significantly different from FE-OP.
FE did not induce any change in the expression of IGF-IEa mRNA levels (Figs. 2 and 3).

3.3. Mgf

The regulation pattern for MGF mRNA was somewhat different from that of IGF-IEa. At baseline (day 2 post-operative) there was no difference between the three intervention groups or the two sides (Figs. 4 and 5). However, after 5 and 12 weeks there was a significant down-regulation of MGF mRNA levels with ES and FE (Fig. 5). In contrast, MGF mRNA levels did not decrease with RT, and the expression of MGF mRNA after 12 weeks of RT was significantly increased compared to FE and ES ($p < 0.05$, Figs. 4 and 5).
3.4. Muscle fiber cross sectional area

There was no difference in muscle fiber cross sectional area between the three intervention groups at baseline (Table 2). However, after 12 weeks of RT there was a significant increase in the cross sectional area of type 1 muscle fibers (+17%, \( p < 0.05 \)) and type 2 fibers (+17%, \( p < 0.05 \)). In contrast, there was no change in FE or ES after 12 weeks (Table 2). Further, there was a significantly difference between RT and FE in the relative changes in both fiber types after 12 weeks of intervention (\( p < 0.05 \)).

4. Discussion

The present study is, to our knowledge, the first to obtain simultaneous measurements of muscle fiber size and the mRNA expression of the IGF-I splice variants IGF-Iεa and MGF in order to address the IGF-I and muscle fiber morphology coupling with different training modalities (resistance training (RT), electrical stimulation (ES), or functional exercises (FE)) in elderly individuals recovering from surgery. The main findings were that prolonged resistance training after surgery led to a substantial hypertrophy in both type 1 and 2 muscle fibers (Table 2) and that these adaptations were accompanied by gains in mRNA expression of the IGF-I splice variants IGF-Iεa and MGF, supporting the idea that IGF-I is playing an important role in regulating muscle hypertrophy in elderly humans. Notably, in contrast to RT, no changes occurred in these parameters following the most commonly employed types of training to elderly patients, i.e. a standard rehabilitation program based on functional exercises (FE) or neuromuscular electric stimulation (ES).

Although, there is a potential impairment in growth factors with aging, resistive types of exercise has emerged as an effective method to induce muscle hypertrophy and increase muscle strength and functional performance in frail elderly [28,29] as well as in patients with chronic diseases [30–32]. Furthermore, it has been demonstrated that resistance training is an effective method to restore muscle function in elderly post-operative patients [21,23,33,34]. However, the most commonly used rehabilitation regimes for elderly individuals are still based on functional types of exercises without external loading, although it has been demonstrated that this type of quite moderate intervention cannot prevent further muscle atrophy [21,35] or fully restore muscle strength and functional performance in elderly post-operative patients [36,37]. These results are further supported by data from the present study demonstrating that 12 weeks of rehabilitation based on functional exercises, in contrast to resistance exercises, do not lead to an increase in myofiber CSA or in the expression of IGF-I mRNA. In animal studies, electrical muscle stimulation is often used as an analog to resistance training [38,39], the stimulation intensity is, however, not comparable to the intensity that can be logistically applied to humans, which might explain the sparse effects on muscle fiber CSA and IGF-I expression with this method in the present study.

It is known that IGF-I has an anabolic action and increases rates of protein synthesis in muscle [40]. In human exercise studies, the expression of IGF-I mRNA has been found to increase after a single bout of resistance exercise [15,16], but not in all studies [16,18]. After prolonged periods of resistance training the results have been more consistent demonstrating an increase for IGF-I at both the mRNA level [19,27,41] and the protein level [20]. However, the activation of satellite cells is also required for hypertrophied fibers to maintain their DNA to protein ratios [42] and it seems that MGF may play a role in the activation of satellite cells [11], thus the two isoforms act in tandem to promote muscle growth and repair.

BTG involved in muscle repair it therefore does not seem surprising that MGF mRNA levels in all three groups are elevated compared to the non-operated-side 48 h post-surgery. However, in contrast to ES and FE MGF mRNA expression levels do not decrease in the RT group, which could support the hypothesis that MGF is involved in both muscle repair (baseline) and hypertrophy (5 and 12 weeks).

Furthermore, the suggestion that the two isoforms are regulated differently, with IGF-Iεa being GH responsive and MGF appearing relatively insensitive to GH would make sense as these isoforms have been reported to play different roles for the satellite cells [11]. In the present study, absolute levels of IGF-Iεa mRNA were low in all three groups with no side-to-side difference at baseline (Fig. 3) and interestingly, there was only observed an increase in the OP-leg that performed RT at 5 and 12 weeks. This finding was in contrast to FE and ES as well as the control legs in all three groups (Figs. 2 and 3). In line with these results, absolute levels of MGF mRNA were also significantly higher with RT at 12 weeks, which was not observed with FE and ES (Fig. 5).

Further, changes in MGF mRNA expressed as fold changes relative to baseline (CON) demonstrated an up-regulation at baseline (48 h post-operative) in all three groups, which in contrast to FE and ES remained up-regulated with RT. The up-regulation of MGF mRNA 48 h after surgery in all three groups supports the hypothesis that MGF is involved in muscle repair and in the initiating phase of muscle hypertrophy by kick-starting the satellite cells to proliferate [11,13,14]. In contrast to the expression level of MGF there was no increase in the IGF-Iεa mRNA expression level at baseline, however, after 5 and 12 weeks of resistance exercise there was a marked increase in the expression of IGF-Iεa mRNA, supporting the idea that the IGF-Iεa splice variant seems to be more involved in the later phase of muscle hypertrophy by

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>FE</th>
<th>ES</th>
<th>RT</th>
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<tbody>
<tr>
<td><strong>Type 1 (µm²)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>4221 ± 610</td>
<td>4846 ± 501</td>
<td>3688 ± 575</td>
</tr>
<tr>
<td>5w</td>
<td>3613 ± 354</td>
<td>4294 ± 432</td>
<td>3210 ± 155</td>
</tr>
<tr>
<td>12w</td>
<td>4099 ± 438</td>
<td>4207 ± 334</td>
<td>3577 ± 199</td>
</tr>
<tr>
<td><strong>Type 2 (µm²)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>3505 ± 654</td>
<td>3424 ± 612</td>
<td>2784 ± 538</td>
</tr>
<tr>
<td>5w</td>
<td>3054 ± 546</td>
<td>3684 ± 470</td>
<td>2352 ± 347</td>
</tr>
<tr>
<td>12w</td>
<td>3315 ± 612</td>
<td>3891 ± 443</td>
<td>2655 ± 364</td>
</tr>
</tbody>
</table>

Measurements of single muscle fiber area from all three intervention groups, functional exercises (FE), electrical muscle stimulation (ES) and resistance training (RT). Data are presented from both sides, the operated-side (OP) and the control-side (CON) from at baseline (PRE), at 5 weeks after the operation (5w) and 12 weeks after the operation (12w). Data are presented as means ± SEM, \( p < 0.05 \) significantly different from baseline. \( \dagger \) \( p < 0.05 \) refers to intergroup differences, RT being significantly different from FE and ES.
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Acknowledgements

We greatly acknowledge the patients who volunteered to participate in this study. The study was supported by grants from the Danish Medical Research Council, The Nordea Foundation, IMK Foundation, The Danish Rheumatism Association and the Maersk McKinney Moller Foundation.
Muscle size, neuromuscular activation, and rapid force characteristics in elderly men and women: effects of unilateral long-term disuse due to hip-osteoarthritis

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Muscle size, neuromuscular activation, and rapid force characteristics in elderly men and women: effects of unilateral long-term disuse due to hip-osteoarthritis

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Suëtta C, Aagaard P, Magnusson SP, Andersen LL, Sipilä S, Rosted A, Jakobsen AK, Duus B, Kjaer M. Muscle size, neuromuscular activation, and rapid force characteristics in elderly men (M: 60–86 yr; n = 19) and women (W: 60–86 yr; n = 20) with unilateral chronic hip-osteoarthritis. Both sides were examined to compare the effect of long-term decreased activity on the affected (AF) leg with the unaffected (UN) side. AF had a significant lower MVC (W: 20%; M: 20%), LCSA (W: 8%; M: 10%), contractile RFD (W: 17–26%; M: 15–24%), impulse (W: 10–19%; M: 19–20%), maximal EMG amplitude (W: 22–25%; M: 22–28%), and an increased muscle activation deficit (~18%) compared with UN. Furthermore, women were less strong (AF: 40%; UN: 39%), had less muscle mass (AF: 33%; UN: 34%), and had a lower RFD (AF: 38–50%; UN: 41–48%) compared with men. Similarly, maximum EMG amplitude was smaller for both agonists (AF: 51–63%; UN: 35–61%) and antagonist (AF: 49–64%; UN: 36–56%) muscles in women compared with men. However, when MVC and RFD were normalized to LCSA, there were no differences between genders. The present data demonstrate that disuse leads to a marked loss of muscle strength and muscle mass in elderly individuals. Furthermore, the data indicate that neuromuscular activation and contractile RFD are more affected by long-term disuse than maximal muscle strength, which may increase the future risk for falls.

aging; rate of force development; neural activity; muscle activation

IT IS WELL KNOWN FROM ANIMAL and human studies that a chronic reduction in neuromuscular activity results in marked muscle atrophy (4, 28), reduced muscle strength (2, 33), and diminished neural drive to muscle fibers (9). For people older than 45 yr of age, >33% suffer from joint pain, and osteoarthritis (OA) has been shown to be the most common cause of inactivity and long-term disability in people aged ≥65 yr (12). However, most of the current knowledge with respect to the effects of inactivity and immobilization on neuromuscular function is based on animal data (2, 8) or on studies performed on healthy young individuals (4, 28). This is contrasted by the fact that the elderly population more often undergoes periods of immobilization and disuse, not only due to joint pain, but also due to a higher degree of comorbidity and hospitalization (32). Studies in healthy young adults have demonstrated that immobilization leads to rapid decreases in maximal muscle strength, muscle mass, and neural activation (4, 19); however, recent studies indicate that skeletal muscle in aged animals and humans is more vulnerable to muscle unloading than that in young individuals (8, 41). Furthermore, recent data from Yasuda et al. (43) show that there might be a gender-specific response to unloading, as evidenced by a more pronounced decrease in specific strength in young women compared with young men after 14 days of unilateral limb immobilization. Yet the lack of investigations into the effect of unloading or disuse in elderly humans makes it difficult to distinguish the extent with which reductions in muscle mass or reduced physical activity level are responsible for the observed decrease in muscle force production with aging.

With increasing age, human skeletal muscle morphology and function decay, which is dramatically evident by the sixth decade and onward (20, 27). This deterioration is known to be caused to a great extent by morphological changes, like decreased muscle mass, both due to a loss of muscle fibers and a decrease in the individual muscle fiber size (29). However, studies comparing groups of young and old human subjects indicate that the loss in muscle force cannot be entirely explained by these quantitative changes (35, 42). Accordingly, it has been suggested that the loss of muscle strength in aging may exceed that of the morphological changes, resulting in a decreased muscle quality in the elderly (7, 42), although it has not been a universal finding (17). In addition, aging is also associated with neurological changes, which affects maximum voluntary force production (34), as well as the capacity for rapid muscle force production; i.e., contractile rate of force development (RFD) (42). The ability to develop force rapidly (i.e., RFD) seems to be an important muscle mechanical performance parameter in aging subjects in several tasks of

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daily life, such as walking and attempting to avoid falls (13, 39). At the same time, reduced muscle strength in older people, for example after a period of immobilization or disuse, may be associated with muscle atrophy (6), a lowered ability to produce force rapidly, and thereby an increased risk of falling (13).

Reduced contractile RFD has been demonstrated in elderly compared with young individuals of both genders (5, 42). However, to what extent reductions in muscle mass or size, suppression in voluntary muscle activation, elevated coactivation of antagonist muscles, or a general reduction in the physical activity level is responsible for the decrease in muscle strength in the elderly remains unclear. It, therefore, appears of paramount importance to gain a better understanding of how prolonged disuse affects mechanical muscle characteristics and neuromuscular activation in the elderly and, furthermore, try to ascertain what effects can be attributed to activity level and the aging process, respectively. In the present study, individuals who suffered from diagnosed hip OA, with symptoms lasting >1 yr, were studied. The fact that OA was unilateral allowed for a comparison between the affected (AF) lower limb and the contralateral unaffected (UN) side.

The aim of the present study was to investigate the side-to-side difference in maximal muscle strength, muscle size, rapid force characteristics, and neuromuscular activation in elderly individuals with long-term unilateral OA. It was hypothesized that maximal muscle strength and muscle mass would be reduced on the AF side compared with the UN side, and furthermore that rapid force characteristics and neuromuscular activation parameters would be affected by the chronic disuse to a greater extent than muscle mass and strength.

METHODS

Subjects. Thirty-nine elderly individuals, 20 women (W; age range 60–86 yr) and 19 men (M; age range 60–79 yr), volunteered to participate in the study. Eligibility criteria included age >60 yr and primary unilateral hip-OA clinical and radiological, verified according to Kellgren and Lawrence grade >2 (36). All subjects had symptoms lasting >1 yr and were scheduled for primary unilateral hip-replacement surgery at Bispebjerg University Hospital, Copenhagen, Denmark. A careful physical examination was obtained by a physician to exclude subjects with cardiopulmonary, neurological, or cognitive problems. All subjects ambulated with a walking aid; however, lower limb problems other than hip-OA and/or pain during testing, as measured on a visual analog scale (VAS, 0–10), was considered an exclusion criteria (VAS > 3). The study was approved by the ethics committee of Copenhagen and Frederiksberg, in accordance with the Helsinki declaration, and written, informed consent was obtained from all participants.

Maximal isometric muscle strength and RFD. Maximal muscle strength was measured as the maximum voluntary isometric knee extension torque [maximum voluntary contraction (MVC)] exerted in an isokinetic dynamometer (KinetCom; Kinetic Communicator, Chattanooga, TN), according to procedures described in detail elsewhere (39). Individual setting of the seat, backrest, dynamometer head, and lever arm length was registered, so identical subject positioning was ensured throughout the study. All torque values were corrected for the effect of gravity (1). Subjects were carefully instructed to contract as fast and hard as possible. Visual feedback was provided to the subjects as real-time display of the dynamometer force output on a computer screen (22). After a standardized warm-up, including dynamic and submaximal isometric contractions, subjects performed three maximal isometric knee extensions of 3-s duration, each separated by a 45-s pause, and after 3-min rest three maximal isometric knee flexions were performed in a similar manner. All MVCs were performed at a knee joint angle of 60° (0° = full knee extension). The trial with the highest maximal knee joint torque for moving symmetric root-mean-square filter with a time constant of 50 ms was performed for both legs separately and were preceded by a familiarization session (2–4 days before test 1). On all test occasions, UN was tested first to minimize subjects’ discomfort with the procedure. Contractile RFD was defined as the average slope of the torque-time curve in the initial contraction phase (Δforce/Δtime) at 0–30, 0–50, 0–100, and 0–200 ms relative to the onset of contraction (1, 39). Onset of contraction was defined as the instant where torque increased 3.5 N·m above the resting baseline level (corresponding to ~2% of the peak torque). Contractile impulse was determined as the area under the torque-time curve (force·dt) in the same time intervals (1). Normalized RFD was calculated as RFD divided by CSA.

Estimation of muscle activation. To evaluate the ability to activate the quadriceps muscle of AF as well as the UN, an electrically evoked muscle twitch was superimposed onto a maximal voluntary muscle contraction. The subjects were seated in an upright position with the thigh placed horizontally and knee flexed at a right angle. A steel cuff was strapped around the lower leg, 1 in. above the malleoli. The cuff was connected via a rigid steel bar to a strain-gauge load cell (Bofors KRG-4, Bofors), which was connected to a preamplifier (BK15, Nobel Elektronik) and an amplifier (Gould 5900, Gould, Valley View, OH). The strain-gauge force signal was sampled at 1,000 Hz. Each test procedure began with the determination of the maximal twitch response. For evoking twitch responses from the knee extensors, percutaneous surface stimulation electrodes (Bioflex, model PE3590) were placed over the distal and proximal muscle belly of the quadriceps femoris. Contractions were evoked using single square-wave pulses of 0.1-ms duration delivered by a direct current stimulator (Digitimer Electronics, model DS7). Before the MVC, a maximal baseline twitch (P₀) was defined where a stepwise increment in current delivered every 30 s resulted in no further increases in force. Following a short rest, three voluntary contractions (with 2-min rest between each contraction) were performed with the addition of supramaximal single pulses. The subject was asked to push as hard and fast as possible and maintain the contraction for 3–5 s. The force recording of each contraction was viewed on a computer screen in real time, which enabled stimuli to be triggered manually on top of a MVC. The height of the superimposed twitch during this peak portion was measured, and an estimate of muscle activation was then calculated as follows: activation (%) = [1 − (P₀/P₁)] × 100, where P₀ is the force from the superimposed twitch, and P₁ is the force from the resting twitch

Electromyogram recordings. After careful preparation of the skin by shaving and cleaning with alcohol, pairs of surface electrodes (Medicotest Q-10-A, 20-mm interelectrode distance) were placed over the belly of vastus lateralis (VL), vastus medialis (VM), rectus femoris (RF), biceps femoris (BF), and semitendinosus (ST). The electromyogram (EMG) electrodes were connected directly to small custom-built amplifiers with a frequency response of 10–10,000 Hz and common mode rejection ratio exceeding 100 dB (1). EMG and dynamometer strain-gauge signals were synchronously sampled at a 1,000-Hz analog-to-digital conversion rate using an external analog-to-digital converter (dt 2801-A, Data Translation, Marlboro, MA). Subsequently, during later offline analysis, EMG signals were digitally high-pass filtered with a fourth-order, zero-lag Butterworth filter with a 5-Hz cut-off frequency, followed by a moving symmetric root-mean-square filter with a time constant of 50 ms (1). Maximum EMG amplitude of the root-mean-square-filtered signal was identified within the entire contraction phase, which included a 70-ms time period prior (1, 39). The magnitude of antagonist muscle cocontraction was calculated by dividing maximal an-
Quadriceps muscle composition. Cross-sectional area (CSA) of the quadriceps femoris muscle was obtained by computed tomography (CT: Picker 5000) with an image matrix of 512 × 512 pixels, slice thickness of 8 mm, and scanning time of 5 s. All CSA scans were obtained at the midpoint between the great trochanter and lateral joint line of the knee. CT scans were analyzed using software developed for cross-sectional CT image analysis (Geanie 2.1, BonAlyse Oy, Jyväskylä, Finland). Quadriceps muscles were encircled manually to exclude subcutaneous fat and other muscles from the region of interest. Lean tissue cross-sectional area (LCSA) and inter- and intramuscular fat CSAs were measured using CT density limits for fat and lean tissue (37). Mean attenuation [Hounsfield unit (HU)] of the lean tissue area was also recorded. Each scan was blinded for both leg and gender, and the coefficient of variation between two consecutive measurements is <1% for lean tissue HU and 1–2% for LCSA (37).

Statistical analysis. Statistical analyses were performed by using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, 2003). Data from maximal muscle strength measurements (MVC), quadriceps muscle composition (total CSA, LCSA, muscle density), MVC/LCSA, muscle activation (interpolated twitch technique), neural activity (EMG), and hamstring contraction were analyzed by a two-factor ANOVA, with gender and side as factors. Data on contractile RFD, contractile impulse, normalized RFD, and muscle activation (interpolated twitch technique) were analyzed with paired Student’s t-test. Data are presented as mean values ± SE, and a P value of <0.05 was considered significant.

RESULTS

Subjects. Men and women were similar in age (M: 69 ± 1 yr, W: 70 ± 1 yr) and body mass index (M: 29 ± 1 kg/m² vs. W: 27 ± 1 kg/m²), and men were significantly taller (M: 173 ± 2 cm, W: 165 ± 1 cm) and had a greater body mass than women (M: 85.8 ± 3.6 kg, W: 71.5 ± 3.6 kg).

Quadriceps muscle composition. Total quadriceps muscle CSA (TCSA), quadriceps muscle LCSA, and mean HU were significant smaller on AF compared with UN, both for men (TCSA: −7.5%, LCSA: −9.5%, HU: −7.0%) and women (TCSA: −6.0%, LCSA: −7.9%, HU: −8.7%) (Table 1). Furthermore, women showed lower CSA compared with men, both in AF (TCSA: −32.0%, LCSA: −33.3%) and UN (TCSA: −33.0%, LCSA: −34.1%). However, there was no gender difference with respect to muscle density expressed in HU on either of the two sides (Table 1).

Maximal isometric strength. Maximal voluntary muscle strength was lower on AF compared with UN in both men (AF: 149.6 ± 8.8 N·m, UN: 186.5 ± 9.1 N·m, reduced 19.8%) and women (AF: 90.6 ± 4.8 N·m, UN: 113.4 ± 6.2 N·m, reduced 20.3%) (Fig. 1). Compared with men, the elderly women had 39.5% less maximal isometric strength on AF and 39.2% on UN (Fig. 1). The self-reported pain score (VAS) obtained during the strength measurements was 1.2 ± 0.3 for men and 1.1 ± 0.2 for women for AF. None of the subjects reported any pain during testing of UN.

Force per unit LCSA. When MVC was expressed relative to LCSA (MVC/LCSA), there was a difference between AF and UN for both men (AF: 2.58 ± 0.14 N·m·cm⁻², UN: 2.96 ± 0.13 N·m·cm⁻², reduced 12.8%, P < 0.05) and women (AF: 2.34 ± 0.14 N·m·cm⁻², UN: 2.73 ± 0.12 N·m·cm⁻², reduced 14.3%, P < 0.05) (Fig. 2). There was no gender difference for specific strength on either side.

Contractile RFD and impulse. In men, contractile RFD was reduced (P < 0.05) on AF compared with UN for peak RFD (15%), and at 0–50 ms (24%), 0–100 ms (19%), and 0–200 ms (18%) (Fig. 3). In women, contractile RFD was lower on AF compared with UN for peak RFD (26%) and at 0–50 ms (18%) and 0–100 ms (17%) (Fig. 3). When RFD was normalized to LCSA (RFD/LCSA), AF of the men showed reduced RFD compared with UN at 0–100 ms (13%) and 200 ms (18%), and for the women, normalized peak RFD (15%) AF remained reduced (Table 2). Similarly, contractile impulse was reduced on AF compared with UN in both men and women at 0–50 ms (M: 19% vs. W: 18%), 0–100 ms (M: 20% vs. W: 19%), and 0–200 ms (M: 19% vs. W: 10%) (Table 2).

Table 1. Quadriceps muscle composition

<table>
<thead>
<tr>
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<th>Women</th>
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<th>Men</th>
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<tr>
<td></td>
<td>AF</td>
<td>UN</td>
<td>AF</td>
<td>UN</td>
</tr>
<tr>
<td>Total CSA, mm²</td>
<td>4,150±261†</td>
<td>4,407±280†</td>
<td>6,108±243*</td>
<td>6,604±217</td>
</tr>
<tr>
<td>Lean CSA, mm²</td>
<td>3,809±239†</td>
<td>4,137±267†</td>
<td>5,684±221*</td>
<td>6,281±221</td>
</tr>
<tr>
<td>Muscle density, HU</td>
<td>46.01±1.45*</td>
<td>50.38±1.07</td>
<td>48.87±1.64*</td>
<td>52.45±1.39</td>
</tr>
</tbody>
</table>

Values are means ± SE. Total muscle cross sectional area (CSA), lean quadriceps muscle CSA, and quadriceps muscle density of the affected (AF) vs. the unaffected (UN) leg in men and women is shown. HU, Hounsfield units. *P < 0.05, AF significantly different from UN. †P < 0.05, women significantly different from men.

Fig. 1. Maximal isometric strength. Maximal isometric strength is shown for the affected (AF) vs. the unaffected (UN) leg for women and men. Data are presented as means ± SE. *P < 0.05, AF significantly different from UN. #P < 0.05, women significantly different from men. Solid bars: AF leg; shaded bars: UN leg.

Fig. 2. Force per unit lean muscle cross-sectional area (LCSA) [maximum voluntary contraction (MVC)/LCSA]. Force per unit LCSA is shown for the AF leg vs. the UN leg for women and men. Data are presented as means ± SE. *P < 0.05, AF leg significantly different from UN leg. No difference was observed between women and men. Solid bars: AF leg; shaded bars: UN leg.
In addition, significant differences emerged between genders (Table 2). Thus, compared with men, the women demonstrated smaller absolute RFD values for peak RFD (AF: 50%; UN: 43%) and at 0–30 ms (AF: 42%; UN: 48%), 0–50 ms (AF: 45%; UN: 48%), 0–100 ms (AF: 42%; UN: 43%), and 0–200 ms (AF: 38%; UN: 41%) (Table 2). However, no statistically significant differences between genders were found when RFD was normalized to LCSA. In a subgroup of 17 subjects (12 men and 5 women), the magnitude of muscle activation was estimated by the superimposed twitch technique. Due to the limited number of subjects, it was not possible to make gender comparisons. There was a remarkable, insufficient muscle activation on both sides (AF: 57.6 ± 5.0%, UN: 70.7 ± 3.6%), although this deficit was more pronounced on AF (-18.5%, P < 0.01).

**DISCUSSION**

The present study investigated maximal muscle strength, muscle size, neural activation, and rapid muscle force characteristics in elderly individuals with unilateral hip-OA. The data demonstrate a marked side-to-side difference with decreased muscle mass, maximal muscle strength, neuromuscular activation, and rapid muscle force characteristics (RFD, impulse) on the arthritic side compared with the healthy side.

**Table 2. Contractile rate of force development and impulse**

<table>
<thead>
<tr>
<th></th>
<th>Women AF</th>
<th>UN</th>
<th>Men AF</th>
<th>UN</th>
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<tbody>
<tr>
<td>RFD, N·m·s⁻¹</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Peak</td>
<td>836±75*†</td>
<td>1,129±101†</td>
<td>1,681±147*</td>
<td>1,970±183</td>
</tr>
<tr>
<td>0–30 ms</td>
<td>415±37†</td>
<td>497±63†</td>
<td>718±109</td>
<td>953±109</td>
</tr>
<tr>
<td>0–50 ms</td>
<td>536±51*†</td>
<td>655±74†</td>
<td>968±105*</td>
<td>1,268±138</td>
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<tr>
<td>0–100 ms</td>
<td>484±37†</td>
<td>583±53†</td>
<td>850±65*</td>
<td>1,020±80</td>
</tr>
<tr>
<td>0–200 ms</td>
<td>343±25†</td>
<td>396±28†</td>
<td>552±38*</td>
<td>676±45</td>
</tr>
<tr>
<td>RFD/MCSA, N·m·s⁻¹·cm⁻²</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Peak</td>
<td>0.22±0.02*</td>
<td>0.26±0.01</td>
<td>0.29±0.02</td>
<td>0.31±0.03</td>
</tr>
<tr>
<td>0–30 ms</td>
<td>0.11±0.01</td>
<td>0.13±0.01</td>
<td>0.12±0.02</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>0–50 ms</td>
<td>0.14±0.01</td>
<td>0.16±0.01</td>
<td>0.17±0.02</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>0–100 ms</td>
<td>0.13±0.01</td>
<td>0.14±0.01</td>
<td>0.14±0.01</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>0–200 ms</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Impulse, N·m·s⁻¹</td>
<td>0.29±0.01†</td>
<td>0.33±0.02†</td>
<td>0.41±0.04</td>
<td>0.48±0.04</td>
</tr>
<tr>
<td>0–50 ms</td>
<td>0.74±0.05*†</td>
<td>0.90±0.08†</td>
<td>1.23±0.13*</td>
<td>1.52±0.14</td>
</tr>
<tr>
<td>0–100 ms</td>
<td>2.89±0.22†</td>
<td>3.58±0.32†</td>
<td>4.97±0.42*</td>
<td>6.20±0.52</td>
</tr>
<tr>
<td>0–200 ms</td>
<td>9.03±0.65†</td>
<td>10.89±0.85†</td>
<td>14.89±1.03*</td>
<td>18.37±1.31</td>
</tr>
</tbody>
</table>

Values are means ± SE. Normalized rate of force development (RFD) [RFD/lean tissue CSA (LCSA)] and contractile impulse (force d) of the AF vs. the UN leg for men and women are shown. *P < 0.05, AF significantly different from UN. †P < 0.01, women significantly different from men.
Despite the fact that elderly persons are particularly exposed to periods of immobilization and disuse, either due to joint pain or hospitalization (12), most of the current knowledge concerning the effect of immobilization on skeletal muscle is based on studies in healthy young individuals (4, 28). Furthermore, comparisons between young and old subjects per se make it difficult to ascertain what effects can be attributed to activity level and the aging process, respectively. The model of disuse in the present study clearly presents some limitations; however, we believe that comparing two legs with a different activity level in the same person can provide new information as to how decreased activity affects muscle function in elderly individuals. One of the limitations of the study was that the exact activity level was not obtained. It could be speculated that subjects with unilateral hip-OA would favor the healthy limb and thereby spare the AF side. Another possibility could be that the overall activity level of these persons would decrease because of joint pain; however, since both MVC and CSA of UN side in these subjects were similar to those of healthy age-matched subjects measured in our laboratory (10, 26) and in other studies (14, 17), we were probably observing a combination of increased loading on the UN side and an overall decreased activity level. Furthermore, supporting our hypothesis that long-term disuse has a similar effect on muscle mass as a standardized period of limb immobilization, both men and women had smaller quadriceps muscle CSA (LCSA) on AF compared with UN (M: −9.5%, W: −7.9%), which is comparable to 4 wk of bed rest in young healthy individuals (28).

Although muscle mass is an important determinant of strength loss with age and immobilization, it is clearly not the sole factor involved in this process. In addition, changes in structural components, such as increased intramuscular fat and connective tissue (29), likely contributes to the strength loss with aging. Previous studies have demonstrated a reduced specific strength (MVC/LCSA) in elderly compared with young individuals (31, 42), although not all studies have been able to detect such a difference (23). While women in the present study had a lower MVC on both sides (AF: −39.5%, UN: −39.2%) compared with men, no gender difference could be detected when corrected for lean muscle tissue (MVC/LCSA), which is in agreement with earlier investigations (30, 42). However, MVC/LCSA on AF was lower compared with UN in both genders (M: −13.7%, W: −11.6%), in line with D’Antona et al. (6), who demonstrated decreased specific force in single muscle fibers of the quadriceps muscle in immobilized elderly individuals, indicating a disuse-related decrease in muscle quality on the immobilized side. These results are supported by Klitgaard et al. (25), who demonstrated that sedentary elderly subjects showed a decline in specific strength, whereas elderly subjects with a long-term history of strength or endurance training demonstrated specific strength that was equal to those of young subjects. Compared with the side-to-side difference in MVC (M: −19.8%, W: −20.3%), the decline in specific strength (M: −12.8%, W: −14.3%) with inactivity and disuse suggests that ~60–70% (M: 12.8/19.8 = 65%, W: 14.3/20.3 = 70%) of this difference may be explained by qualitative changes within the muscle tissue; i.e., changes in neuromuscular activation, fiber type or muscle architectural components, and/or increased ratio of noncontractile to contractile tissue (6, 31).

To the best of the author’s knowledge, no other study has investigated rapid muscle force characteristics in the elderly after a period of disuse or immobilization. In healthy elderly individuals, it has been demonstrated that the ability to develop force rapidly (i.e., contractile RFD) is reduced compared with that in young individuals of both genders (5, 42), likely due to the decreased number and size of type II muscle fibers in the elderly (29) and an increased amount of noncontractile intramuscular tissue (29). However, when RFD is normalized...
relative to MVC, a difference between young and old subjects is not a universal finding (5, 40). In the present study, absolute contractile RFD was lower on AF compared with UN in both men (~18%) and women (~25%). Notably, AF remained reduced compared with UN when RFD was normalized to LCSA in women (W: ~17.6%), indicating qualitative changes with prolonged disuse. Although only rarely reported in the literature (1, 39), contractile impulse is an important strength parameter, since it reflects the specific time history of contraction by providing a measure of the accumulated area covered by the moment-time curve (1). In the present study, contractile impulse was reduced in the female subjects compared with male subjects on both the AF (28–41%) and UN (31–42%). Furthermore, contractile impulse was reduced on the AF compared with the UN in both men (19%) and women (10–19%).

In agreement with Berg et al. (4), who investigated the effect of bed rest on lower limb muscle function in young healthy individuals, there was reduced EMG response during maximal voluntary knee extensor MVC in the quadriceps muscle of AF compared with UN, in both men (VL, VM) and women (RF). These data suggest a general suppression in neuromuscular activity during maximum quadriceps contraction for men and women, indicating that the decreases in maximal isometric strength (MVC) and rapid force capacity (RFD, impulse) observed in AF were at least partially explained by changes in neuromuscular activation. However, it should be recognized that a multitude of confounding factors exist that may compromise the information that can be extracted from surface EMG data (11, 21). Thus side-to-side differences in EMG signal amplitude could also have been caused by a variety of nonneural factors, such as differences in limb fat distribution, muscle fiber size, and muscle fiber pennation angle. The amount of hamstring antagonist cocontraction was comparable to that previously reported for elderly individuals (24, 31). Notably, no difference was found in the magnitude of hamstring activity during maximum voluntary contraction strength and neuromuscular activity, since antagonist muscle coactivation is known to increase in the presence of muscle and/or joint pain (16). Notably, the subjects were well familiarized with the test procedure. More importantly, this finding supports that the subjects did not experience pain on AF during the recording of maximal voluntary contraction strength and neuromuscular activity, since antagonist muscle coactivation is known to increase in the presence of muscle and/or joint pain (16).

It should be noted, however, that single-twitch muscle stimulation, as used in the present study, is more susceptible to muscle fatigue than stimulation using paired twitches (15) and, furthermore, that the present resting twitches were recorded in an unpotentiated state (before contraction). Both of these factors are known to result in smaller resting twitches and thereby larger activation deficits (3, 15, 18). This does not, however, explain the difference in muscle activation observed between the AF and contralateral UN limb. Moreover, the observed AF activation deficit is very much in line with that observed by Stevens et al. (38) after 7 wk of cast immobilization in young subjects. In summary, the present study indicates that long-term limb disuse in the elderly is associated with marked decreases in maximal muscle strength, anatomical CSA of the quadriceps femoris muscle, maximal EMG amplitudes, and rapid muscle force characteristics (RFD, impulse). Furthermore, a side-to-side difference was observed in specific strength (MVC/LCSA) and normalized RFD (RFD/LCSA), which indicate that 40–70% of the observed changes with disuse may be explained by qualitative changes. The present results underline the need of effective neuromuscular rehabilitation regimes for the elderly after a period of immobilization. This need becomes particular important when considering the importance of restoring symmetry of lower limb strength and rapid muscle force capacity to avoid decremental impairments in postural balance, maximal walking speed, and other functional tasks of daily life.

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Effects of aging on human skeletal muscle after immobilization and retraining

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THE LOSS OF MUSCLE MASS WITH AGING, i.e., sarcopenia, and the concomitant decline in muscle strength are associated with increased disability and mortality (36, 50). In addition, elderly individuals are more prone to periods of bed rest due to a higher degree of comorbidity and hospitalization (47), which, per se, result in a rapid and accelerated loss of skeletal muscle mass (34, 55). Despite this, very little is known about the physiological consequences of unloading on muscle mass and neuromuscular function in the elderly, while even less is known about the regenerative capacity of skeletal muscle in the elderly human being.

The negative effects of unloading on skeletal muscle in young individuals are well elucidated (8, 18, 45). Furthermore, chronic disuse in old individuals seems to accelerate the age-related decrease in the contractile capacity of the quadriceps muscle (69), as well as in single muscle fibers (17). However, only very few studies have investigated the effects of immobilization in old compared with young humans (21, 72), and, so far, none have addressed the atrophy response to unloading of weight-bearing muscles in aging individuals. Thus the present knowledge is primarily based on animal data, where hindlimb suspension (HS) has been used as a model of muscle unloading to investigate the underlying mechanisms associated with disuse muscle atrophy in aging (3, 4, 9–11, 13, 19, 20, 22). However, the data obtained by HS in young vs. old animals are somewhat inconsistent. The majority of studies have reported young animals to be more affected by HS (13, 59), while others find a similar degree of muscle atrophy between young and old animals (67) or even greater magnitude of muscle atrophy in old animals following HS (20). Further, there are substantial indications that the muscle tissue of old animals demonstrates an attenuated recovery response after immobilization and injury (12, 19, 66). Although it is evident that aging leads to a multitude of changes in the neuromuscular system that are similar to those evoked by unloading (73), the lack of research into the effect of unloading in elderly humans makes it difficult to ascertain what effects can be attributed to a decreased physical activity per se and which to the aging process, as such.

The purpose of the present study was to investigate the effects of unilateral lower limb immobilization and subsequent retraining on muscle mass, muscle architecture, neuromuscular activation, and resting twitch characteristics in young and aged human individuals. By assessing these changes, we also aimed to study the potential interaction between changes in muscle contractile properties, specific force (Spforce), and muscle mass characteristics after immobilization, and also to examine the regenerative capacity of old compared with young individuals. Based on the literature, it was difficult to hypothesize if aging would affect the response to disuse muscle atrophy; however, due to the more consistent data regarding the capacity for regrowth in aging muscle, we hypothesized that old individuals would display an attenuated response to subsequent retraining.

METHODS

Subjects and Study Design

Twenty healthy men, 9 old (OM: 67.3 yr, range 61–74 yr) and 11 young (YM: 24.4 yr, range 21–27 yr), volunteered to participate in the study. Before inclusion, subjects were screened by a physician to exclude subjects with cardiovascular disease, diabetes, neural- or musculoskeletal disease, inflammatory or pulmonary disorders, and any known predisposition to deep venous thrombosis. Only healthy, nonmedicated individuals were included in the study. Physical activity during work and leisure time was graded in four levels based on

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questionnaire assessment (65). All subjects were moderately active (OM: 5.2 ± 1.4 h/wk, YM: 5.0 ± 0.9 h/wk) with no difference between the groups, and none of the subjects had previously participated in systematic strength training. The local Ethics Committee approved the conditions of the study (KF01–322606), and all experimental procedures were performed in accordance with the Declaration of Helsinki. Written, informed consent was obtained from all participants before inclusion in the study. After separate familiarization trials and baseline test procedures, all subjects were subjected to unilateral (randomly selected limb) lower limb casting from the hip to the ankle for 2 wk. All measurements were conducted at baseline previous to the immobilization procedure (Pre), after 2 wk of immobilization, and again after 4 wk of heavy resistance training (6 wk). All measurements were performed on both sides, with the nonimmobilized side serving as within-subject control.

Immobilization Protocol

Immobilization was accomplished by 2 wk of randomized, unilateral, whole leg casting using a lightweight fiber cast applied from just above the malleoli to just below the groin, which previously has proven to induce substantial muscle atrophy in short-term immobilization studies in young individuals (33, 35, 37). The cast was positioned in 30° of knee joint flexion to circumvent walking ability of the casted limb, and the subjects were carefully instructed to perform all ambulatory activities on crutches and abstain from ground contact, as well as performing isometric contractions of quadriceps of the immobilized leg. During the 2-wk immobilization period, the subjects were contacted on a regular basis and carefully instructed to contract the muscles around the ankle joint (venous pump exercises) several times a day to prevent potential formation of deep venous thrombosis.

Retraining Procedure

After removal of the whole leg cast, the subjects received manual mobilization of their immobilized leg by a trained physiotherapist. This was carried out to ensure that minimal pain was present and that normal range of motion could be obtained at the knee joint. The retraining protocol was accomplished by 4 wk of surveyed and supervised unilateral strength training on the immobilized leg, with three sessions each week, and has previously proven to elicit increases in muscle size and maximal muscle strength in elderly individuals (27, 71). After a 5-min warm-up on a stationary bike, the subjects performed knee extension, leg press, and knee flexion, with all of the machines being adjustable (Technogym International). To induce a sufficient response in the thigh musculature, the training intensity was being adjustable (Technogym International). To induce a sufficient response in the thigh musculature, the training intensity was sufficient. Repeated surveys and retraining protocol was accomplished by 4 wk of surveyed and subsequent filtered with a 20-Hz Butterworth low-pass filter (∼3-dB gain) (52). Twitch contractions were evoked in the passive muscle using electrical stimulation consisting of single square-wave pulses of 0.1-ms duration delivered by a direct current stimulator (Digitimer 10E)

Body Composition

Dual-energy X-ray absorptiometry (Lunar DPX, version 3.6Z software) was used to estimate whole body composition and percent body fat.

Maximal Muscle Strength and Neuromuscular Activation

Maximal voluntary and evoked muscle force was measured in a custom-made setup, where the subjects were seated in an upright position with back support and the hip and knee joint were flexed at 90° (69). A steel cuff was strapped around the lower leg, ~2 cm above the medial malleoli, and was connected via a rigid steel bar to a strain-gauge load cell (Bofors KRG-4, Bofors, Sweden), which was connected to an instrumentation amplifier (Gould 5900, Gould, Valley View, OH).
Quadriceps Muscle Volume

Muscle volume of the quadriceps muscle (\(Q_{\text{vol}}\)) was obtained by use of axial magnetic resonance imaging measurements (1). Imaging was performed in a body array coil with the subject in a supine position with both limbs extended and relaxed. Before the first scan, a localizing scan centered midfemur was conducted to ensure the knee joint was included in the field (field of view 48). The following first scan was centered just below the femur condyles to ensure the same scan position at all time points. Dependent on the femur length of the subject, seven to eight transverse scans were carried out with a slice thickness of 10 mm and an interisocap gap of 50 mm. The scans were T1-weighted with a field of view 42 and matrix 512 × 512. The anatomic cross-sectional area of each scan was measured three times by a blinded trained person using a Web1000 imaging software. The mean coefficient of variation between consecutive measurements was &lt;5%, with the latter being used as the resting reference twitch (Fig. 1B). The force recording of each contraction was displayed online on a computer screen, which enabled stimuli to be triggered manually on top of a MVC. The height of the superimposed and resting doublet twitches were measured, and central activation (CA) (i.e., neuromuscular activation) was calculated as (49, 68):

\[
CA(\%) = 100 - \left\{ \left[ (D + Tb/T_{\text{max}})^{-1} \right] + \left[ (T_{\text{m}}/T_{\text{ref}}) \right] \right\}
\]

where \(D\) is the difference between \(T_{\text{b}}\) and the maximum torque attained during the superimposed doublet stimulation; \(T_{\text{b}}\) is the torque recorded just before the instant of doublet stimulation; \(T_{\text{max}}\) is the maximal attained torque measured during the preceding MVC trials; and \(T_{\text{ref}}\) is the torque recorded during the potentiated resting doublet twitch. \(D\) is indicative of additional activity from motor units not fully activated at the time of stimulus. Correction of \(D\) (\(-T_{\text{b}}/T_{\text{max}}\)) was included in the equation, since the manually controlled doublet twitch stimulation was not always perfectly timed at \(T_{\text{max}}\) (49, 68).

Muscle Architecture

Sagittal ultrasound images of the quadriceps femoris muscle were recorded with the use of a Siemens real-time scanner with a 7.5-MHz linear array transducer. Images were obtained with the subject in a seated position (90° flexion in the hip and knee joint) at 50% of femur length over the midbelly of the vastus lateralis (VL) muscle, according to the procedures described previously (70). To ensure identical scan position at each time point, the specific scan position was marked with traces drawn on acetate paper, which was aligned relative to individual skin marks and anatomical landmarks. The pennation angle (\(\theta_p\)) of the VL fascicles was measured as the angle between the VL muscle fascicles and the deep aponeurosis of the insertion, i.e., the fascia separating VL and the vastus intermedius muscle (70). Two images from each limb were obtained from each subject. Each image was evaluated three times, and the mean value was recorded as the average fiber \(\theta_p\). The coefficient of variation between consecutive measurements was &lt;5%.

\(S_{\text{fors}}\)

To quantify the relative contributions to changes in muscle mass and muscle architecture with immobilization in old and young individuals, \(S_{\text{fors}}\) of the quadriceps muscle was calculated by dividing fascicle force (\(F_f\)) by the physiological cross-sectional area (PCSA) of the quadriceps muscle (46, 63):

\[
S_{\text{fors}} = \frac{F_f}{PCSA}
\]

As previously described (1), quadriceps contraction force (\(F_q\)) was estimated from measurements of the maximal voluntary isometric knee extension torque (MVC), assuming that patella tendon moment arm length (\(M_p\)) was 4.0 cm (53, 54), and that the ratio of patella tendon force to quadriceps tendon force was 0.7 (53, 54).

\[
F_q = \frac{MVC/M_p}{0.7}
\]

FF was then calculated as the \(F_q\) divided by cosine to the \(\theta_p\) of the VL fascicles:

\[
FF = \frac{F_q}{\cos(\theta_p)}
\]

Correlation Analyses

Correlation analysis between premuscle volume and the relative decrease after immobilization was performed to investigate the importance of habitual muscle mass on the individual responses to immobilization. Furthermore, to investigate the association between the individual responsiveness to immobilization and subsequent retraining, correlation analysis was performed on the individual (relative) decreases in muscle size after immobilization and the subsequent individual (relative) increase after retraining.

Statistics

Changes in muscle strength, neuromuscular activation, muscle volume, \(\theta_p\) of the VL fascicles, and \(S_{\text{fors}}\) were evaluated by using the Friedmann two-way analysis of variance by ranks of related samples with subsequent analysis using the Wilcoxon signed rank test for paired samples and presented as group means ± SE. Intergroup differences were evaluated using Kruskal-Wallis signed-rank test. Spearman’s rho (\(r_s\)) was used to determine the presence of any rank-order association. A 0.05 level of statistical significance (two-tailed) was used.

RESULTS

Subjects

At baseline, there was no difference in body mass (OM: 84.8 ± 3.4 kg, YM: 72.2 ± 2.3 kg) or height (OM: 178.7 ± 2.6 cm, YM: 181.4 ± 1.8 cm), whereas OM had a larger percentage of body fat (OM: 26.0 ± 3.9%, YM: 14.7 ± 5.7%) and a...
higher body mass index (OM: 26.3 ± 0.5 kg/m², YM: 22.1 ± 0.5 kg/m²) than their young counterparts.

**Maximal Muscle Strength and Central Activation**

At baseline, maximal quadriceps strength was 31% (P < 0.05) lower in OM compared with YM (Table 1). After 2 wk of immobilization, OM and YM lost 15.7% and 19.8% of the maximal quadriceps strength, respectively (P < 0.05); however, after 4 wk of retraining, both OM and YM had regained their baseline MVC (Table 1). No changes were observed in the control leg (Con) in either OM or YM. Before immobilization, OM and YM showed similar levels of central activation (OM, Pre: 88.6 ± 1.6%; YM, Pre: 91.6 ± 1.6%, nonsignificant, Table 1). However, while the activation level of YM remained unchanged, OM experienced a 9.9% (P < 0.05) decline after immobilization. After 4 wk of subsequent strength training, OM returned to the initial activation level, whereas YM reached values above the baseline activation level (P < 0.05). There was no change in the activation level of the Con leg in any of the two groups.

**Resting Twitch Characteristics**

Before intervention, peak twitch torque (PTT: −42.6%, P < 0.05) and twitch RFD at 0–30 ms (OM: −46.3%, P < 0.05) and 0–50 ms (−44.9%, P < 0.05) were reduced in OM compared with YM (Fig. 1 and Table 2). In contrast, there was no difference between young and old in the TPT. After immobilization, peak twitch torque decreased in both young and old (OM: −27.7%, YM: −22.2%, P < 0.05) with subsequent increases after retraining (OM: +30.7%, YM: +21.5%, P < 0.05). Furthermore, twitch RFD decreased after immobilization in time intervals of 0–30 ms (OM: −30.7%, YM: −21.7%, P < 0.05) and 0–50 ms (OM: −30.4%, YM: −21.5%, P < 0.05), while subsequently increasing after retraining at 0–30 ms (OM: +38.3%, YM: +16.8%, P < 0.05) and 0–50 ms (OM: +36.5%, YM: +17.8%, P < 0.05), respectively. Moreover, there was no change in TPT in either old or young individuals in response to immobilization or retraining (Table 2). No changes were observed in the Con leg. All relative changes reported above did not differ between OM and YM.

**Quadriceps Muscle Volume**

Before intervention, quadriceps muscle volume (Qvol) was 11% reduced in OM compared with YM (P < 0.05, Table 1). After immobilization, muscle volume decreased more in young than old (YM: −8.9%, OM: −5.2%, P < 0.05). Moreover, YM showed greater increases in Qvol in response to retraining (YM: +8.2%, OM: +3.8%, P < 0.05) and reached the initial baseline level (Fig. 2). In contrast, OM did not fully recover their Qvol after retraining (P < 0.05) (Fig. 2). No change was observed in the Con leg. Moreover, correlations emerged

| Table 1. Effects of immobilization and retraining on muscle contractile properties, specific force, and muscle mass characteristics |
|---------------------------------------------------|------------------|------------------|------------------|------------------|
| | Immobilized | Control | Immobilized | Control |
| MVC, N×m | | | | |
| Pre | 214 ± 27* | 212 ± 44 | 139 ± 21† | 142 ± 30‡ |
| 2 wk immobilization | 171 ± 23† | 218 ± 39 | 118 ± 24‡ | 135 ± 11‡ |
| 4 wk Retraining | 226 ± 30* | 229 ± 30 | 145 ± 31‡ | 152 ± 30‡ |
| Muscle activation, % | | | | |
| Pre | 91.6 ± 1.6 | 92.3 ± 1.5 | 88.6 ± 1.6 | 86.9 ± 3.2 |
| 2 wk immobilization | 90.6 ± 2.8 | 89.6 ± 2.9 | 80.2 ± 2.8† | 83.2 ± 2.5 |
| 4 wk Retraining | 95.2 ± 1.5†‡ | 92.4 ± 1.7 | 90.6 ± 2.8‡ | 90.8 ± 2.8 |
| Quadriceps volume, cm³ | | | | |
| Pre | 1,841.3 ± 62.2 | 1,829.5 ± 72.4 | 1,633.1 ± 46.3‡ | 1,580.1 ± 61.3‡ |
| 2 wk immobilization | 1,676.9 ± 47.3‡ | 1,824.3 ± 78.5 | 1,154.0 ± 39.7‡ | 1,577.1 ± 65.1‡ |
| 4 wk Retraining | 1,813.7 ± 61.6* | 1,814.7 ± 73.0 | 1,605.5 ± 45.3‡ | 1,558.7 ± 61.1‡ |
| PCSA, cm² | | | | |
| Pre | 164.4 ± 7.1 | 160.3 ± 7.2 | 135.8 ± 6.8‡ | 145.5 ± 7.8‡ |
| 2 wk immobilization | 157.5 ± 7.0 | 158.1 ± 8.5 | 139.7 ± 8.6‡ | 149.4 ± 9.6‡ |
| 4 wk Retraining | 173.1 ± 7.1* | 161.2 ± 6.8 | 143.0 ± 8.3‡ | 146.5 ± 8.9‡ |
| Penetration angle, ° | | | | |
| Pre | 10.4 ± 0.4 | 9.8 ± 0.3 | 9.0 ± 0.5‡ | 9.0 ± 0.5‡ |
| 2 wk immobilization | 9.4 ± 0.4† | 10.0 ± 0.2 | 8.4 ± 0.5‡ | 8.7 ± 0.4‡ |
| 4 wk Retraining | 10.5 ± 1.5* | 10.1 ± 0.4 | 8.6 ± 0.4‡ | 8.9 ± 0.5‡ |
| Fascicle length, mm | | | | |
| Pre | 11.7 ± 0.3 | 11.9 ± 0.3 | 11.9 ± 0.5 | 11.7 ± 0.8 |
| 2 wk immobilization | 10.5 ± 0.4† | 11.6 ± 0.5 | 11.1 ± 0.5 | 11.4 ± 0.7 |
| 4 wk Retraining | 11.0 ± 0.3 | 11.7 ± 0.5 | 11.4 ± 0.5 | 11.3 ± 0.5 |
| Specific force, N/cm² | | | | |
| Pre | 47.9 ± 1.8 | 48.7 ± 3.5 | 33.0 ± 1.9‡ | 34.5 ± 2.0‡ |
| 2 wk immobilization | 40.1 ± 2.4* | 51.3 ± 3.9 | 25.5 ± 2.0†‡ | 32.4 ± 1.2§ |
| 4 wk Retraining | 48.4 ± 2.6*§ | 51.4 ± 2.4 | 32.2 ± 3.3‡ | 34.0 ± 1.41 |

Values are means ± SE. Changes are shown for maximal muscle strength, quadriceps muscle volume, quadriceps activation, muscle architecture, and specific force with unloading and retraining in old and young men. Measurements were conducted at baseline (Pre), after 2 wk of unilateral immobilization, and following 4 wk of retraining on both limbs, immobilized and control. MVC, maximal voluntary contraction; PCSA, physiological cross-sectional area. †Significant different from Pre. *significant different from 2-wk immobilization. ‡Old men significant different from young men: P < 0.05.
between the individual $Q_{\text{vol}}$ at baseline and the resultant individual decrease after immobilization in young ($r = -0.669, P < 0.05$) but not old subjects ($r = -0.429, \text{NS}$) (Fig. 3). Furthermore, the relative decrease in muscle volume after immobilization was correlated to the corresponding increase after retraining in both young ($r = -0.702, P < 0.05$) and old subjects ($r = -0.778, P < 0.05$) (Fig. 4).

Muscle Architecture

Before immobilization, OM had smaller $\theta_p$ than YM (Table 1) and a tendency toward smaller fascicle lengths ($P = 0.08$). However, after immobilization, the $\theta_p$ decreased to a larger extent in YM than in OM (OM: $-6.5\%$, YM: $-9.3\%, P < 0.05$). In both young and old, there was a tendency toward a decrease in fascicle length after immobilization (OM: $-6.1\%, P = 0.08$; YM: $-9.7\%, P = 0.06$, both groups collapsed: $P < 0.05$). After retraining, $\theta_p$ increased in YM by $12.0\%$ ($P < 0.05$), whereas the $4.5\%$ increase observed in OM did not reach statistical significance. No change was observed for the Con leg in YM or OM.

Physiological CSA and Specific Force

Physiological CSA (PCSA) and specific force ($S_{\text{force}}$) were reduced by $17$ and $31\%$ in OM compared with YM ($P < 0.05$), respectively, before immobilization. PCSA remained unaltered in both OM and YM after immobilization; however, the retraining regime led to a $10.6\%$ increase in PCSA in YM ($P < 0.05$), whereas no change was observed in OM. Moreover, there was a marked decrease in $S_{\text{force}}$ after immobilization in both groups (OM: $-23.1\%$, YM: $-16.6\%, P < 0.05$), where YM tended to decrease more than OM ($P = 0.09$). After $4$ wk of retraining, $S_{\text{force}}$ returned to baseline level in both old and young individuals (OM: $+34.4\%$, YM: $+23.7\%, P < 0.05$). There were no changes in PCSA or $S_{\text{force}}$ in the Con leg in either of the two groups.

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**Table 2. Effects of immobilization and retraining on resting twitch characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Young Men</th>
<th>Control</th>
<th>Old Men</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak torque, N·m</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>47.72±3.81</td>
<td>44.99±3.77</td>
<td>27.40±1.95</td>
<td>26.42±1.54</td>
</tr>
<tr>
<td>2-wk Immobilization</td>
<td>35.79±2.07</td>
<td>45.38±3.70</td>
<td>19.80±3.65</td>
<td>22.84±2.56</td>
</tr>
<tr>
<td>4-wk Retraining</td>
<td>43.32±3.11*</td>
<td>45.24±3.03</td>
<td>25.87±1.64*</td>
<td>26.29±1.80*</td>
</tr>
</tbody>
</table>

**Time to peak tension, ms**

|                      |           |         |         |         |
| Pre                  | 88±2      | 88±2    | 89±2    | 89±2    |
| 2-wk Immobilization  | 88±2      | 89±2    | 90±2    | 88±2    |
| 4-wk Retraining      | 89±2      | 88±2    | 87±2    | 86±1    |

**RFD 0–30 ms, N·m·s⁻¹**

|                      |           |         |         |         |
| Pre                  | 1.375±0.99| 1.286±102| 738±48†| 784±68‡|
| 2-wk Immobilization  | 1.053±0.72†| 1.301±105| 546±97†‡| 678±103‡|
| 4-wk Retraining      | 1.226±0.86*| 1.328±96 | 756±65*‡| 788±70*‡|

**RFD 0–50 ms, N·m·s⁻¹**

|                      |           |         |         |         |
| Pre                  | 1.778±1.26| 1.673±132| 980±66†| 1,033±87‡|
| 2-wk Immobilization  | 1.361±0.90†| 1.692±136| 734±131†‡| 896±136‡|
| 4-wk Retraining      | 1.598±1.10*| 1.705±121| 1,001±86*‡| 1,047±93‡|

Values are means ± SE. Changes are shown for peak twitch torque, time to peak tension, and twitch rate of force development in 0–30 ms (RFD 0–30) and in 0–50 ms (RFD 0–50) with unloading and retraining in old and young men. Measurements were conducted at Pre, after 2 wk of unilateral immobilization, and following 4 wk of retraining on both limbs, immobilized and control. *Significant different from Pre, †significant different from 2-wk immobilization, ‡old men significant different from young men: $P < 0.05$.  

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**Fig. 2.** Relative changes in quadriceps muscle volume after 2 wk of immobilization (Immob) and subsequent 4 wk of retraining. $Q_{\text{vol}}$, quadriceps muscle volume.

**Fig. 3.** Correlation analysis between premuscle volume and the relative decrease after immobilization. $Q_{\text{vol}}$, quadriceps muscle volume; ns, nonsignificant.
was the purpose to address the effect of aging on retraining in old vs. young human individuals, respectively. It activation, and muscle architecture induced by unloading and change in muscle contractile function, muscle size, central results reported by Deschenes et al., who examined the effects between the two age groups, which is in line with previous muscle strength after 2 wk of immobilization did not differ humans. In the present study, the average decrease in maximal and old animals, these aspects remain to be elucidated in effects of Immobilization

Multiple interrelated factors appear to contribute to the deterioration of muscle mechanical function with aging as a result of changes in both quantitative and qualitative factors. In addition to these changes in intrinsic factors, the level of physical activity has been shown to modify the age-related loss in muscle size and function. However, as there was no difference in the activity level between old and young individuals examined in the present study, we believe the observed differences were mainly attributable to the effect of aging per se.

Effects of Immobilization

It is evident that aging and disuse bring about analogous impairments in the neuromuscular system, and, although there are indications of different strategies to muscle disuse in young and old animals, these aspects remain to be elucidated in humans. In the present study, the average decrease in maximal muscle strength after 2 wk of immobilization did not differ between the two age groups, which is in line with previous results reported by Deschenes et al., who examined the effects of 7 days of limb immobilization in young and old men, and by Urso et al., who examined the effects of 2-wk immobilization of the adductor pollicis muscle in young and old human individuals, respectively. Furthermore, immobilization led to significant reductions in $Q_{vol}$, comparable to previous data obtained in young individuals after 2 wk of immobilization. Interestingly, the observed decrease in muscle volume in old subjects was significantly smaller than the decrease observed in young subjects, in contrast to earlier findings obtained in the human adductor pollicis muscle. This discrepancy could, however, be due to the different muscle groups investigated, as different adaptation strategies have been suggested for small muscle groups compared with that of bigger muscle groups. Animal data are somewhat inconsistent as well. Some studies report old animals to be more affected by hindlimb suspension, while others, in line with findings from the present study, find a higher magnitude of muscle atrophy in young compared with old animals. The attenuated decline in muscle size in OM following immobilization could hypothetically be related to a reduced muscle protein breakdown rate with aging concurrent with the observed decrease in muscle protein synthesis rate. In some support of this notion, MuRF1 and atrogin-1 that drive ubiquitin-proteasome-mediated myofibrillar proteolysis were downregulated in skeletal muscle of old rats, although not consistently shown to differ between young and old humans, comparable to the age of the present subjects. Furthermore, the observed correlation between baseline muscle volume and relative decrease after immobilization in young but not old subjects in the present study suggests that the habitual muscle volume predicts the individual response to muscle disuse in young but not aged individuals. This finding indicates qualitative differences in the myogenic response to immobilization between young and old individuals, with old subjects being more affected on the efferent neuronal function, while young individuals were more affected at the muscle protein level.

In accordance with the changes in muscle volume, the present decrease in pennation angle of the VL fascicles observed in young subjects after immobilization was larger than that observed in old subjects, underlining the importance of muscle architecture to explain part of the discrepancy between the average relative decrease in muscle strength (OM: $-15.7\%$, YM: $-19.8\%$, $P < 0.05$), being about twice as large compared with the average relative decrease in muscle mass (YM: $-8.9\%$, OM: $-5.2\%$, $P < 0.05$). Although $S_{force}$ of the old individuals was markedly reduced compared with young subjects before immobilization, the relative decrease in $S_{force}$ was similar in old and young subjects following immobilization. Similarly, the present study demonstrated comparable decreases in resting twitch PT and twitch rate of force development between young and old individuals after immobilization, which indicates that the change in intrinsic (“qualitative”) mechanical muscle function (including muscle phenotype expression and/or tendon stiffness) did not differ between old and young individuals. The age-related differences in twitch PT and twitch RFD observed before immobilization may be due to potential changes in muscle fiber composition and/or tendon stiffness with aging, whereas the effect of immobilization in both young and old subjects demonstrated a diminished capacity to restore muscle size and muscle architecture during subsequent retraining; and 3) immobilization led to reduced muscle activation in old but not young subjects. Thus the present data suggest that the adaptive plasticity in skeletal muscle mass and central nervous system function associated with unloading and subsequent remobilization, respectively, may differ substantially between old and young individuals.


discussion

In the present study, concurrent data were obtained on the change in muscle contractile function, muscle size, central activation, and muscle architecture induced by unloading and retraining in old vs. young human individuals, respectively. It was the purpose to address the effect of aging on 1) the magnitude of acute muscle disuse atrophy, and 2) the adaptive plasticity of subsequent exercise rehabilitation. The main and novel findings were 1) that young subjects showed a greater magnitude of muscle atrophy and more marked changes in VL fascicle pennation angle after immobilization compared with old subjects; 2) old subjects demonstrated a diminished capacity to restore muscle size and muscle architecture during subsequent retraining; and 3) immobilization led to reduced muscle activation in old but not young subjects. Thus the present data suggest that the adaptive plasticity in skeletal muscle mass and central nervous system function associated with unloading and subsequent remobilization, respectively, may differ substantially between old and young individuals.

Fig. 4. Correlation analysis between the individual (relative) decreases in muscle size after immobilization and the subsequent individual (relative) increase after retraining.

\[ \text{Relative increase in } Q_{vol} \text{ after retraining} \]

\[ \text{Relative decrease in } Q_{vol} \text{ after immobilization} \]

\[ (\text{YM: } r = -0.702, p < 0.05) \]

\[ (\text{OM: } r = -0.778, p < 0.05) \]
old individuals may have included changes in sarcoplasmic Ca\textsuperscript{2+} kinetics (44).

Importantly, old subjects demonstrated an impaired ability to activate the quadriceps muscle after immobilization (OM: $-9.9\%$, $P < 0.05$), whereas young subjects, latter in line with recent findings, remained unaffected (18). This finding could partly be explained by a potential age-related decline in somatosensory afferent inflow on motor unit activation (30) and a reduced maximal motor unit firing rate at MVC (39, 41, 56) that occasionally is accompanied by reduced levels of muscle activation (42). It is possible that immobilization leads to an amplified age-related gap in these parameters that could, at least in part, explain the present findings, which should be examined in future experiments.

Effects of Retraining

The capacity for muscle regrowth in elderly human individuals after immobilization has not previously been investigated, despite the obvious clinical importance of such knowledge. Importantly, the present data demonstrate that old subjects, in contrast to young, did not fully recover from the decrease in muscle volume and pennation angle, despite 4 wk of intensive resistive exercises. These findings corresponds well with previous animal data, indicating an attenuated response to reloading in old animals (12, 48, 77), and, although the knowledge about the molecular processes involved in muscle regrowth is limited, there are indications that aging impairs the activation of satellite cells (28). Furthermore, animal data from the Conboy group indicate that the impaired regenerative capacity in old skeletal muscle is due to a diminished activation of the Notch signaling pathway with aging (16). Moreover, it has been put forward that the satellite cell pool, and thereby the myogenic potential, is reduced with aging (31, 38), although this is not a universal finding (15, 64). The attenuated rate of muscle size gain in old compared with young during the period of subsequent retraining could also be due to a lesser or delayed rise in protein synthesis rate, and/or an increased protein breakdown rate during the acute training sessions in OM. Furthermore, attenuated exercise-induced changes in myogenic regulatory factor expression may have been involved in the smaller gain in muscle size in OM observed after the period of retraining (40, 48).

Even though the present data suggest that the rate and magnitude of change in muscle mass is attenuated in old compared with that of young individuals after 4 wk of resistance training, more prolonged regimes of resistance training have revealed marked increases in muscle size and muscle architecture in old individuals (62, 70), comparable to that of young individuals (1). These data are further supported by recent data from Carey and colleagues (14), who have elegantly demonstrated that the expression of Notch genes is reduced in aged human skeletal muscle compared with that of young; however, after 12 wk of resistance training, there was no difference in basal Notch gene expression between young and old subjects.

While long-term resistance training may lead to changes in resting twitch TPT (7, 24), no longitudinal changes were observed in the present study, suggesting that the present 2 wk of immobilization followed by 4 wk of retraining were too short in duration to elicit any major change in muscle fiber-type composition and/or tendon stiffness properties. Consequently, the differential age-related changes in muscle size and muscle activation likely were the major factors to explain the present changes in mechanical muscle function induced by immobilization and subsequent retraining. On the other hand, the correlations observed in the present study between the individual relative decrease in muscle volume after immobilization and the subsequent individual relative gain after retraining (YM: $r = -0.702$, OM: $r = -0.778$, $P < 0.05$) indicate that, besides aging per se, genetic factors are also likely to play an important role for the myogenic potential, in line with recent findings by Petrella et al. (58).

In conclusion, the present data shows that aging is accompanied by an attenuated rate of muscle atrophy in response to immobilization compared with that of young individuals, and importantly that old subjects demonstrate a diminished capacity to restore muscle size and muscle architecture during subsequent retraining. Moreover, immobilization led to reduced muscle activation in old but not young subjects. Thus the present data suggest that the adaptive plasticity in skeletal muscle mass and central nervous system function associated with unloading and subsequent remobilization, respectively, may differ between old and young individuals. Collectively, these findings suggest that old individuals may be more affected with respect to neural function, and young individuals more affected in terms of muscle size, in response to short-term immobilization. Furthermore, the present data indicate that aging is accompanied by an impaired ability to recover from disuse muscle atrophy, and, consequently, old individuals may need a longer time to recover from periods of disuse compared with young individuals.

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EFFECTS OF IMMobilIZATION and RETRAINING in OLD AGE


Aging Affects the Transcriptional Regulation of Human Skeletal Muscle Disuse Atrophy

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Abstract

Important insights concerning the molecular basis of skeletal muscle disuse-atrophy and aging related muscle loss have been obtained in cell culture and animal models, but these regulatory signaling pathways have not previously been studied in aging human muscle. In the present study, muscle atrophy was induced by immobilization in healthy old and young individuals to study the time-course and transcriptional factors underlying human skeletal muscle atrophy. The results reveal that irrespective of age, mRNA expression levels of MuRF-1 and Atrogin-1 increased in the very initial phase (2–4 days) of human disuse-muscle atrophy along with a marked reduction in PGC-1α and PGC-1β (1–4 days) and a ~10% decrease in myofiber size (4 days). Further, an age-specific decrease in Akt and S6 phosphorylation was observed in young muscle within the first days (1–4 days) of immobilization. In contrast, Akt phosphorylation was unchanged in old muscle after 2 days and increased after 4 days of immobilization. Further, an age-specific down-regulation of MuRF-1 and Atrogin-1 expression levels was observed following 2 weeks of immobilization, along with a slowing atrophy response in aged skeletal muscle. Neither the immediate loss of muscle mass, nor the subsequent age-differentiated signaling responses could be explained by changes in inflammatory mediators, apoptosis markers or autophagy indicators. Collectively, these findings indicate that the time-course and regulation of human skeletal muscle atrophy is age-dependent, leading to an attenuated loss in aging skeletal muscle when exposed to longer periods of immobility-induced disuse.

Introduction

Skeletal muscle wasting is a common debilitating condition associated with human immobilization and aging resulting in a reduced muscle function [1,2]. In animal models, loss of muscle mass with immobilization or unloading has been suggested primarily to occur through an accelerated degradation of myofibrillar proteins via the ubiquitin-proteasome pathway [3–6], although rapid decreases in protein synthesis has also been shown [7,8]. Somewhat in contrast, studies in young human individuals have suggested that a decline in protein synthesis rather than accelerated protein breakdown is responsible for the muscle loss observed with disuse [9–11]. With aging, muscle loss is suggested to be associated with increased inflammation [12], decreased anabolic signaling [13], increased apoptosis [14,15], impaired myogenic responsiveness [16,17] as well as decreased mitochondrial function [18]. Moreover, aging has been found to affect signaling pathways that regulate myogenic growth factors and myofibrillar protein turnover in skeletal muscle of rodents [19,20]. However, the differential involvement and time course of such signaling pathways remains undescibed in elderly humans exposed to immobilization.

We therefore set to investigate the modulation in cellular signaling pathways involved in the initiation and temporal development of human disuse muscle atrophy, and specifically examine if aging affects the molecular regulation of human disuse related muscle loss. Recent data from our group indicate that, although immobility induces muscle atrophy in both young and old individuals, the loss in muscle mass was more pronounced in young [21], as also demonstrated in rodent models [22]. An age-specific regulation of the signaling pathways orchestrating the initiation and time-course of human disuse muscle atrophy was therefore hypothesized and a range of genes from signaling pathways previously demonstrated to play a central role in the regulation of skeletal muscle atrophy and hypertrophy in a variety of animal models was profiled [4,6,23–32].

From the ubiquitin-dependent proteolytic system expression levels of Muscle-specific muscle Ring Finger 1 (MuRF-1) and Atrogin-1 was assessed as they have been demonstrated to play a key role in the induction of muscle atrophy in multiple animal
disuse models [4,5,26], although data from human in vivo studies have been less consistent [9,33–36]. As aging and muscle loss is associated with a decrease in the activation and sensitivity of the IGF-1/Akt signaling pathway [37] gene expression profiles of Insulin-like Growth Factor 1 Ea (IGF-1Ea) and Mechano growth factor (MGF: IGF-1Ec) were assessed, along with protein levels of total and phosphorylated Akt as well as total and phosphorylated ribosomal protein S6. Furthermore, since autophagy in parallel with proteolysis, has been demonstrated to be an important stimulator of muscle atrophy in animal models [25,32,38], isoforms of the FoxO family (FoxO3 and possibly FoxO1) along with markers of autophagy (GABARAPL, ATG4 and microtubule-associated protein 1 light chain 3 beta, MAP1LC3B) [39,40] were examined. Further downstream, a range of genes of importance for oxidative phosphorylation and glycolysis are known to be coordinately suppressed in a variety of models for muscle wasting in rodents [6,27] and recently also in young human individuals following short term immobilization [35]. Over expression of two of the master genes of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) and the close homolog PGC-1β, has been shown to prevent muscle atrophy by inhibiting muscle proteolysis [41], and the expression levels of PGC-1α and PGC-1β were therefore assessed to investigate the potential age-specificity of this signaling pathway in human disuse muscle atrophy.

Although, the importance of apoptosis in human skeletal muscle atrophy has been regarded as controversial, we investigated the importance of this pathway by assessing the expression levels of the Bcl-2–associated X protein (Bax), Bcl-2-like protein 1 (BCL2L1) and tumor protein 53 (p53), as apoptosis seems to play an important role in the development of muscle atrophy in aged animal models [14,42–46]. Furthermore, the mRNA expression level of Nuclear Factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB) along with the upstream pro-inflammatory cytokine Tumor Necrosis Factor α (TNF-α) were profiled to study the effect of immobility-induced disuse on the induction of the NF-κB pathway [24]. In addition, expression levels of the pro-inflammatory cytokine IL-6 was profiled as an elevated expression of this cytokine along with an increased expression level of TNF-α has been linked to various diseases as well as aging [47]. Collectively, these transcriptional data were combined with measures of contractile capacity, morphology of the immobilized muscle and protein quantification in order to gain a more thorough understanding of the pathways regulating muscle protein degradation with disuse in old versus young human adults and further to examine the influence of these molecular regulatory pathways on muscle function and muscle size.

![Figure 1. Immobility-induced skeletal muscle atrophy causes an age-specific decline in muscle size. A. Scheme of experimental setup, including the time points of muscle biopsy procedure. B. Percentage decreases in muscle size (mean muscle fiber area) after 4 days of immobility in young (n = 11) and old (n = 9) as well as after 14 days of immobilization in young (n = 11) and old (n = 12), respectively. * Time effect, p < 0.05 compared to pre, # Age effect, p < 0.05 young compared to old within time point. Group mean data ± SEM. Mean myofiber area was assessed in the quadriceps femoris muscle, by muscle biopsy sampling. C. Muscle histology from resting state (pre) and immobility (14 d) was analyzed by myofibrillar ATPase at pH 10.3 preincubations demonstrating type I (white) and type II muscle fibers (black) [52]. doi:10.1371/journal.pone.0051238.g001](https://www.plosone.org/doi/10.1371/journal.pone.0051238.g001)
Materials and Methods

Study design

In the present manuscript results from two human intervention studies are reported. At first, myofiber atrophy was induced for a period of 2 weeks to investigate the signaling pathways regulating disuse skeletal muscle atrophy in young and aged individuals, respectively (Figure 1). Muscle biopsies of the vastus lateralis muscle were collected prior to the intervention and immediately after cast removal. In order to study the time-course and identify signaling pathways involved in the initiation of disuse muscle atrophy, two additional groups of young and aged individuals were recruited and immobility was induced for 4 days. These subjects were biopsied after 24 h (~1 d), 48 h (~2 d) and 96 h (~4 d) of immobility (Figure 1A). Within age groups, subjects recruited for the two immobilization protocols did not differ with respect to age, weight, BMI and activity level (Table S1).

Human subjects

A total of 43 healthy men volunteered to participate in the two immobilization studies, 20 persons were recruited for the 14 d immobilization protocol (11 young: 24.4, range 21–27 yrs; 9 old: 67.3, range 61–74 yrs) while 23 persons were recruited for the 4 d immobilization protocol (11 young: 24.3, range 21–30 yrs; 12 old: 66.8, range 60–72 yrs) (Table S1). Prior to inclusion a trained physician screened all subjects to exclude individuals with cardiovascular disease, diabetes, neural or musculoskeletal disease, inflammatory or pulmonary disorders or any known predisposition to deep venous thrombosis. The local Ethics Committee of Copenhagen and Frederiksborg municipality approved the study (KF01-322606) and all experimental procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants before inclusion in the study.

Immobilization protocols

14 d immobilization.

This protocol has been described in detail before [21] along with results on muscle contractile properties and morphology [21,48] but in brief immobilization was accomplished by 2 weeks of randomized unilateral whole-leg casting using a lightweight fiber cast applied from just above the malleoli to just below the groin. The cast was positioned in 30° casting using a lightweight fiber cast applied from just above the malleoli to just below the groin. The cast was positioned in 30° of knee joint flexion to circumvent walking ability of the casted limb and the subjects were carefully instructed to perform all ambulatory activities on crutches and abstain from ground contact during the immobilization period. Muscle biopsies were divided into two separate pieces, one oriented in embedding medium (Tissue Tec®) frozen in isopentane cooled with liquid nitrogen and stored at ~80°C and one piece directly frozen in liquid nitrogen and stored at ~80°C until further analyses. Subsequently, serial transverse sections (10 μm) were cut in a cryostate at ~20°C and stained for myofibrillar ATPase at pH 9.4 after both alkaline (pH 10.3) and acid (pH 4.3) preincubations [52]. All samples of each individual person were stained in the same batch to avoid interassay variation. Based on the ATPase staining pattern muscle fibers were characterized as type I and type II and an average of 277+/−49 fibers were analyzed in each biopsy. For the determination of muscle fiber size only horizontally fibers were used, with a minimum of 100 fibers included for the analysis. A videomicroscope consisting of a microscope (Olympus BX 50) and color video camera (Sonya high resolution CCD) in combination with Tema Image-analyses System (Scanbeam Denmark) were used to calculate the mean muscle fiber area.

RNA purification

Total RNA was isolated from ~20 mg of frozen muscle biopsy by phenol extraction (TriReagent; Molecular Research Center, OH, USA) as previously described [53]. Intact RNA was confirmed by denaturing agarose gel electrophoresis.

Real-time PCR

Total RNA (500 ng) was converted into cDNA in 20 μl using the Omniscript reverse transcriptase (Qiagen, CA, USA) according to the manufacturer’s protocol. The mRNA expression of FoxO1, FoxO3, FoxO4, PGC-1α, PGC-1β, IL-6, MGF, IGF-1Ea, GADPH and RPLP0 were analyzed by quantitative real-time RT-PCR. For each of the mRNA targets, 0.25 μl cDNA was amplified in a 25 μl SYBR Green PCR reaction containing 1 × Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (Table S2A). The amplification was monitored real-time using the MX3000P real-time PCR machine (Stratagene, CA, USA). The threshold cycle (Ct) values were related to a standard curve made with the cloned PCR products and specificity ensured by melting curves analysis and the quantities were normalized to RPLP0. TaqMan based quantitative real-time RT-PCR of MuRF-1, Atrogin-1, NF-κB, Bax, BCL2L1, p53, TNF-α, ATG4B, GA-BARAPL1, and RPLP0 mRNA (Table S2B) were performed in

Assessment of contractile muscle strength

Maximal muscle contraction strength was measured for the quadriceps muscle in a custom made setup where the subjects were seated in an upright position with back support and the hip and knee joint flexed at 90° [49]. A steel cuff was strapped around the lower leg, approximately 2 cm above the medial malleoli and was connected via a rigid steel bar to a strain gauge load cell (Bofors KRG-4, Bofors, Sweden), which was connected to an instrumentation-amplifier (Gould 5900, Gould Inc. Valley View, OH USA).
Figure 2. Immobility results in an increase in the transcriptional status and immunodetection of Atrogin-1 and MuRF-1 in the early phase of disuse muscle atrophy. 

**A & B.** The mRNA level of Atrogin-1 and MuRF-1 was determined using qRT-PCR and significant increases were found in the early phase (2–4 days) of immobility, in both young and old skeletal muscle. At the later time-point (14 d) Atrogin-1 and MuRF-1 expression levels decreased in aged muscle, whereas the expression levels of Atrogin-1 and MuRF-1 mRNA were unchanged from baseline in young muscle. Data are geometric means ± back-transformed SEM. 

**C.** Immunodetection of DAPI (blue), and MuRF-1 (red) are shown for 10 μm skeletal muscle cryosections. Potential muscle fiber specificity was analyzed by simultaneous incubation of primary antibodies for MuRF-1 mixed with either anti-skeletal Myosin-Fast or anti-skeletal Myosin-Slow (green), demonstrating an almost 100% affinity of MuRF-1 for type 1 muscle fibers. 

**D.** Total numbers of MuRF-1 positive myofibers were quantified for both young and old muscle and a significant increase was detected in the number of MuRF-1 positive fibers after 4 days of immobility. * Time effect, p < 0.05 compared to pre. * Time effect, p < 0.05 bar indicates young and old combined compared to pre. Data are means ± SEM. 

**E.** Immunodetection of DAPI (blue), Laminin (green) and MuRF-1 (red) are shown for 10 μm skeletal muscle cryosections at pre, 1 d, 2 d and 4 d of immobilization. doi:10.1371/journal.pone.0051238.g002
Western blotting analyses

From each muscle biopsy 150 cryosections (10 μm) were homogenized in a micro vial containing 1 silicium carbide crystal, 5 steel beads (2.3 mm) and 250 μl ice-cold homogenization buffer (50 mM Tris-base, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 0.1% v/v Triton-X, 0.1% v/v mercaptoethanol and protease inhibitor (Complete, Roche, Basel, Switzerland), pH 7.5) using a FastPrep-24 (MP Biomedicals, Solon, OH, USA) homogenizer. Laemmli buffer was added and protein concentrations were determined with the EZQ Protein Quantitation Kit according to the manufacturer’s protocol (Molecular Probes, Eugene, OR, USA). Then, samples were heated at 90°C for 4 min, shortly vortexed and spun in a microcentrifuge and equal amounts (10 mg/ml) were separated by SDS-PAGE using a 4–12% Bis-Tris gel (Criterion, Bio-Rad, Hercules, CA, USA) at 200 V for 1 h. Gels were blotted (Trans-blot cell, Bio-Rad, 400 mA, 2 h) to polyvinylidene difluoride membranes (Amersham Hybond LFP, GE Healthcare, Buckinghamshire, UK), which were blocked for 30 min with 20% Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) in phosphate-buffered saline, incubated overnight at 4°C with primary antibody, incubated for 1 h in fluorophore-conjugated with secondary antibody and visualized with the Odyssey Infrared Imaging System (Li-Cor Biosciences). Total and phosphorylated protein pairs were detected simultaneously on the same membrane. Band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA). Total and phospho (serine 473) Akt primary antibodies (Cell Signaling Technology, Danvers, MA, USA, no. 2920 and 4060) were diluted 1:2,000 and actin (Sigma, Saint Louis, MO, USA, no. A2066) primary antibody was diluted 1:10,000. Due to low tissue availability n equals 3 in each age group for the measurement of total and phospho Akt. Western blot analysis for LC3B, as well as S6 ribosomal protein and phospho-S6 ribosomal protein (Ser235/236) were performed on frozen tissue homogenized in 10 volumes (wt/vol) of ice-cold buffer (300 mM Sucrose, 1 mM EDTA, 10 mM NaN3, 40 mM tris-base and 40 mM histidine at pH 7.8 with protease inhibitors, #05892791001, Roche Inc.) using a 1 ml glass homogenizer with a glass pestle (Kontes Glass Industry, Vineland, NJ). Protein content in the muscle homogenate was measured in triplicates using a standard kit (Pierce BCA protein reagent no. 23225, Pierce Inc.). Samples were heated at 90°C for 4 min, shortly vortexed and spun in a microcentrifuge and equal amounts (20 μg) were separated by SDS-PAGE using a 4–15% Tris/glycine gel (Mini-protein TGX, Bio-Rad, Hercules, CA, USA) at 200 V for 35 min. Gels were blotted (Trans-blot cell, Bio-Rad, 250 mA, 1 h) to polyvinylidene difluoride membranes (Immun-Blot PVDF,

Figure 4. Immobility induced skeletal muscle atrophy results in an age-specific decrease in Akt and ribosomal protein S6 phosphorylation. A. Western blotting of whole muscle protein homogenates of phosphorylated Akt and total Akt. B. Immobility decreased levels of phosphorylated Akt/total Akt ratio (p-Akt/Akt) at the early (2–4 days) phase of immobility in young but not aged skeletal muscle. * Time effect, p<0.05, compared to pre. # Age effect, p<0.001 young compared to old within time point. Due to lack of muscle tissue n = 6 (3 young and 3 old) in these analyses. C. Western blotting of whole muscle protein homogenates of total and phosphorylated S6 ribosomal protein. D. The percentage of the total number of subjects at each time point where p-S6 could be detected. Chi-square: Young p<0.001, Old p = 0.44. In a high number of especially young subjects phosphorylated S6 ribosomal protein (but not total S6 ribosomal protein) became non detectable after immobilization which made an exact quantification impossible.

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This document contains detailed information on the study of muscle atrophy in human subjects undergoing immobilization. The study includes various experimental procedures such as mRNA and Western blot analyses, histological assessments, and statistical analyses to understand the regulatory mechanisms of disuse atrophy.

### Statistical Analyses

A part from total and phosphorylated S6 ribosomal protein, all mRNA and Western blot data were log-transformed prior to statistical analyses and are presented as geometric means ± back-transformed SEM. To test for changes over time (mRNA, TUNEL, Immunohistochemistry and Western blot analyses) one-way Bonferroni corrected repeated-measures ANOVA was performed separately for young and old individuals, respectively, as well as for young and old combined (SPSS). Pairwise multiple comparison procedures were evaluated using Student-Newman-Keuls Method post-hoc testing. Independent-samples t-testing were used to test for differences between groups’ with a subsequent Bonferroni correction. As phosphorylated S6 ribosomal protein (but not total S6 ribosomal protein) became non-detectable after immobilization in a high number of especially young subjects, quantification of phosphorylated S6 ribosomal protein/total S6 ribosomal protein was not possible and we therefore decided to perform a Chi-square test (young p<0.001, old p = 0.44). The percentage of the total number of subjects where p-S6 could be detected, at each time-point, are visualized in figure 4D. Non-parametric statistics were used to analyze changes in muscle fiber CSA, since not all of these data were normally distributed. To evaluate the effect of intervention over time a repeated-measures Friedman test was used with post-hoc Wilcoxon testing. Between-group differences were analyzed with Kruskall-Wallis tests and subsequent Mann-Whitney U testing. Data are presented as mean values ± SEM. A p-value of less than 0.05 was considered statistically significant.

### Results

**Maximal contractile muscle strength**

Maximal contractile muscle strength declined in both young (13%) and old (14%) after 4 days of unloading, with no difference between age groups (Figure S1A). Following 14 days of immobilization the loss in mean myofiber size was greater in young (219.9%) than old individuals (212.6%, p<0.01) (Figure 1B). There was no difference between the decline in type 1 fibers (O: −7.1%, Y: −8.1%) and type 2 (O: −10.9%, Y: −12.6%) fibers at the 4 d time-point, however after 14 d the decline in type 2 fibers (O: −17.1%, Y: −26.5%) was significantly larger than in type 1 fibers (O: −8.9%, Y: −14.3%) in both young and old (p<0.05) (Figure S1B).

**Muscle fiber cross sectional area**

Despite the very limited period of immobilization, our histological analyses revealed significant decreases in mean myofiber area of approximately 10% in both young and old subjects after 4 days of immobility (Figure 1B). However, following 14 days of immobilization the loss in mean myofiber size was greater in young (−19.9%) than old individuals (−12.6%, p<0.01) (Figure 1B). There was no difference between the decline in type 1 (O: −7.1%, Y: −8.1%) and type 2 (O: −10.9%, Y: −12.6%) fibers at the 4 d time-point, however after 14 d the decline in type 2 fibers (O: −17.1%, Y: −26.5%) was significantly larger than in type 1 fibers (O: −8.9%, Y: −14.3%) in both young and old (p<0.05) (Figure S1B).

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**Figure 5. Changes in the transcriptional status of FoxO1, FoxO3 and FoxO4 as a result of immobility-induced muscle disuse. A–C.** The mRNA level of FoxO1, FoxO3 and FoxO4 was determined using qRT-PCR. No up-regulation in the mRNA expression levels in any of these three genes was observed during the initial phase of immobility, in contrast, a general down-regulation in all three genes was observed in both young and aged muscle at the 4 d time point, potentially reflecting a negative feedback signal from high presence of active FoxO protein in the muscle cell. However, it is difficult to interpret the role of FoxO1 in the present study since the phosphorylated forms of FoxO were not measured. * Time effect, p<0.05 bar indicates young and old within time point. Data are geometric means ± back-transformed SEM.

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0.2 μm, BioRad, Hercules, Ca, USA), which were blocked for 60 min with 5% Blotting–Grade Blocker (#170-6404, BioRad, USA) in phosphate-buffered saline with 0.05% Tween 20. Membranes were subsequently incubated overnight at 4°C with primary antibody LC3B (1:1000, #2775, Cell Signaling Technology) as well as S6 ribosomal protein (1:1000, #2217, Cell Signaling Technology Inc.) and phospho-S6 ribosomal protein (Ser235/236) (1:2000, #4858, Cell Signaling Technology Inc.). Membranes were subsequently washed and incubated for 1 h with HRP-conjugated secondary antibody (Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate, #170-5046, BioRad, Hercules, Ca, USA) and visualized with Immun-star western kit (170-5070, BioRad, Hercules, Ca, USA). After detecting the phosphorylated protein, the membrane was stripped and the total protein was detected. Band exposure and visualization were quantified using ChemiDoc XRS with Image Lab Software (Bio-Rad Laboratories, Inc.)

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Ubiquitin ligases

The present data revealed a rapid up-regulation of MuRF-1 and Atrogin-1 mRNA expression levels after 48 h in both young and old subjects (Figure 2, A–B). These data were further supported by immunohistochemical staining for MuRF-1 (Figure 2C), which demonstrated an acute increase in the number of MuRF-1 positive fibers and nuclei within the first days of immobility in both young and old muscle (Figure 2D). Notably, there was an almost 100% affinity of MuRF-1 for type 1 muscle fibers (Figure 2C), although this was not accompanied by a selective type 1 muscle fiber atrophy at the 4 d time point (Figure S1B).

IGF-1/Akt signaling

The data revealed an age-specific (old subjects only) up-regulation of IGF-1Ea and MGF at 1 d and 2 d of immobilization, while a general up-regulation was observed in both age groups after 14 days of immobility (Figure 3, A–B). Furthermore, protein analysis by Western blotting revealed selective decreases in phosphorylated Akt/total Akt ratio (2 d and 4 d) in young individuals. In addition, the number of subjects, where phosphorylated S6 ribosomal protein could be detected within the very first days of immobilization was reduced in young compared to old individuals (1 d and 2 d) (Figure 4, A–D). No change was observed in protein levels of Actin in neither young nor old muscle (Figure S3).

Forkhead box O (FoxO) transcription factors

There was no up-regulation in the mRNA expression levels of any of the three isoforms of the FoxO family in skeletal muscle (FoxO1, FoxO3 and FoxO4) after 4 days or 14 days of immobilization. In fact, a general down-regulation in all three genes was observed in both young and aged muscle after 4 days of unloading but not at the 14 days time point (Figure 5, A–C). No age related differences were observed at any time point (Figure 5, A–C).

Autophagy

The mRNA expression profiles of cysteine protease ATG4B and GABARAPL1 were examined, and a significant up-regulation of ATG4B was observed after 14 days of immobilization and old (Figure 6A), whereas no changes were observed for the expression level of GABARAPL1 (Figure 6B). However, using immunohistochemical targeting of ATG4B no visible autophagosomes could be observed in biopsies obtained from young and old individuals (data not shown). However, protein analysis of microtubule-associated protein 1 Light Chain 3 beta (LC3B) I and II by Western blotting (LC3B II/I ratio) tended to increase at 1 day (p = 0.093) and 4 days (p = 0.066) of immobilization in both young but not aged skeletal muscle (compared to pre) (Figure 6, C–D).

PGC-1 co-activators

The expression levels of PGC-1α mRNA revealed a marked down-regulation at 1 d in young muscle (Figure 7A) and a down-
regulation in both young and old muscle at the later time points (2 d, 4 d and 14 d). In contrast to PGC-1α, the expression levels of PGC-1β mRNA returned to basal levels at 14 days of immobilization, indicating that the two genes may play different roles in the later stages of muscle unloading. * Time effect, p<0.05 compared to pre. Data are geometric means ± back-transformed SEM. doi:10.1371/journal.pone.0051238.g007

**Figure 7. Changes in the transcriptional status of PGC-1α and PGC-1β as a result of immobility-induced disuse-muscle atrophy.** The mRNA level of PGC-1α and PGC-1β was determined using qRT-PCR. A. The results revealed a marked down-regulation of PGC-1α at 24 h in young muscle and a down-regulation in both young and old muscle at the later time points (2 d, 4 d and 14 d). B. The expression levels of PGC-1β mRNA also revealed an age-specific down-regulation after 24 h in young muscle and a down-regulation in both young and old muscle at 2 d and 4 d. In contrast to PGC-1α, the expression levels of PGC-1β mRNA returned to basal levels at 14 days of immobilization, indicating that the two genes may play different roles in the later stages of muscle unloading. * Time effect, p<0.05 compared to pre. Data are geometric means ± back-transformed SEM. doi:10.1371/journal.pone.0051238.g007

Apoptosis

Our data revealed an age-specific up-regulation of Bax and p53 in aged muscle after only 2 days of immobility, with further increasing levels after 4 days in both young and old muscle (Figure S3, A & C). These data were supported by a significant increase in TUNEL-positive nuclei (Figure S3, D & E). However, very few TUNEL-positive myonuclei were observed in both young and old muscle and the expression pattern was not related to the immobilization intervention (data not shown). TUNEL-positive nuclei were mainly localized outside the plasma membrane in the interstitial space between muscle cells, potentially comprised by satellite cells, endothelial cells and leukocytes. Double immuno-

NF-κB signaling and pro-inflammatory cytokines

Apart from a small increase in the expression of NF-κB at 14 d, there was no change in the mRNA expression of NF-κB or TNF-α at any time-point in neither young nor old muscle. Further, there was a small increase in the expression level of IL-6 mRNA in both young and aged muscle after 4 days of immobility, but beside this no major induction of this pro-inflammatory cytokine was observed (Figure S2).

**Discussion**

The mechanisms underlying human skeletal muscle atrophy in aged muscle are largely unknown. In the present study, we report transcriptional data from regulatory signaling pathways related to skeletal muscle disuse-atrophy, which has not previously been studied in aging human muscle. The main findings were that irrespective of age the ubiquitin-proteasome pathway was activated in the very initial phase (2–4 days) of human disuse-muscle atrophy along with a marked reduction in markers of oxidative metabolism. Moreover, an age-specific regulation of Akt and S6 phosphorylation was observed with a decrease in young muscle within the first days (1–4 days) of immobilization. In contrast, aged muscle demonstrated a rise in Akt phosphorylation at 4 days along with a decrease in mRNA expression levels of MuRF-1 and Atrogin-1 after 14 days of leg muscle immobilization. Furthermore, elderly individuals demonstrated less overall muscle loss with disuse than their young counterparts after 14 days (but not 4 days) of muscle disuse. Neither the immediate loss in muscle mass, nor the subsequent age-differentiated signaling responses could be explained by changes in inflammatory mediators or markers of apoptosis.

Certain controversy exists in the literature regarding whether muscle atrophy in human skeletal muscle is regulated primarily via an increase in protein degradation or a decrease in protein synthesis. In animal models, evidence has pointed at protein degradation as the main driving factor, with the ubiquitin-dependent proteolytic system being rapidly activated [3–6] in relation to unloading and various disease states [4,6,26], although decreases in protein synthesis also have been demonstrated [7,55,56]. In contrast, the role of the ubiquitin-proteasome pathway in human in vivo studies has been less consistent [9,33–36,57]. Our data revealed a significant up-regulation in MuRF-1 and Atrogin-1 within the initial days of immobilization (~2–4 days), with no difference between young and aged muscle. Similar results have recently been observed after 48 h and 72 h of unloading in young human individuals [35,57], which could suggest a role for the ubiquitin-proteasome pathway in the initiation of human skeletal muscle atrophy. The fact that we observed more modest changes compared to previous animal reports may reflect that more drastic and/or systemic wasting models were used in these animal studies [4–6] compared to human immobilization models. Notably, the present data revealed that expression levels of Atrogin-1 and MuRF-1 returned to basal levels after 14 days of immobility in young individuals and was further down-regulated in old individuals, along with a (compared to young) smaller decrease in muscle fiber area. These findings may indicate that the ubiquitin-proteasome pathway mediate a transient rise in protein degradation in human skeletal muscle important for the initial and
rapid loss of muscle mass with disuse but may not be important for a more prolonged atrophy response [35]. Notably, a similar time-course of MuRF-1 and Atrogin-1 expression levels has been demonstrated in the rat model after denervation and spinal cord injury [26].

A transient rise in signaling markers of protein degradation does, however, not exclude a simultaneous down-regulation of protein synthesis with immobilization which has been demonstrated to occur in young individuals [9–11,58,59]. In line with these results, as well as previous data shown by Booth and co-workers in a rat model [7], a decline was observed in phosphorylated Akt and phosphorylated ribosomal protein S6 in the initial phase of immobility (day 1–4) in the present study. In addition to being a central regulator of muscle protein synthesis and muscle hypertrophy the IGF-1/Akt signaling pathway has been proposed to be a potent suppressor of myofibrillar proteolysis and atrophy related ubiquitin ligases, respectively [23,39,60,61].

**Figure 8. Immobility increases the transcriptional status of Bax, BCL2L1 and p53 and the immunodetection of TUNEL.** A–C. The mRNA level of Bax, BCL2L1 and p53 was determined using qRT-PCR and significant increases were found in the early phase (0–4 days) as well as later phase (14 days) of disuse-muscle atrophy. D. Skeletal muscle cryosections were immunostained for nNOS (red), DAPI (blue) and TUNEL (green). E. Total numbers of TUNEL-positive nuclei were quantified for both young and old muscle and significant increases of TUNEL-positive nuclei were detected in old muscle in the early phase of immobility (1–2 days) and in both young and old after 4 days of immobility. F. Double immunohistochemical staining for TUNEL and the muscle satellite cell marker Pax7 did not reveal any TUNEL-positive muscle satellite cells. Additional green fluorescent expression on the shown image is due to autofluorescence by lipofuscin and this is considered non-specific in our analysis. *Time effect, p<0.05 compared to pre. † Time effect, p<0.05 bar indicates young and old combined compared to pre. Data are means ± SEM.

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in Akt and ribosomal protein S6 phosphorylation suggests that
immobility leads to reduced protein synthesis in young skeletal
muscle, in line with previous findings [9–11,58,59]. In contrast,
the relative to young - higher Akt phosphorylation in elderly in
combination with an early up-regulation of MGF and IGF-1Ea
expression is potentially contributing to the attenuated atrophy
response in aging skeletal muscle observed in the present study.
In support of these findings, the expression of molecular markers for
anabolic signaling (mTOR and S6K1) and elevated protein
synthesis rate either remained unchanged or increased in 24 and
27 months old sarcopenic rats compared to young animals [62].
Although a coordinated regulation of the ubiquitin-proteasome
and the autophagy-lysosome pathways has been shown to exist in mice [25,32,63], the present study did not demonstrate an increase in expression levels of ATG4, GABARAPL or FoxO3 mRNAs
(Figure 6, A–B and Figure 5B). However, we did see a trend
away from an increase in LC3B II/I protein ratio selectively in young muscle after 1 d and 4 d of immobility, which suggests that the
autophagic process (lipidation) was initiated at least in the young
myofibers and thus, crosstalk between the ubiquitin-proteasome
and the autophagy-lysosome pathways may also exist in the
human model. However, more detailed studies investigating both
upstream and downstream regulators of the autophagic and
proteolytic processes in humans are needed to elucidate these
signaling pathways. Further, the present data revealed that disuse
of skeletal muscle resulted in a marked down-regulation of genes
signaling pathways. These findings may be important for the
initiation and regulation of human skeletal muscle atrophy, as also observed in rodents [27,41]. Although only minor transcriptional changes of FoxO were noted in the present study, we found a rapid increase in atrogenes downstream of FoxO (Figure 2). However, as protein
levels of FoxO were not determined, it is not possible to exclude
that high levels of FoxO in the cell and the nucleus will feedback
upon the FoxO signaling it self. If so, the present finding of
reduced FoxO after 4 days could reflect a feedback phenomenon.

Another topic of debate has been the role of apoptosis in human
skeletal muscle atrophy and sarcopenia. There are a significant
amount of data indicating an important role for apoptosis in the
development of muscle atrophy observed with aging in animal
models [14,42–46], whereas human data have been more
inconsistent [64–66]. Notably, despite an increase in TUNEL-
positive nuclei was observed primarily in aging muscle after
immobility (Figure 8, D–E), there were limited signs of specific
TUNEL-positive myonuclei in young as well as old
muscle, in contrast to previous findings in the murine model [67].
The TUNEL-positive nuclei observed in the present study were
primarily localized in the interstitial space between muscle cells
and the cellular origin and were neither macrophages, endothelial
cells nor muscle satellite cells. Thus myofiber as well as muscle
satellite cells apoptosis seems not to play a key role for the
initiation of human disuse-muscle atrophy in agreement with
recent mouse studies [68].

In conclusion, the present findings collectively demonstrate that
a number of signaling pathways related to both muscle atrophy
and muscle hypertrophy are activated in the initial phase of disuse
along with a rapid atrophy response in skeletal muscle of both
young and old individuals. Importantly, activation of the
ubiquitin-proteasome pathway was observed along with a down-
regulation of PGC-1α and PGC-1β during the first 1–2 days of
disuse, suggesting that proteolysis may play an important role in
the initiation of human disuse atrophy in both young and old
muscle. These changes were accompanied by rapid decreases in
phosphorylated Akt and phosphorylated ribosomal protein S6
selectively in young muscle. In contrary, aged muscle selectively
showed an elevated Akt phosphorylation and up-regulation of
IGF-1Ea and MGF in combination with a decrease in Atrogin-1
and MuRF-1 mRNA expression levels and a less marked atrophy
response in the later phase of disuse.

Although several fundamental mechanistic questions in regards
to muscle loss remains to be answered, the present data provide
novel insights into the molecular regulation of human skeletal
muscle disuse-atrophy and its modulation by aging. Our findings
indicate that the initiation and regulation of human skeletal muscle
atrophy is age dependent and involves a number of independent
signaling pathways. These findings may be important for the
identification of biomarkers and future therapeutic intervention
paradigms that can be used to counteract human skeletal muscle
atrophy in relation to aging and disuse.

Supporting Information

Figure S1 Immobility-induced decrease in maximal contractile muscle strength and atrophy of type I and type II muscle fibers. A. Four days of immobility revealed a rapid decrease in maximal contractile muscle in both young and

old. The rate of loss in muscle strength seemed to slow down in both groups at 14 d. B. The relative decreases in muscle fiber area of type I and type II fibers after 4 d and 14 d of immobility in young and old individuals, revealed a rapid decrease in muscle fiber area of type I as well as type II fibers, respectively. In contrast
to young subjects, the rate of muscle loss slowed down in old
individuals after 14 d of immobility. * Time effect, p<0.05 compared to pre. # Age effect, p<0.05 difference between young
and old within time point. Data are means ± SEM. (TIF)

Figure S2 Changes in the transcriptional status of NF-

κB, TNF-α and IL-6 as a result of immobility induced
disuse muscle atrophy. The mRNA level of NF-κB, TNF-α and IL-6 was determined using qRT-PCR. A–B. A part from a small increase in the expression of NF-κB at 14 d, we did not find
any change in the mRNA expression of NF-κB or TNF-α at any
time-point in neither young nor old muscle. C. A part from a small increase in the expression level of IL-6 mRNA in both young and
aged muscle after 4 days of immobility, no major induction of this
pro-inflammatory cytokine was observed. * Time effect, p<0.05 compared to pre. * Time effect, p<0.05 bar indicates young
and old combined compared to pre. Data are geometric means ±
back-transformed SEM. (TIF)

Figure S3 Unchanged actin protein levels during skel-
etal muscle disuse-atrophy. A. Western blotting of whole
muscle protein isolates; quantified in B. Protein levels of actin
were unchanged at the early (2–4 days) phase of immobility
in both young and aged skeletal muscle. Data are geometric means ±
back-transformed SEM. Due to lack of muscle tissue n = 6 (3 young and 3 old) in these analyses. (TIF)

Figure S4 Changes in the transcriptional status of GAPDH as a result of immobility induced disuse muscle
atrophy. mRNA expression levels of GAPDH were determined
using qRT-PCR. * Time effect, p<0.05 compared to pre. Data are geometric means ± back-transformed SEM.

(TIF)

Table S1  Subject characteristics. There was no difference between subjects from the 14 days and 4 days immobilization study with respect to age, weight and BMI, young and old respectively, however, old subjects weighed more and had a higher body mass index (BMI) than young subjects. # Age effect, p<0.05 old compared to young within time point. Data are means ± SEM.

(TIF)

Table S2  Primers for the qRT-PCR and TaqMan Low Density array (LDA) analysis. A. The mRNA expression of FoxO1, FoxO3, FoxO4, PGC-1α, PGC-1β, IL-6, MGF, IGF-1Ea and RPLP0 were analyzed by quantitative real-time RT-PCR. B. TaqMan based quantitative real-time RT-PCR of MuRF-1, Atrogin-1, NF-xB, Bax, BCL2L1, p53, TNF-α, ATG16B, GA-BARAPL1, and RPLP0 mRNA were performed in the ABI Prism 7900HT Sequence System (Applied Biosystems) using ABI TaqMan Low Density Arrays (Applied Biosystems).

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Author Contributions

Conceived and designed the experiments: CS UF PAA MK. Analyzed the data: LJ MJM GJ MB. Wrote the paper: PAA HS KH PS. Plos ONE 12 December 2012 | Volume 7 | Issue 12 | e51238

References


Ageing is associated with diminished muscle re-growth and myogenic precursor cell expansion early after immobility-induced atrophy in human skeletal muscle

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Key points

• Elderly individuals require a prolonged recovery phase in order to return to initial muscle mass levels following short-term immobilisation.
• The cellular mechanisms responsible for the attenuated re-growth and associated molecular signalling processes in ageing human skeletal muscle are not fully understood.
• The main study finding was the observation of a less marked muscle mass recovery after immobilisation in elderly compared to young individuals that was paralleled by an elevation in myogenic precursor cell content in young individuals only, whereas the elderly failed to demonstrate any change in myogenic precursor cells.
• No age-related differences were observed in the expression of major myogenic regulating factors known to promote skeletal muscle hypertrophy or satellite cell proliferation (IGF-1Ea, MGF, MyoD1, myogenin, HGF gene products).
• In contrast, the expression of myostatin demonstrated a more pronounced up-regulation following immobilisation along with an attenuated down-regulation in response to reloading in older compared to young individuals, which may have contributed to the present lack of satellite cell proliferation in ageing muscle.

Abstract Recovery of skeletal muscle mass from immobilisation-induced atrophy is faster in young than older individuals, yet the cellular mechanisms remain unknown. We examined the cellular and molecular regulation of muscle recovery in young and older human subjects subsequent to 2 weeks of immobility-induced muscle atrophy. Retraining consisted of 4 weeks of supervised resistive exercise in 9 older (OM: mean age) 67.3, range 61–74 yrs) and 11 young (YM: mean age 24.4, range 21–30 yrs) males. Measures of myofibre area (MFA), Pax7-positive satellite cells (SCs) associated with type I and type II muscle fibres, as well as gene expression analysis of key growth and transcription factors associated with local skeletal muscle milieu, were performed after 2 weeks immobility (Imm) and following 3 days (+3d) and 4 weeks (+4wks) of retraining. OM demonstrated no detectable gains in MFA (vastus lateralis muscle) and no increases in number of Pax7-positive SCs following 4wks retraining, whereas YM increased their MFA (P < 0.05), number of Pax7-positive cells, and had more Pax7-positive cells per type II fibre than OM at +3d and +4wks (P < 0.05). No age-related differences were observed in mRNA
expression of IGF-1Ea, MGF, MyoD1 and HGF with retraining, whereas myostatin expression levels were more down-regulated in YM compared to OM at +3d ($P < 0.05$). In conclusion, the diminished muscle re-growth after immobilisation in elderly humans was associated with a lesser response in satellite cell proliferation in combination with an age-specific regulation of myostatin. In contrast, expression of local growth factors did not seem to explain the age-related difference in muscle mass recovery.

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Abbreviations CDKN1A (p21), cyclin-dependant kinase inhibitor 1A; CDKN1B (p27), cyclin-dependant kinase inhibitor 1B; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HGF, hepatocyte growth factor; IGF-1Ea, insulin-like growth factor-1Ea; MFA, mean fibre area; MGF, mechano growth factor; MFA, myofibre area; OM, older males; RM, repetition maximum; SC, satellite cell; YM, young males.

Introduction

Human skeletal muscle is a highly plastic tissue, which is reflected by its ability to rapidly adapt to short-term changes in habitual loading intensity (Hespel et al. 2001; Jones et al. 2004; Hvid et al. 2011) and it has been demonstrated that elderly individuals require a prolonged recovery phase in order to return to initial muscle mass levels following short-term immobilisation (Suetta et al. 2009; Hvid et al. 2010). Yet, there is a paucity of studies examining the cellular mechanisms responsible for the attenuated re-growth and associated molecular signalling processes in ageing human skeletal muscle.

The regulation of muscle growth and maintenance of muscle mass are known to be influenced by a unique population of muscle resident stem cells referred to as satellite cells (SCs) or myogenic stem cells (Mauro, 1961; Moss & Leblond, 1970; Hespel et al. 2001). Notably, an impaired response to muscle damage has been documented as a consequence of ageing in mice (Conboy et al. 2003) and recently also demonstrated in human individuals when examining a subpopulation of individuals from the present intervention (Carlson et al. 2009). As suggested by the latter data, the age-related impairment in muscle re-growth following disuse could, at least in part, reside in an impaired capacity for myogenic stem cell proliferation and activation in aged myofibres (Carlson et al. 2009), but it is not known whether such changes are related to muscle fibre phenotype (type I vs. type II fibres). Further, systemic factors appear to play an important role in explaining the impaired proliferative capacity of SCs cultured from old vs. young human adults (Carlson et al. 2009) in close accordance with previous findings using the murine model (Conboy et al. 2005). There is, however, also evidence of local mechanisms influencing satellite cell activation (Sheehan et al. 2000; Horsley & Pavlath, 2003; Lorenzon et al. 2004; Mitchell & Pavlath, 2004) and recent data suggest a close relation between various systemic and local factors in the regulation of SC function in vivo (Chakkalakal et al. 2012). Furthermore, myogenic regulatory factors such as MyoD and myogenin, the growth and differentiation factor myostatin, as well as growth factors like hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF-1) have been shown to be involved in the regulation of muscle mass with changes in mechanical muscle loading while also affecting satellite cell activation, proliferation and differentiation (Mezzogiorno et al. 1993; Adams & Haddad, 1996; McPherron et al. 1997; McCroskery et al. 2003; Gopinath & Rando, 2008). However, it is not known to what extent the expression of these factors are associated with any age-related differences in recovery of muscle mass after a period of muscle immobilisation. Based on the previous findings, we hypothesised that satellite cell proliferation would be impaired especially in relation to type II myofibres along with a reduced expression of key anabolic genes in elderly compared to young individuals during rehabilitation after immobilisation of skeletal muscle.

Methods

Subjects

Twenty healthy males, 11 young males (YM; 24.4 years, range 21–30 years) and 9 older males (OM; 67.3 years, range 61–74 years) volunteered to participate in the study. Prior to inclusion all subjects were screened by a physician to exclude individuals with cardiovascular disease, diabetes, neural or musculoskeletal disease, inflammatory or pulmonary disorders or any known pre-disposition to deep venous thrombosis. The local ethics committee of Copenhagen and Frederiksberg approved the conditions of the study (KF01-322606) and all experimental procedures were performed in accordance with the Declaration of Helsinki. Written informed consent
Ageing affects human skeletal muscle recovery

was obtained from all participants before inclusion in the study.

### Intervention procedures

The intervention protocol along with data on changes in muscle contractile function and morphology have been described previously (Suëta et al. 2009; Hvid et al. 2010). In brief, immobility was accomplished by 2 weeks of randomised unilateral whole-leg casting using a lightweight whole-leg fibre cast extending from the malleoli to the proximal groin region. The retraining protocol comprised 4 weeks of surveyed and supervised unilateral strength training for the immobilised leg, with three sessions performed per week using a protocol consistently proven effective for inducing substantial gains in muscle size in elderly individuals with 12 weeks of training in our laboratory (Esmaeili et al. 2001; Lange et al. 2002; Suëta et al. 2004a,b). After a 5–8 min warm-up on a stationary bike, subjects performed isolated knee extension and flexion, and leg press exercises. Each exercise was performed in 3–4 sets × 12 repetitions (reps) (at 15 rep maximum (RM)) in week 1, followed by 5 sets × 10 reps (at 12RM) in weeks 2 and 3, and 4 sets × 10 reps (at 12RM) in week 4. Training loads were determined at baseline and loads were progressively adjusted on a weekly basis by use of 5RM testing.

### Muscle biopsy sampling

Muscle biopsies were obtained at ~1 week prior to the immobilisation (Pre), immediately after 2 weeks of immobilisation (Imm), following 3 days of retraining (+3d) and finally after 28 days of retraining (+4wks). Subjects were prohibited from exercise for at least 2 days before the first biopsy and were carefully instructed only to eat a light meal in the morning of the day of biopsy sampling. The intervention protocol along with data on changes in muscle damage from repeated biopsies (Guerra et al. 2011). After dissecting the muscle samples of visible blood, adipose and connective tissue, samples were divided into two separate pieces, one oriented in embedding medium (Tissue-Tek, Sakura Finetek, USA) frozen in isopentane cooled with liquid nitrogen and stored at −80°C and the other directly frozen in liquid nitrogen and stored at −80°C until further analyses.

### ATPase staining and muscle fibre area

Subsequently serial transverse sections (10 μm) were cut in a cryotome at −20°C and stained for myofibrillar ATPase at pH 9.4 after both alkaline (pH 10.3) and acid (pH 4.3 and 4.6) preincubations (Brooke & Kaiser, 1970). All samples of each individual person were stained in the same batch to avoid interassay variation. Based on the ATPase staining pattern muscle fibres were characterised as type I and type II and an average of 213 ± 39 fibres were analysed in each biopsy. For the determination of muscle fibre size only truly horizontal fibres were used, with a minimum of 50 fibres included for the analysis. A videoscope consisting of a microscope (Olympus BX 50) and colour video camera (Sanyo high resolution CCD) in combination with Tema Image Analysis System (Scanbeam, Denmark) were used to determine the mean fibre area of the muscle fibres.

### Satellite cell analysis

SC analysis was carried out by microscopic evaluation of cryosections that had been immunohistochemically stained for Pax7, as previously described in detail (Mackey et al. 2010). A combination of immunoenzymatic and immunofluorescence methods was employed to allow the staining of Pax7, type I myosin and laminin on the same section. Sections were fixed for 5 min with a 5% formaldehyde solution (Histofix, Histolab, Gothenburg, Sweden), followed by incubation for 1 h with blocking buffer (0.05 M Tris-buffered saline (TBS) containing 0.01% Triton X-100, 1% bovine serum albumin, 1% skimmed milk powder and 0.1% sodium azide). Satellite cells were labelled with a mouse anti-Pax7 antibody (cat. no. MO15020; Neuromics, Edina, MN, USA), diluted 1:500 in the blocking buffer, and incubated overnight at 4°C. The next day, the slides were washed in two changes of TBS containing 0.01% Triton. A biotinylated goat anti-mouse secondary antibody (cat. no. E0433; Dako Denmark, Glostrup, Denmark) was then applied, followed by Vector Elite ABC kit (cat. no. PK6100; Vector Laboratories, Peterborough, UK). Horseradish peroxidase activity was visualised with the ImmPACT diaminobenzidine substrate (cat. no. SK-4105; Vector yes Laboratories). The sections were then incubated for 2 h at room temperature with a mixture of the two primary antibodies, mouse anti-A4.951 (cat. no. A4.951; Developmental Studies Hybridoma Bank, Iowa, IA, USA) and rabbit anti-laminin (cat. no. Z0098; Dako, Denmark), for visualisation of type I myosin and laminin, respectively. A mixture of Alexa Fluor 488 goat anti-rabbit (Molecular Probes cat. no. A11034; Invitrogen, Taastrup, Denmark)
and Alexa Fluor 568 goat anti-mouse secondary antibodies (Molecular Probes, cat. no. A11031) was applied for 45 min. Washing in two changes of TBS was carried out between all steps, except for between the blocking and Pax7 incubation steps, where no washing was performed. 4’,6-Diamidino-2-phenylindole (DAPI) in the mounting medium (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36935) stained the nuclei, rendering nuclei blue, type I myosin red and laminin green. Sites of Pax7 antigenicity were stained brown, visible by light microscopy. The number of Pax7 cells associated with type I (A4.951-positive) or type II (A4.951-negative) fibres was counted separately and expressed relative to the total number of type I or type II fibres included in the assessment, as described in detail (Mackey et al. 2010; Fig. 2).

Real-time PCR

The mRNA expression of IGF-1Ea, mechano growth factor (MGF, also known as IGF-1Ec) and RPLP0 was analysed by real-time PCR as described previously (Suett et al. 2012). Total RNA (500 ng) was converted into cDNA in 20 μl using the OmniScript reverse transcriptase (Qiagen, CA, USA) according to the manufacturer’s protocol. For each of the mRNA targets, 0.25 μl cDNA was amplified in a 25 μl SYBR Green PCR reaction containing 1 × Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (Table 1A). The amplification was monitored real-time using the MX3000P real-time PCR machine (Stratagene, CA, USA). The threshold cycle (Ct) values were related to a standard curve made with the cloned PCR products and specificity ensured by melting curves analysis; the quantities were normalised to RPLP0. Quantitative real-time PCR of myostatin, Pax7, MyoD1, myogenin, FGF2, fibroblast growth factor receptor 1 (FGFR1), HGF, c-Met, CDKN1A (p21), CDKN1B (p27) and RPLP0 mRNA were performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, UK) using ABI TaqMan Low Density Arrays (Applied Biosystems; Table 1B). Each sample was run in triplicate with four samples per card. cDNA, 250 ng, was mixed with 100 μl TaqMan Gene Expression Mastermix and loaded into two ports (2.5 ng cDNA per reaction). Raw data were extracted and analysed using the SDS 2.1 software (Applied Biosystems, UK) and qBasePlus (Biogazelle) was used to quality-check Ct values, assess triplicates, exclude runs when the difference among triplicates exceeded 0.5Ct and finally to normalise data to RPLP0 using the 2Ct method (Livak & Schmittgen, 2001).

Correlation analyses

Correlation analyses were performed at post immobilisation (Imm) to examine the relationship between myofibre area (fibre type I and type II) and number of Pax7-positive (+) cells (total, fibre type I and type II), for young and older individuals combined. Furthermore, correlation analyses were performed between changes during the retraining (4wks relative to Imm) in myofibre area (MFA, fibre type I and type II) and number of Pax7+ cells (total, fibre type I and type II) for young and older individuals combined.

Statistical analyses

Statistical analyses were performed with SigmaPlot v11.0. Interaction between Age and Time was tested with a two-way repeated measures ANOVA. Differences over time were tested with one-way repeated measures ANOVA for Young and Old separately. If an overall time difference was found, the different time points were compared using Student–Newman–Keuls post hoc test. Pair-wise comparisons between Young and Old at each time point were obtained from the two-way repeated measures ANOVA (to include global variance) and Bonferroni corrected.

Non-parametric statistical analysis was used to evaluate changes in muscle fibre cross-sectional area, since not all data were normally distributed. To evaluate the effect of intervention over time, the Friedman two-way analysis of variance by ranks of related samples was used with subsequent analysis using the Wilcoxon signed rank test for paired samples. Intergroup differences were evaluated using the Kruskal–Wallis signed rank test. Correlation analysis was performed using the Spearman’s rho (r_s) method. Data are presented as mean values ± SEM or for mRNA data geometric means ± back-transformed SEM. A P value of less than 0.05 was considered significant.

Results

Muscle fibre cross-sectional area

Young individuals showed a 21.3% increase in type I fibre area (4830.4 ± 499.9 μm², P  0.05) after retraining (+4wks; Fig. 1). In contrast, no increases in type II fibre area (4830.3 ± 517.5 μm², P  0.05) along with a 35.5% increase in type II myofibre area (4347.5 ± 419.5 μm², 508.9 μm², P  0.05) after retraining (+4wks; Fig. 1). In contrast, no increases in type II fibre area (4830.3 ± 517.5 μm², P  0.05) after retraining (+4wks; Fig. 1).

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Table 1. Primers for qRT-PCR and TaqMan low density array (LDA) assay ID

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<tr>
<th>Gene products</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<td>A. Primers for qRT-PCR using SYBR Green assay</td>
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<td>MGF</td>
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A. mRNA expression of MGF (IGF-1Ec), IGF-1Ea and RPLP0 was analysed by SYBR Green-based quantitative real-time RT-PCR. B. TaqMan-based quantitative real-time RT-PCRs of MyoD1, myogenin, HGF, c-Met, FGF2, FGFR1, Pax7, myostatin, CDKN1A, CDKN1B and RPLP0 mRNA were performed using ABI TaqMan Low Density Arrays.

4848.2 ± 336.9 μm² or type II fibre area (4004.3 ± 467.4 vs. 4225.1 ± 276.2 μm²) were observed in the older individuals. Furthermore type II fibre area was restored with retraining in young individuals to reach higher levels than observed in older individuals (YM: 5386.3 ± 508.9 μm², OM: 4225.1 ± 276.2 μm², P < 0.05). No difference was observed in fibre-type distribution between YM (type I: 55.5%; type II: 44.5%) and OM (type I: 56.1%; type II: 43.9%) at Pre or following the interventions (Hvid et al. 2010).

Satellite cells: association with fibre type

To analyse for satellite cells, Pax7+ cells were assessed for type I and II fibres separately, as illustrated in Fig. 2. An overall effect of age was found for Pax7+ cells in relation to type II (P < 0.01), but not type I fibres (Fig. 3). The number of Pax7+ cells in the young subjects increased compared to Pre in both type I and II fibres at all time points. No changes in the elderly individuals were seen over time and in the type II fibres at +3d and +4wks; this was significantly different to the young individuals.

mRNA expression levels at baseline

At baseline (Pre) there was no age-related differences in the expression levels of IGF-1Ea, MGF, MyoD1, myogenin, HGF, c-Met, FGF2, FGFR1, Pax7, CDKN1A (p21) or CDKN1B (p27) whereas the expression levels of myostatin mRNA and GAPDH were lower in old compared to young (Fig. 4, P < 0.05).

IGF-1Ea and MGF

Expression levels of IGF-1Ea and MGF (IGF-1Ec) mRNA increased in young individuals with immobilisation (Fig. 5A and B). Expression levels of IGF-1Ea demonstrated a subsequent marked decrease after 3 days of retraining in young individuals and after 4 weeks of retraining mRNA expression levels were increased compared to baseline levels as well as post immobilisation (Imm) in both young and old (Fig. 5A, P < 0.05). Expression levels of MGF mRNA remained elevated after 3 days of retraining in the young (P < 0.05), and after 4 weeks of retraining mRNA expression levels were further increased compared to baseline and post immobilisation (Imm) levels, respectively, in the young (Fig. 5B, P < 0.05).

MyoD1 and myogenin

MyoD1 expression increased in both young and older males with immobilisation, and in addition showed a marked decrease following 3 days of retraining in both young and old (P < 0.05, Fig. 5C). After 4 weeks of retraining MyoD1 mRNA levels returned to baseline levels in both young and old (Fig. 5C). Myogenin mRNA expression increased markedly in both young and old, while decreasing after 3 days of retraining in both young...
and old (Fig. 5D, \( P < 0.05 \)). After 4 weeks of retraining myogenin expression levels remained reduced compared to post immobilisation levels (Fig. 5D).

**HGF and c-Met**

Expression levels of HGF and c-Met mRNA increased in young individuals in the early phase of retraining (+3d, \( P < 0.05 \); Fig. 5E and F) but remained unaltered after immobilisation and more prolonged retraining. In old individuals no change in expression levels of HGF and c-Met was observed at any time point.

**FGF2 and FGFR1**

FGF2 mRNA levels remained unaltered at all time points in young as well as older individuals (\( P < 0.05 \), Fig. 5G). Conversely, FGFR1 mRNA expression increased in both young and old following immobilisation, followed by marked decreases after 3 days of retraining in both young and old individuals in the early phase of retraining (\( P < 0.05 \); Fig. 5J). After 4 weeks of retraining, FGFR1 mRNA expression increased compared to baseline and the initial training phase (+3d) approaching values similar to the expression levels observed following immobilisation (Fig. 5H).

**Pax7**

The expression of Pax7 mRNA following immobilisation was higher in the older males compared to the young males (Fig. 5I). Although there was an interaction (Age \( \times \) Time, \( P < 0.05 \)), it was not possible to pinpoint whether this difference was due to an increase in the elderly or a decrease in the young, or both. No differences with time or age were detected at the retraining time points, except for a lower level at +3d compared to +4wks in the young.

**Myostatin**

Myostatin mRNA expression increased in the elderly with immobilisation, and showed a marked decrease after 3 days of retraining (+3d) in both young and old (\( P < 0.05 \), Fig. 5J). After 4 weeks of retraining, myostatin expression levels returned to baseline levels in both young and old. The temporal changes in myostatin mRNA expression differed between young and old individuals, with a less pronounced down-regulation of myostatin expression levels in the initial phase of retraining (+3d) in older individuals (\( P < 0.05 \), Fig. 5J).

**CDKN1A (p21) and CDKN1B (p27)**

Expression levels of CDKN1A (p21) remained unchanged with immobilisation (Imm) while decreasing in both young and older males after 3 days of retraining (\( P < 0.05 \); Fig. 5K). After 4 weeks of retraining CDKN1A mRNA expression returned to baseline levels in both young and old (\( P < 0.05 \), Fig. 5K). No change in the expression level of CDKN1B (p27) was observed with immobilisation; however, a marked decrease was observed in both young and old after 3 days of retraining (\( P < 0.05 \), Fig. 5L). After 4 weeks of retraining CDKN1B mRNA expression levels returned to baseline levels in both young and older individuals (Fig. 5L).

**Correlation analysis**

After muscle disuse, the number of Pax7+ cells was positively related to myofibre area \( (r_s = 0.712, P < 0.01; n = 16) \). Comparison within fibre type showed that this relationship was true for both type I \( (r_s = 0.779; P < 0.001; n = 16) \) and type II fibres \( (r_s = 0.694; P < 0.01; n = 16) \). Notably, analysing the retraining phase, relative
Membranes were subsequently incubated overnight at 4°C with primary antibody LC3B (1:1000, #2775, Cell Signaling Technology) as well as S6 ribosomal protein (1:1000, #2217, Cell Signaling Technology Inc.) and phospho-S6 ribosomal protein (Ser235/236) (1:2000, #4858, Cell Signaling Technology Inc.). Membranes were subsequently washed and incubated for 1 h with HRP-conjugated secondary antibody (Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate, #170-5046, BioRad, Hercules, Ca, USA) and visualized with Immun-star western kit (#170-5070, BioRad, Hercules, Ca, USA). After detecting the phosphorylated protein, the membrane was stripped and the total protein was detected. Band exposure and visualization were quantified using ChemiDoc XRS with Image Lab Software (Bio-Rad Laboratories, Inc.)

Statistical analyses
A part from total and phosphorylated S6 ribosomal protein, all mRNA and Western blot data were log-transformed prior to statistical analyses and are presented as geometric means ± back-transformed SEM. To test for changes over time (mRNA, TUNEL, Immunohistochemistry and Western blot analyses) one-way Bonferroni corrected repeated-measures ANOVA was performed separately for young and old individuals, respectively, as well as for young and old combined (SPSS). Pair wise multitude comparison procedures were evaluated using Student-Newman-Keuls Method post-hoc testing. Independent-samples t-testing were used to test for differences between groups’ with a subsequent Bonferroni correction. As phosphorylated S6 ribosomal protein (but not total S6 ribosomal protein) became non-detectable after immobilization in a high number of especially young subjects, quantification of phosphorylated S6 ribosomal protein/total S6 ribosomal protein was not possible and we therefore decided to perform a Chi-square test (young p, 0.001, old p = 0.44). The percentage of the total number of subjects where p-S6 could be detected, at each time-point, are visualized in figure 4D. Non-parametric statistics were used to analyze changes in muscle fiber CSA, since not all of these data were normally distributed. To evaluate the effect of intervention over time a repeated-measures Friedman test was used with post-hoc Wilcoxon testing. Between-group differences were analyzed with Kruskall-Wallis tests and subsequent Mann-Whitney U testing. Data are presented as mean values ± SEM. A p-value of less than 0.05 was considered statistically significant.

Results
Maximal contractile muscle strength
Maximal contractile muscle strength declined in both young (13%) and old (14%) after 4 days of unloading, with no difference between age groups (Figure S1A). Following 14 days of unloading maximal isometric muscle strength was reduced by 20% in young and by 16% in old, again with no age-related differences (Figure S1A).

Muscle fiber cross sectional area
Despite the very limited period of immobilization, our histological analyses revealed significant decreases in mean myofiber area of approximately 10% in both young and old subjects after 4 days of immobility (Figure 1B). However, following 14 days of immobilization the loss in mean myofiber size was greater in young (−19.9%) than old individuals (−12.6%, p<0.01) (Figure 1B). There was no difference between the decline in type 1 (O: −7.1%, Y: −8.1%) and type 2 (O: −10.9%, Y: −12.6%) fibers at the 4 d time-point, however after 14 d the decline in type 2 fibers (O: −17.1%, Y: −26.3%) was significantly larger than in type 1 fibers (O: −8.9%, Y: −14.3%) in both young and old (p<0.05) (Figure S1B).
response to short-term reloading in old compared to young humans. However, due to the relatively short observation period, we cannot conclude to what extent the recovery of skeletal muscle mass in elderly undergoing short-term immobilisation is impaired or just occurs more slowly than in young counterparts. In support of the second view, observations over more prolonged periods of reloading (e.g. 12 weeks) have demonstrated that the elderly can fully recover whole muscle cross-sectional area and myofibre area after long-term (months to years) muscle disuse due to hip osteoarthritis and subsequent elective hip-replacement surgery, but only if the reloading phase comprises a systematic use of resistance-based exercise (Suetta et al. 2004b, 2008).

Changes in myogenic progenitor cells with reloading

In skeletal muscle the myogenic stem cells, also referred to as satellite cells (SCs), are known to play a key role in the maintenance, growth and repair of myofibres (Mauro, 1961; Moss & Leblond, 1970; Heslop et al. 2001; Pallafacchina et al. 2012). During the process of load-induced muscle hypertrophy, satellite cells are thought to proliferate, differentiate and eventually fuse with existing myofibres (McCormick & Schultz, 1994). The resulting donation of new myonuclei by the fusion of SCs with existing myofibres is thought to ensure that myonuclear domain size stays within certain functional limits in situations of marked myofibre hypertrophy (Kadi et al. 2004b; Petrella et al. 2008). In humans, skeletal muscle SCs seems to be maintained into the seventh decade of life (Roth et al. 2000; Petrella et al. 2006; Hikida, 2011), with a decline in content and activation capabilities with progressive ageing (Renault et al. 2002; Kadi et al. 2004a; Verdijk et al. 2007) accompanied by a reduced migration capacity of existing SCs in turn resulting in a reduced regeneration potential after muscle injury and disuse (Carlson & Faulkner, 1989; Mitchell & Pavlath, 2004; Conboy et al. 2005; Carlson & Conboy, 2007). Despite a mean age of only ∼70 years in our aged individuals signs of impaired SC proliferation with immobilisation and subsequent retraining were observed compared to young subjects (∼25 years old), indicating an attenuated myogenic response to changes in exercise pattern (Fig. 3A and B). Interestingly, compared to the changes induced by 2 weeks of disuse, more accentuated age-related differences in SC activation were observed in the acute (+3d) as well as the prolonged (+4wk) phase of reloading (Fig. 3A and B). The latter trend is in line with Dreyer et al. (2006) reporting a greater increase in SC content in young compared to aged skeletal muscle within 24 h following 92 maximal eccentric muscle contractions (Dreyer et al. 2006). The importance of SC number in relation to muscle size across the age-span was underlined by Kim and co-workers who demonstrated a positive linear association between muscle size and SC number in baseline muscle biopsies obtained from young and older human individuals (Kim et al. 2005b). Extending those data, here we report for the first time in human individuals that a positive relationship exists between SC number and myofibre area following disuse atrophy (Fig. 5A) as well as in response to subsequent reloading (Fig. 5B).

Since human ageing is associated with a preferential reduction in muscle fibre type II size (Andersen, 2003; Aagaard et al. 2010) it has been speculated that SC content might decrease more in type II fibres compared to type I fibres with ageing (Kim et al. 2005b). In young human individuals, SC appears to be similar between type I and type II muscle fibres (Kadi et al. 2006; Verdijk et al. 2007). In contrast, age-related type II muscle fibre atrophy is accompanied by a type II muscle fibre-specific reduction in SC content (Verdijk et al. 2007; Verney et al. 2008). In support of these observations, young and old adults demonstrating marked exercise-induced gains in myofibre area (i.e. ‘hypertrophy responders’) are characterised by a greater concurrent up-regulation in myogenic SCs compared to individuals with a less robust hypertrophy response (Petrella et al. 2008). However, although the
regenerative capacity of human skeletal muscle seems to decline at a more advanced age (reflected by a decline in SC number and/or proliferative capacity), the impairment in SC function does not seem to prevent a significant capacity for muscle hypertrophy provided that the intervention period is sufficiently long (months), as reported even at very old age (Thornell et al. 2003; Dedkov et al. 2003; Shefer et al. 2006).

Changes in IGF-1 expression with reloading

Numerous growth factors are known to regulate satellite cell activity, among which insulin-like growth factor 1 (IGF-1) is known to play an essential role in the process of muscle hypertrophy (Rosenblatt et al. 1994; Adams & Haddad, 1996; Suetta et al. 2010). The discovery of distinct IGF-1 isoforms (mechano growth factor (MGF) and IGF-1 Ea) has suggested different roles for IGF-1, namely that MGF mainly triggers satellite cell activation and proliferation, while IGF-1 Ea is thought to mainly promote differentiation of proliferating SCs (Yang & Goldspink, 2002). In line with those findings the present study demonstrated a differentiated regulation in IGF-1 Ea and MGF expression, with both an acute and a sustained up-regulation of MGF mRNA expression in response to retraining (+3d and +4wks), whereas IGF-1 Ea expression was up-regulated in the later phase of reloading (+4wks) only, suggesting a supportive role of paracrine/autocrine IGF signalling in the process of human muscle hypertrophy, at least when recovering from short-term muscle disuse. In contrast to earlier findings in rodent and human skeletal muscle (Owino et al. 2001; Hameed et al. 2003) the present up-regulation in MGF mRNA expression with reloading (post 4 weeks resistance training) was not attenuated in older vs. young individuals in the present study.

Changes in MyoD1 and myogenin expression with reloading

In the present study MyoD1 and myogenin expression were markedly up-regulated with immobilisation in both age groups, whereas subsequent reloading led to both an acute (+3d) and a sustained (+4wks) decrease in MyoD1 and myogenin expression in young as well as aged skeletal muscle. The rise in MyoD1 and myogenin mRNA expression following immobilisation independently of age may represent a compensatory signalling pathway for partial muscle retention during periods of acute muscle loss, while also observed in other atrophy situations (Alway et al. 2001). Following resistance-type exercise increased expression of MyoD1 and myogenin mRNA has been observed in both young and older adults (Psilander et al. 2003; Kim et al. 2005a; Kosek et al. 2006; Raue et al. 2006; Costa et al. 2007; McKay et al. 2008). In the present study, however, only a modest up-regulation in myogenin expression compared to the basal non-immobilised state was observed following the reloading phase consisting of 4 weeks of resistance training, suggesting that retraining after short-term disuse atrophy may evoke different molecular signalling stimuli compared to regular resistance exercise intervention.

Changes in HGF and c-Met expression with reloading

Hepatocyte growth factor (HGF) is generally considered to be one of the most important growth factors involved in organ regeneration (Zarnegar, 1995) as well as a key regulator of satellite cell activity during muscle regeneration (Jennische et al. 1993). The presence of HGF transcripts in newly regenerated myotubes and in satellite cells suggests that HGF activity is mediated primarily through paracrine/autocrine mechanisms (Anastasi et al. 1997; Sheehan & Allen, 1999). Furthermore, HGF is a
potent growth factor that has the ability to stimulate quiescent satellite cells to enter the cell cycle early in vitro as well as in vivo (Allen et al. 1995; Tatsumi et al. 1998) and is therefore considered most important during the early phase of re-growth (Tatsumi et al. 1998). In support of this, we observed a significant increase in HGF mRNA expression in young individuals in response to early retraining (+3d) but not after more sustained retraining.

**Figure 5.** mRNA expression levels relative to baseline following 2 weeks of immobilisation and 4 weeks of retraining

- A, IGF-1Ea; B, MGF; C, MyoD1; D, myogenin; E, HGF; F, c-Met; G, FGF2; H, FGFR1; I, Pax7; J, myostatin; K, CDKN1A (p21); L, CDKN1B (p27). *Time effect, \( P < 0.05 \) compared to Pre. #Time effect, \( P < 0.05 \) compared to Imm. §Time effect, \( P < 0.05 \) compared to +3d. ∞Age effect, \( P < 0.05 \) young compared to old. Data are geometric means ± back-transformed SEM.
In contrast to HGF, a marked decrease in the HGF receptor c-Met expression was observed in response to early retraining (+3d) in young individuals, which may support the hypothesis previously proposed that increasing concentrations of HGF reduces c-Met which forms a negative feedback mechanism inducing SC quiescence in regenerating muscle (Tatsumi et al. 2009; Yamada et al. 2010).

Changes in FGF2 and FGFR1 expression with reloading

In recent years, fibroblast growth factors (FGFs) and their receptors (FGFRs) have gained increased focus as major players in both embryonic development and skeletal muscle tissue repair (Coutu & Galipeau, 2012). Moreover, somatic stem cells have been suggested as major targets of FGF signalling in both tissue homeostasis and repair where FGFs appear to promote self-renewing proliferation and inhibit cellular senescence in nearly all tissues tested to date (Coutu & Galipeau, 2012). Fibroblast growth factor 2 (FGF2) is a polypeptide growth factor that stimulates SC proliferation in already activated SCs (Allen & Boxhorn, 1989; Mezzogiorno et al. 1993). However, despite our findings of marked SC proliferation with reloading, expression levels of FGF2 remained unchanged at all time points examined, suggesting that FGF may be less important for SC proliferation in human skeletal muscle, at least in relation to reloading subsequent to immobilisation.

Changes in Pax7 expression with reloading

The observed differences in immunohistochemical Pax7+ cell content of the vastus lateralis muscle in young and older individuals during immobilisation and subsequent retraining (Fig. 3) were not associated with corresponding changes in mRNA for Pax7. In fact, the expression level of Pax7 was down-regulated in young and up-regulated in old individuals following 2 weeks of immobilisation (Fig. 5I), indicating that factors other than Pax7 mRNA levels may influence the content of Pax7+ SCs during immobilisation and retraining conditions in humans.

Changes in myostatin expression with reloading

Myostatin is a member of the transforming growth factor-β superfamily and a strong negative regulator of skeletal muscle mass, known to inhibit myogenic SC activation (McPherron et al. 1997; Trendelenburg et al. 2009). Although the mechanisms are not fully understood, myostatin is thought to modulate key regulators of the cell cycle such as cyclin-dependent kinase inhibitors p21cip and p27kip (Kim et al. 2005a), thereby inhibiting SC cycle progression from G0 to S phase (McCroskery et al. 2003). Down-regulated myostatin mRNA expression has been observed in response to exercise training in both young and elderly individuals (Roth et al. 2003; Kim et al. 2005a; Raue et al. 2006; Costa et al. 2007), with some studies reporting an attenuated response with ageing (Kim et al. 2005b; Haddad & Adams, 2006). In line with the latter findings, McKay and colleagues recently demonstrated a...
markedly blunted myogenic response in older compared to younger individuals (McKay et al. 2012). Although stem cell-specific myostatin levels did not appear to differ at baseline, acute resistance exercise (75% 1RM) was found to evoke ∼70% greater content of myostatin-positive type II-associated SCs in old versus young adults at 24 h post exercise, suggesting that the greater co-localisation of myostatin with SCs may provide a mechanism for the impaired myogenic capacity of aged muscle (McKay et al. 2012). Somewhat unexpectedly, the expression level of myostatin mRNA was lower in old compared to young at baseline in the present study, which might reflect the rather high activity level (equal to that of young) of the present group of elderly individuals. Despite this, an age-specific difference in the regulation of myostatin was also observed in the present study, manifested by a reduced suppression with reloading in aged vs. young individuals (Fig. 5J). This observation may explain, at least in part, the impaired capacity for SC proliferation and re-growth in aged skeletal muscle observed in the present study, although the interpretation of these data is limited if only assessing transcript levels.

Changes in cyclin-dependent kinase inhibitors with reloading

In line with the regulation in myostatin mRNA, expression levels of cyclin-dependent kinase inhibitors CDKN1A (p21) and CDKN1B (p27) decreased in response to acute loading in the present study. Both CDKN1A and CDKN1B are known to block cell cycle progression and induce SC cell cycle withdrawal (Coqueret, 2003). Furthermore, ectopic expression of CDKN1B has been shown to block the IGF-I-induced increase in satellite cell proliferation (Chakravarthy et al. 2000) and thus CDKN1B is considered a key regulatory factor in the regulation of satellite cell cycle progression (Machida et al. 2003).

Conclusions

Collectively, the present data suggest that significant age-specific differences may exist for the ability of human skeletal muscle to regenerate after immobility-induced muscle atrophy. More specifically, our results indicate an attenuated – or at least delayed – response in aged individuals to active reloading subsequent to short-term

![Figure 6. Association between changes in myofibre area and number of Pax7+ cells](https://example.com/figure6.png)

A, number of Pax7+ cells versus fibre area for all fibres collapsed or separated into type I or II fibres post immobilisation (Imm). B, relative changes in myofibre area (MFA, type I or type II) following 4 weeks of retraining (relative to post immobilisation) versus the change in number of Pax7+ cells (total, type I associated and type II associated). Open triangles, young individuals; filled triangles, older individuals.

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disuse, as reflected by attenuated gains in myofibre area and SC number despite no age-related differences being observed in local growth factors responsible for promoting skeletal muscle hypertrophy (IGF-I, MyoD, myostatin). These disparate trends may partly reside on a reduced cellular sensitivity to paracrine/autocrine growth factors as basal MRF mRNA expression appears to be chronically up-regulated in senescent muscle (Edström & Ulfhake, 2005; Kim et al. 2005a; Kosek et al. 2006; Raue et al. 2006). Our findings of an age-specific regulation in myostatin expression levels may also have contributed to the apparent lack of increase in SC number and myofibre area with ageing in response to reloading. Gaining an improved understanding of the ability of human skeletal muscle to recover from atrophy has important implications for the development of effective molecular and rehabilitative countermeasures against physical frailty in the continuously growing population of elderly adults.

References


Aging affects human skeletal muscle recovery


Additional information

Competing interests

None.

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Translational perspective

We report measures of myofiber area and myogenic stem cell number (SC) associated with type I and type II muscle fibres in young and older humans, in combination with transcriptional data from regulatory pathways related to skeletal muscle re-growth in immobilised and re-trained aging human muscle. The main study finding was a less marked muscle mass recovery after immobilisation in elderly compared to young individuals that was paralleled by an elevation in SC content in young only, whereas elderly failed to demonstrate any change in SC’s. This potential coupling of SC proliferation and recovery in myofiber area in young individuals occurred despite no age related differences in the expression of myogenic regulating genes normally known to promote skeletal muscle hypertrophy or SC proliferation. However, expression of myostatin was more pronounced after immobilisation along with an attenuated down-regulation in response to re-loading in older compared to young individuals, which may have contributed to the lack of SC proliferation in aging muscle. The age-specific regulation in myostatin expression may also have contributed to the apparent lack of increase in SC number and myofiber area with aging in response to re-loading. Collectively, the present findings underlines that elderly have an impaired ability to recover from disuse muscle atrophy and thus, elderly may need longer time to recover from periods of disuse or disease compared to younger ones.

Gaining insight in the ability to recover muscle from atrophy has implications for effective molecular and rehabilitative countermeasures against frailty in the growing population of elderly.
Aging impairs the recovery in mechanical muscle function following 4 days of disuse

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ABSTRACT

As aged individuals are frequently exposed to short-term disuse caused by disease or musculoskeletal injury, it is important to understand how short-term disuse and subsequent retraining affect lower limb mechanical muscle function. The purpose of the present study was, therefore, to investigate the effect of 4 days of lower limb disuse followed by 7 days of active recovery on mechanical muscle function of the knee extensors in young (24.3 ± 0.9 years, n = 11) and old (67.2 ± 1.0 years, n = 11) recreationally active healthy males. Slow and moderate dynamic muscle strength were assessed using isokinetic dynamometry (60 and 180° s−1, respectively) along with isometric muscle strength and rapid muscle force capacity examined as contractile rate of force development (RFD), Impulse, and relative RFD (rRFD) during the initial phase of contraction (100 ms time interval relative to onset of contraction). Prior to disuse, marked age-related differences (p < 0.05) were observed in isometric and dynamic muscle strength (~35%) as well as in RFD and Impulse (~39%). Following disuse, young and old individuals experienced comparable decrements (p < 0.05) in isometric strength (~9%), slow dynamic strength (~13%), and RFD and Impulse (~19%), whereas old individuals only experienced decrements (p < 0.05) in moderate dynamic strength (12%) and rRFD (~17%). Following recovery, all measures of mechanical muscle function were restored in young individuals compared to pre-disuse values, while isometric, slow and moderate dynamic muscle strength remained suppressed (p < 0.05) in old individuals (~8%) along with a tendency to suppressed RFD100 (p = 0.068). In conclusion, 4 days of lower limb disuse led to marked decrements in knee extensor mechanical muscle function in both young and old individuals, yet with greater decrements observed in moderate dynamic strength and rapid muscle force capacity in old individuals. While 7 days of recovery – including free ambulation, one test session and a single session of strength training – was sufficient to restore mechanical muscle function in young individuals, old individuals appeared to have an impaired ability to fully recover as evidenced by suppressed values of isometric and dynamic muscle strength and rapid muscle force capacity.

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1. Introduction

Disuse has been shown to induce marked impairments in lower limb mechanical muscle function of both young and old individuals, characterized by decrements in isometric and dynamic muscle strength as well as in rapid muscle force capacity (e.g. rate of force development (RFD) and contractile Impulse) (Deschenes et al., 2008; Hvid et al., 2010; Kortebein et al., 2008; LeBlanc et al., 1992; Narici and de Boer, 2011; Suetta et al., 2009). As these specific measures of muscle function have been shown to be strong predictors of general functional status, quality of life, and risk of mortality in aged individuals (Buchman et al., 2007; Cesari et al., 2009; Newman et al., 2006; Pijnappels et al., 2008; Wyszomierski et al., 2009), even short periods of disuse may lead to severe consequences for a single individual. Specifically, rapid muscle force capacity (i.e. the ability to produce as much force as possible within fractions of a second, e.g. 100 ms) has been advocated to be highly important for the ability to counteract unexpected perturbations during walking and thus avoiding falling (Aagaard et al., 2002; Caserotti et al., 2008; Wyszomierski et al., 2009). In support, having a low vs. a high rate of force development (RFD) has been shown to discriminate older fallers from non-fallers, respectively (Pijnappels et al., 2008).

Based on existing data, impairments in lower limb mechanical muscle function appear to occur very fast within the initial phase (days) of disuse followed by an attenuated rate of decline as time progresses (Deschenes et al., 2008; Deutz et al., 2013; Hvid et al., 2010, 2013; Kortebein et al., 2008; LeBlanc et al., 1992; Narici and de Boer, 2011; Suetta et al., 2009, 2012). Moreover, the subsequent recovery in mechanical muscle function following cessation of disuse appears to be compromised in older compared to younger individuals, at least after more prolonged periods (14 days) of disuse (Hvid et al., 2010;
Suetta et al., 2009), altogether contributing to an increased risk of functional impairments and disability.

Yet, no previous studies have comprehensively investigated lower limb mechanical muscle function in response to disuse lasting less than 7 days and subsequent recovery in aged individuals. As aged individuals are frequently exposed to short periods of disuse due to disease or musculoskeletal injury occasionally involving hospitalization (Cookson and Laudicella, 2011; Covinsky et al., 2003; Saczynski et al., 2010), knowledge of the potential disuse-induced impairments in mechanical muscle function as well as of the responsible underlying mechanisms is of major importance.

The purpose of the present study was, therefore, to investigate the effect of 4 days of lower limb disuse followed by 7 days of recovery on mechanical muscle function of the knee extensors (isometric and dynamic strength as well as rapid muscle force capacity) in young and old healthy men. It was hypothesized that young and old individuals would show a comparable decline in mechanical muscle function with disuse, and that old individuals would have an impaired ability to recover compared to young individuals.

2. Material and methods

2.1. Study design

While the present study focuses on the effects of short-term disuse and subsequent recovery on lower limb muscle mechanical function in old individuals vs. young individuals, detailed information on the disuse design and additional data comprising disuse-induced effects on single fiber contractile function and molecular signaling pathways have previously been published (Hvid et al., 2013; Suetta et al., 2012). In brief, young and old healthy men underwent 4 days of unilateral lower limb disuse followed by 7 days of active recovery. In addition to a familiarization session (~2 weeks prior to the start of the study (Fam), tests of knee extensor mechanical muscle function as well as body weight and height were performed ~1 week before (Pre) and 24 hrs after disuse (Post), as well as 7 days after recovery (Rec) (Fig. 1). Tests of mechanical muscle function were performed in both limbs, thus with the contra-lateral “non-disused” leg serving as an internal control. Muscle biopsies were obtained from the vastus lateralis muscle at Pre, Post, and Rec, in order to examine myosin heavy chain (MHC) isoform composition as and muscle fiber cross-sectional area (CSA) of the disused leg. To minimize the influence of discomfort from the biopsy and particularly the risk of severe muscle damage in the older subjects, tests of mechanical muscle function were performed the day following the muscle biopsy (Fig. 1) according to previous procedures (Hvid et al., 2010; Suetta et al., 2009). Subjects were instructed not to engage in any vigorous activities 48 hrs prior to all test sessions. To minimize the influence from diurnal variation, each subject was tested at the same time of day (~±2 hrs).

2.2. Subjects

While twenty-three healthy men were initially recruited for the study, one subject did not adhere to the disuse protocol for which reason his data was excluded from the analysis. Altogether, 11 young men (24.3 ± 0.9 yrs, 180.4 ± 2.7 cm, 74.3 ± 2.4 kg) and 11 old men (67.2 ± 1.0 yrs, 178.8 ± 1.7 cm, 87.7 ± 3.0 kg) participated in the study, with body mass differing between young and old individuals (p < 0.05). Care was taken to include young and old individuals with comparable levels of physical activity. This was done by using a questionnaire to estimate the amount (hours per week) of occupational (groups I–IV, ranging from predominately sedentary work to heavy manual work) and recreational activities (groups I–IV, ranging from almost completely inactive to regular high-intense physically active, including description of the activities) on a general basis (Saltin and Grimby, 1968). Based on the questionnaire data, physical activity levels were similar in young and old individuals (4.3 ± 0.6 vs. 4.4 ± 0.6 hrs wk−1, respectively) with no differences in low-to-moderate intensity occupational (group II sitting or standing, some walking) and recreational activities (group II some physical activity during at least 4 h per week). None of the subjects had previous experience with systematic resistance training.

Sample size was calculated based on findings from previous short-term disuse studies (Deschenes et al., 2008; Hvid et al., 2010; Suetta et al., 2009) using a statistical power (β) of 0.80, level of significance (α) = 0.05, and an expected range of change of ~10% in maximal knee extensor isometric strength.

All subjects were thoroughly informed of the details of the study and gave written, informed consent prior to participation. The study was approved by the Ethical Committee of Copenhagen and Frederiksberg in accordance with the Helsinki declaration (KF01-322606).

2.3. Disuse protocol

Details of the disuse protocol have been described elsewhere (Hvid et al., 2013). Briefly, 4 days of randomized unilateral lower limb disuse was conducted using a knee brace (DonJoy, DJO Global Inc., USA) fixed in a 30° knee angle (0° = full extension), with plastic strips applied to ensure that the brace was not removed during the disuse period. All ambulatory activities were carried out on the contralateral limb using crutches. Subjects were carefully instructed to refrain from weight-bearing activities (~ground contact) using the disused leg, but were encouraged to perform unloaded ankle flexion–extension on a daily basis in order to reduce the potential risk of venous thrombosis. Daily contact was kept with all subjects to ensure optimal compliance and to avoid health implications.

2.4. Recovery protocol

Immediately after the removal of the knee brace, subjects were biopsied and subsequently received manual mobilization of the disused leg to ensure that minimal pain was present and that normal range of motion could be obtained at the knee joint. In addition to returning to free ambulation and the test session of mechanical muscle function the day after the removal of the knee brace, subjects performed one session of supervised unilateral resistance training (including 5RM testing) of the disused leg three days after the removal of the brace (Fig. 1). Exercises included knee extension, leg press, and knee flexion using load adjustable machines (Technogym Global, Italy). Following a brief warm-up (cycle ergometer, 5 min, 50–150 W) and determination of loading intensity of the chosen exercises by use of the 5RM testing, subjects performed 3 sets × 12 reps (at 15RM) using moderate (~1–2 s) and slow movement speeds (~3–4 s) in the concentric and eccentric contraction phases, respectively.
2.5. Assessment of knee extensor mechanical muscle function

Maximal voluntary isometric (MVC) and dynamic muscle strength as well as rapid muscle force capacity (RFD and Impulse) were obtained for the knee extensors using an isokinetic dynamometer (Kinetics Communicator – Chattecx, USA) (Aagaard et al., 2002; Hvid et al., 2010).

2.5.1. Dynamic and isometric muscle strength

Following several warm-up trials, subjects performed a number of maximal dynamic concentric knee extensions at slow (60° s⁻¹) and at moderate angular speed (180° s⁻¹) in a range of 90 to 20° (0° = full extension). Subsequently, subjects performed three maximal isometric knee extensions at a knee joint angle of 70°, while instructed to contract as fast and forcefully as possible, and maintain maximal force exertion for 2–3 s. All knee extension trials were separated by a 1 min rest period. For both slow and moderate dynamic strength as well as isometric strength, the trial with the highest force (–torque) was selected for further analysis (including rapid muscle force capacity, see below). Strong verbal encouragement along with online visual feedback was given to the subjects during all testing. Onset of contraction was defined as the instant when force production exceeded the baseline level force by 3% of the maximal force value. All trials with a visible initial countermovement were discarded from the analysis. Individual dynamometer settings were registered to ensure identical subject positioning at all test sessions (Fig. 1). Measures of knee extensor muscle strength were gravity corrected and subsequently normalized to body mass, thus reflecting how well an individual would cope with whole body movement tasks (Hvid et al., 2010). Yet, to enable comparison to previously published data, absolute values of isometric strength are presented also.

2.5.2. Rapid muscle force capacity

Rapid muscle force capacity was assessed as the contractile rate of force (torque) development (RFD), Impulse, and relative RFD (rRFD) (Aagaard et al., 2002). RFD was derived as the average tangential slope of the torque–time curve (ΔTorque/Δtime) calculated in the time interval 0–100 ms relative to the onset of contraction (RFD₁₀₀), and contractile Impulse as the area under the torque–time curve (∫ Torque dt) in the same time interval (Impulse₁₀₀), thus reflecting the initial phase of rising muscle force. In comparison to RFD, contractile Impulse additionally reflects the entire time history of contraction and consequently the velocity that the limb segment would achieve at that specific time instant (100 ms) if allowed to move (Aagaard et al., 2002). Measures of rapid muscle force capacity were normalized to body mass, thus reflecting how well an individual would cope with performing rapid whole body movement tasks (Hvid et al., 2010). We chose to analyze rapid muscle force capacity in the time interval 0–100 ms, as this has previously been argued to be functionally important (Aagaard et al., 2002; Wyszomierski et al., 2009) e.g. in discriminating older fallers from non-fallers (Pijnappels et al., 2008). The rRFD was calculated by expressing absolute RFD values relative to maximal isometric strength (MVC) (Aagaard et al., 2002). This measure can be used to examine whether intervention-induced changes in rapid muscle force capacity is due to changes in ‘qualitative’ factors (e.g. initial motor unit firing rate, MHC isoform composition, single muscle fiber contractile function, tendon mechanical properties) (Aagaard et al., 2002).

2.6. MHC isoform composition and muscle fiber cross-sectional area

Muscle biopsies (Bergstrom, 1962) were obtained from the middle portion of the vastus lateralis muscle approximately one week prior to disuse (Pre), immediately after removal of the knee brace (Post), and following 6 days of recovery (Post). This was carried out by the same investigator in order to minimize variability due to muscle depth and vicinity of location.

MHC isoform composition was analyzed as previously described using gel electrophoresis (Andersen and Aagaard, 2000; Daniell Betto et al., 1986; Hvid et al., 2011, 2013). Briefly, one part of the biopsy muscle sample was manually homogenized, mixed with ice-cold buffer in a 1:10 ratio (in mM: 300 sucrose, 1 EDTA, 10 NaCl, 40 Tris-base and 40 l-histidine, pH 7.8), frozen in liquid nitrogen, and subsequently stored at −80 °C. At the day of analysis, muscle homogenates were mixed with 200 μL of sample-buffer (10% glycerol, 5% 2-mercaptoethanol and 2.3% SDS, 62.5 mM Tris and 0.2% bromphenol blue at pH 6.8), boiled in water at 100 °C for 3 min, and loaded in 3 protein amounts (10–40 μL) on a SDS-PAGE gel (6% polyacrylamide/100:1 acrylamide:bisacrylamide), 30% glycerol, 67.5 mM tris-base, 0.4% SDS, and 0.1 M glycine). Gels were run at 80 V for 42 hrs at 4 °C, MHC bands were visualized with Coomassie staining, gels were scanned (Linoscan 1400 scanner, Germany), and MHC bands quantified densitometrically (Phoretix 1D, nonlinear, UK) as an average of the three loaded protein amounts.

Muscle fiber cross-sectional area (CSA) was analyzed as previously described using immunohistochemistry to identify type I (stained) and type II ( unstained ) fibers (Suetta et al., 2012, 2013). Briefly, one part of the biopsy muscle was oriented in the embedding medium (Tissue-Tek, Sakura Finetek, USA), frozen in liquid nitrogen and subsequently stored at −80 °C. At the day of analysis, transverse serial sections (8 μm) of each sample were cut at −22 °C using a cryostat (HM560; Microm, Walldorf, Germany) and mounted on glass slides. Following rehydration of the slides, fixation and permeabilization in 4% normal buffered formalin and triton X-100 (0.1%) for 10 min, immunofluorescent stainings were performed by incubation with rabbit anti-laminin (rabbit anti-laminin, cat. no. 20097, DakoCytomation) and mouse anti-skeletal myosin slow (M8421, 1:2000, St Louis, MO, USA). After washing, slides were then incubated with the secondary antibodies (Alexa-555 goat anti-mouse (Invitrogen, A21424, Life Technologies Denmark, Naerum, Denmark, 1:1000) and Alexa-488 goat anti-rabbit (Invitrogen, A11034, 1:1000)). Finally, sections were mounted with cover glass and stainings visualized on a computer screen using a fluorescent microscope (Carl Zeiss Axio Imager M1, Germany) and a high-resolution AxioCam (Carl Zeiss). A digital analysis program (Carl Zeiss, AxioVision 4.6) was used for the determination of muscle fiber CSA, including truly horizontally cut fibers only, with a minimum of 50 fibers (range 50–200) analyzed per time point.

2.7. Statistical analysis

Statistical analysis was performed as previously described using linear mixed model (STATA 10.1, StataCorp, USA) (Hvid et al., 2011, 2013). Data were tested for normal distribution, and if absent, appropriate transformations were carried out prior to analysis (all measures of rapid muscle force capacity were square-root transformed). Measurements of mechanical muscle function, MHC isoform composition as well as muscle fiber CSA were analyzed with Group (Young, Old) and Time (Pre, Post, Rec) as fixed effects, and with Subject ID as random effect. Relationships between measures of mechanical function and MHC isoform content or muscle fiber CSA were investigated using Pearson’s correlation analysis. Data are presented as mean ± se unless otherwise stated. n denotes number of subjects. Level of statistical significance was set at p < 0.05.

3. Results

3.1. Maximal isometric and dynamic strength

Prior to disuse, marked age-related differences were observed in knee extensor muscle strength, as evidenced by lower levels of isometric and dynamic strength in old individuals vs. young individuals (~35%; p < 0.05) (Table 1). Following disuse, young and old individuals experienced comparable changes in isometric (~10 ± 2 and ~8 ± 4%)}

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respectively; p < 0.05) and slow dynamic strength (−10 ± 4% and −16 ± 4%, respectively; p < 0.05), whereas moderate dynamic strength changed in old individuals only (−12 ± 4%; p < 0.05) (Table 1). Following recovery, isometric and slow dynamic strength returned to pre-disuse values in young individuals, whereas isometric as well as slow and moderate dynamic strength remained suppressed in old individuals (−7 ± 3, −9 ± 2 and −9 ± 2%, respectively; p < 0.05) (Table 1).

In the control leg, measures of knee extensor muscle strength in both young and old individuals were not different from those observed in the disused leg prior to disuse (Table 1). No changes were observed following disuse or following recovery (Table 1).

As shown in Table 1, absolute values of isometric strength (in Nm), follow the same pattern of percentage changes as the body mass normalized values.

### 3.2. Rapid muscle force capacity

Marked age-related differences were also observed in knee extensor rapid muscle force capacity, as evidenced by lower RFD and Impulse in the initial phase of rising muscle force (0–100 ms) in old individuals vs. young individuals (−39%; p < 0.05) (Table 1). However, no age-related differences were observed in rRFD (Table 1).

### Fig. 2.

Group mean torque–time curves obtained during maximal voluntary isometric contraction (MVC) of the knee extensors prior to disuse (Pre, solid line), following 4 days of disuse (Post, dashed line), and following 7 days of recovery (Rec, dash-dotted line) in young (black lines) and old men (gray lines). Data are provided for the first 300 ms of contraction only. Torque is displayed normalized to body mass. Solid circles denote onset of contraction (t = 0 ms). RFD and Impulse was calculated in the time interval 0–100 ms relative to onset of contraction, i.e. reflecting the initial phase of rising muscle force (see Table 1 for specific values).

### Table 1

Knee extensor mechanical muscle function prior to (Pre) and following 4 days of disuse (Post), and following 7 days of recovery (Rec) in young and old healthy men. Assessment of strength encompasses isometric strength, slow dynamic strength (60° s⁻¹), and moderate dynamic strength (180° s⁻¹), while rapid muscle force capacity encompasses RFD, relative RFD (rRFD), and Impulse in the initial 100 ms of rising muscle force. Values are given for both the disused leg and the contra-lateral control leg as group mean ± se. p < 0.05; a: different from young individuals, b: different from Pre (at Post or at Rec), c: different from Post (at Rec), and d: relative changes different from those observed in young individuals. In addition to the body mass normalized values, absolute values are presented for isometric strength enabling comparison to previous published data. See Fig. 2 for a graphical display of mean torque–time curves.

<table>
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<tr>
<th></th>
<th>Disused leg</th>
<th>Control leg</th>
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<td></td>
<td>Young (n = 11)</td>
<td>Old (n = 11)</td>
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<tr>
<td><strong>Strenght</strong></td>
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<td>Isometric (Nm)</td>
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<tr>
<td>Pre</td>
<td>2.93 ± 0.11</td>
<td>2.06 ± 0.19</td>
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<tr>
<td>Post</td>
<td>2.84 ± 0.10</td>
<td>3.10 ± 0.16</td>
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<tr>
<td>Rec</td>
<td>2.92 ± 0.13</td>
<td>3.18 ± 0.18</td>
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<tr>
<td>Isometric (Nm)</td>
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<tr>
<td>Pre</td>
<td>219 ± 13</td>
<td>229 ± 15</td>
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<tr>
<td>Post</td>
<td>197 ± 10</td>
<td>233 ± 17</td>
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<tr>
<td>Rec</td>
<td>217 ± 12</td>
<td>238 ± 17</td>
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<td>Dynamic slow (Nmkg⁻¹)</td>
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<tr>
<td>Pre</td>
<td>2.69 ± 0.07</td>
<td>2.68 ± 0.15</td>
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<td>Post</td>
<td>2.42 ± 0.11</td>
<td>2.75 ± 0.16</td>
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<tr>
<td>Rec</td>
<td>2.67 ± 0.12</td>
<td>2.81 ± 0.17</td>
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<tr>
<td>Dynamic slow (Nmkg⁻¹)</td>
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<tr>
<td>Pre</td>
<td>1.95 ± 0.05</td>
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<tr>
<td>Post</td>
<td>1.88 ± 0.11</td>
<td>1.99 ± 0.12</td>
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<tr>
<td>Rec</td>
<td>1.89 ± 0.08</td>
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<td>RFD (Nms⁻¹kg⁻¹)</td>
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<tr>
<td>Pre</td>
<td>162.6 ± 0.9</td>
<td>18.0 ± 1.0</td>
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<td>Post</td>
<td>138.0 ± 0.7</td>
<td>17.8 ± 1.3</td>
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<tr>
<td>Rec</td>
<td>150.0 ± 1.0</td>
<td>18.9 ± 1.0</td>
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<td>rRFD (NMS⁻¹)</td>
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<tr>
<td>Pre</td>
<td>558 ± 35</td>
<td>590 ± 27</td>
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<tr>
<td>Post</td>
<td>528 ± 28</td>
<td>496 ± 25</td>
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<tr>
<td>Rec</td>
<td>545 ± 33</td>
<td>508 ± 16</td>
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<tr>
<td>Impulse (Nms⁻¹kg⁻¹)</td>
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<tr>
<td>Pre</td>
<td>96.3 ± 6.7</td>
<td>107.4 ± 7.3</td>
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<tr>
<td>Post</td>
<td>77.8 ± 4.9</td>
<td>107.8 ± 8.9</td>
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| MHC isoform composition.

<table>
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<tr>
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<th>Young (n = 11)</th>
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<tr>
<td></td>
<td>MHC %</td>
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<tr>
<td>Pre</td>
<td>51 ± 3</td>
<td>47 ± 4</td>
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<tr>
<td>Post</td>
<td>50 ± 4</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Rec</td>
<td>48 ± 4</td>
<td>48 ± 4</td>
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</table>

Percentage MHC isoform composition (vastus lateralis muscle) in young and old men prior to (Pre) and following 4 days of disuse (Post), and following 7 days of recovery (Rec). Values are group mean ± se. p < 0.05; a: different from young individuals.
Following 4 days of disuse, the isometric torque–time curves appeared less steep in both young and old individuals (Fig. 2). Consequently, RFD and Impulse decreased in young (RFD100 − 13 ± 5% and Impulse100 − 17 ± 6%; p < 0.05) and old (RFD<sub>100</sub> − 23 ± 5% and Impulse<sub>100</sub> − 21 ± 8%; p < 0.05) individuals (Table 1). Moreover, rRFD decreased in old individuals (rRFD<sub>100</sub> − 17 ± 3%; p < 0.05), showing a greater relative decrease than that observed in young individuals (p < 0.05) (Table 1). Following 7 days of recovery, isometric knee extensor torque–time curves became steeper as reflected by gains in RFD, Impulse, and rRFD to reach pre-disuse values (Table 1). However, as suggested by visual inspection of the torque–time curves (Fig. 2), old individuals appeared to demonstrate an attenuated recovery of rapid muscle force capacity (trendy observed for RFD<sub>100</sub>; p = 0.068). When using absolute values (inNm), an identical pattern of torque–time curves was seen as that displayed in Fig. 2 (data not shown).

In the control leg, measures of knee extensor rapid muscle force capacity in both young and old individuals were not different from those observed in the disused leg prior to disuse (Table 1). No changes were observed following disuse or recovery (Table 1).

3.3. MHC isoform composition

Prior to disuse, the vastus lateralis muscle showed a higher type I MHC isoform content along with a lower type IIa MHC isoform content in old individuals compared to young individuals (Table 2). In contrast, the composition of MHC I, IIa, and IIx isoforms remained unaltered following the periods of disuse and recovery, respectively (Table 2).

Prior to disuse, correlations between MHC isoform content and measures of mechanical muscle function were observed when data were combined for young and old individuals (MHC IIa and Impulse<sub>100</sub>, r² = 0.22, p < 0.05; MHC IIa and RFD<sub>100</sub>, r² = 0.16, p = 0.07), but not separately. With both disuse and recovery, no correlations were observed between the relative (percentage) changes in MHC isoform content and measures of mechanical muscle function, combined or separately (data not shown).

3.4. Muscle fiber cross-sectional area

Prior to disuse, muscle fiber CSA of type I fibers was similar when comparing young and old individuals, whereas CSA of type II fibers was smaller in old individuals vs. young individuals (Fig. 3). Following 4 days of disuse, type I muscle fiber CSA decreased in young individuals (−8.1%, p < 0.05) and tended to decrease in old individuals (−7.1%, p = 0.073), while type II muscle fiber CSA decreased in both young and old individuals (−12.6 and −10.5%, respectively, p < 0.05). Following 7 days of recovery, type I and type II CSA returned to pre-disuse values in both young and old individuals, except for type I CSA in old individuals which remained suppressed (−7.2%, p < 0.05) (Fig. 3).

Prior to disuse, correlations between muscle fiber CSA and measures of mechanical muscle function were observed in old individuals (type II CSA and isometric strength, r² = 0.69), but not in young individuals or when combining data for both young and old individuals (data not shown). With both disuse and recovery, no correlations were observed between the relative (percentage) changes in muscle fiber CSA and measures of mechanical muscle function, combined or separately (data not shown).

4. Discussion

As the main overall finding in the present study, only 4 days of lower limb disuse induced marked decrements in mechanical muscle function both in young and old healthy individuals. Furthermore, while mechanical muscle function was fully restored in young individuals following 7 days of active recovery it remained suppressed in old individuals.

Fig. 3. Cross-sectional areas of type I and II muscle fibers (vastus lateralis muscle) in young and old men prior to (Pre) and following 4 days of disuse (Post), and following 7 days of recovery (Rec). Values are group mean ± se. p < 0.05; a: different from young individuals, b: different from Pre (at Post or at Rec).

Fig. 4. Decrease in maximal isometric knee extensor strength (MVC) plotted against the duration of lower limb disuse up to 45 days (n = 27 studies). While a majority of studies have been carried out in young subjects (~25 years), only four studies have examined old subjects (~67 years) including the present study. Notably, in addition to the present experiments, only a single previous study has been conducted to investigate the subsequent ability to recover following the disuse period in old individuals (Hvid et al., 2010). A least-square curve fit has been applied for illustrative purposes (hyperbolic regression, best non-linear fit). ULLS: unilateral lower limb suspension. Studies including young subjects (Adams et al., 1994; Bamman et al., 1998; Berg and Tesch, 1996; Berg et al., 1997, 2007; Clark et al., 2006; de Boer et al., 2007; Deschenes et al., 2002, 2008; Dudley et al., 1992; Hespel et al., 2001; Hvid et al., 2012; Hortobagyi et al., 2000; Hvid et al., 2010; Jones et al., 2004; Kawakami et al., 2001; Kortebein et al., 2008; Kubo et al., 2000, 2004; Labarque et al., 2002; Rozier et al., 1979; Schulze et al., 2002; Yasuda et al., 2005), and studies including old subjects (Deschenes et al., 2008; Hvid et al., 2010; Kortebein et al., 2008).
4.1. Effects of aging

Prior to disuse, age-related reductions in knee extensor muscle strength and rapid muscle force capacity were observed (~35 and ~39%, respectively) in agreement with previous observations (Ditroilo et al., 2010; Hvid et al., 2010; Kubo et al., 2007; Macaluso et al., 2002). As suggested by correlation analyses, this may in part be explained by the slower MHC isoform composition (higher MHC I isoform content, lower MHC Ila isoform content) as well as by the smaller type II fiber CSA shown in old individuals compared to young individuals, factors that has previously been shown to affect muscle force/RFD output (Clarkson et al., 1981; Harridge et al., 1996; Hvid et al., 2010; Kiltgaard et al., 1990; Korhonen et al., 2006; Larsson et al., 1979).

In the present study, young and old subjects spend the same amount of hours doing occupational and recreational activities at an intensity corresponding to low-to-moderate, thus indicating that the observed findings were due to the effect of aging per se and not caused by potential differences in physical activity levels. Nevertheless, as the questionnaire assessed physical activity on a general basis, age-related differences may potentially have existed in patterns of habitual physical activity in old individuals (continuous, shifting between low and moderate intensities) vs. young individuals (intermittent, shifting between low, moderate, and high intensities), thus hypothetically influencing the observed findings in mechanical muscle function.

4.2. Effects of disuse

In the present study 4 days of lower limb disuse led to marked decrements in maximal knee extensor isometric and dynamic muscle strength as well as in rapid muscle force capacity in both young and old healthy individuals. The magnitude of changes was in strong agreement with previous reports, collectively confirming that disuse-induced decrements in lower limb mechanical muscle function occur very fast within the initial phase (days) of disuse irrespectively of age, followed by an attenuated rate of decline with more prolonged periods of disuse (weeks) (see Fig. 4 for references). Thus, collapsed data from known studies (including the present data) reveal average decreases per day in isometric muscle strength of 2.05, 1.53, and 1.37% during 10, 15, and 20 days of disuse, respectively (Fig. 4). Although less often examined, greater decrements in rapid muscle force capacity (RFD) appear to occur (Bamman et al., 1998; de Boer et al., 2007; Horstman et al., 2012; Hvid et al., 2010; Kubo et al., 2000). Intriguingly, even greater decrements may therefore have been observed in both young and old individuals if testing of mechanical muscle function had been carried out on the day of cast removal, and not the following day (see Section 2.1 Study design).

While the present data suggest that disuse-induced decrements in lower limb isometric and slow dynamic strength occur independently of age (at least up to ~70 years), moderate dynamic strength and to some extent rapid muscle force capacity revealed disproportionally greater impairments in old individuals compared to young individuals, also in support of previous observations (Deschenes et al., 2008; Hvid et al., 2010; Suetta et al., 2009). This may be explained by different physiological variables involved in slow and moderate-to-fast muscle contractions, respectively. The latter are specifically influenced by muscle quantity particularly of fast type II muscle fibers (Clarkson et al., 1981; Hvid et al., 2010; Kiltgaard et al., 1990; Larsson et al., 1979), yet also by qualitative factors such as fiber type composition (higher proportions of MHC II vs. I isoforms favor high muscle force/RFD output during moderate-to-fast contractions) (Clarkson et al., 1981; Harridge et al., 1996; Hvid et al., 2010; Kiltgaard et al., 1990; Korhonen et al., 2006; Larsson et al., 1979), intrinsic contractile properties of fibers of a given MHC isoform composition (Bottinelli et al., 1999; Hvid et al., 2011, 2013; Metzger and Moss, 1990) and specific patterns of efferent neural input (Aagaard et al., 2002; Klass et al., 2008). In the present study, no disuse-induced changes in fiber type (MHC isoform) composition were observed in either young individuals or old individuals (Table 2), decrements in type I and type II muscle fiber CSA as well as decrements in specific force (force per cross-sectional area) of isolated single muscle fibers occurred independently of age (Hvid et al., 2013). However, the fact that relative RFD decreased with disuse in old individuals only (Table 1) suggests that qualitative changes, likely involving modulations in efferent neural input, were responsible for the age-dependent decrements observed in moderate dynamic strength as well as in rapid muscle force capacity (Fig. 2, Table 1).

4.3. Effects of recovery

In conjunction with previous observations (Hvid et al., 2010; Suetta et al., 2009, 2013), the findings of the present study suggest that the magnitude and time-course of changes in mechanical muscle function during recovery following short-term disuse are compromised in old individuals compared to young individuals.

While the recovery period (including free ambulation, a high-intensity test session, and a single high-intensity training session) appeared effective of restoring knee extensor mechanical muscle function in the young study participants in accordance with previous reports (Berg and Tesch, 1996; Hespel et al., 2001; Hortobagyi et al., 2000; Hvid et al., 2010; Jones et al., 2004; Labarque et al., 2002), knee extensor mechanical muscle function remained suppressed in the old study participants, as also observed following 14 days of disuse (Hvid et al., 2010; Suetta et al., 2009). Yet, it remains unknown whether old individuals would have benefitted from an even stricter scheme of recovery (e.g. including an additional session of strength training), or whether skeletal muscles of old individuals simply failed to tolerate the regime of active recovery chosen in the present study.

The observed impairment in restoring lower limb mechanical muscle function following short-term disuse in old adults may in part reside from qualitative muscle factors as discussed above (RFD<sub>100</sub> tended to remain reduced in old individuals following 7 days of recovery), while likely also from an age-related impairment in the capacity for muscle re-growth as shown in the present (type I CSA remained suppressed in old individuals following 7 days of recovery) as well as in previous studies (Carlson et al., 2009; Tanaka et al., 2004).

4.4. Functional implications

The present findings showing that old individuals experienced marked decrements in knee extensor mechanical muscle function following only 4 days of disuse, combined with an attenuated/slower rate of recovery, are likely to have important functional consequences. In particular since the selected measures of knee extensor mechanical muscle function (isometric and dynamic muscle strength as well as rapid muscle force capacity) have been shown to be a strong predictor of functional status including fundamental elements required to maintain independent living in older individuals (e.g. mobility and ability to counteract falls) while also influencing quality of life and risk of mortality (Buchman et al., 2007; Cesari et al., 2009; Newman et al., 2006; Wyszomierski et al., 2009). As the age of the present older study participants was ~67 years, we can only speculate to what extent similar disuse and retraining periods would have affected mechanical muscle function of very old individuals (i.e. > 80 years). It may be speculated that substantially greater disuse-induced decrements in mechanical muscle function would have been observed as indicated by data from animal disuse studies (Arora et al. 2008), accompanied by an even slower rate of training-induced recovery as observed in human training studies comprising very old individuals (Laroche et al., 2008; Raue et al., 2009).

Altogether, it seems of paramount importance to develop effective preventive and/or rehabilitative intervention strategies to counteract the deleterious impact of short-term disuse in aged individuals, in particular when considering the rapid rate of decline observed in the initial
days of disuse (cf. Fig. 3). For this purpose, we specifically suggest use of exercise interventions based on high-intensity resistance training, as this has previously been proven highly effective in improving mechan-ical muscle function (particularly through improvements in neural input) in old and very old individuals while at the same time being safe and well tolerated (Caserotti et al., 2008; Hvid et al., 2010). Notably, the impaired ability to recover following short-term disuse in aged indi-

5. Conclusions

Lower limb mechanical muscle function decreased markedly in both young and old males following short-term (4 days) disuse. The observed decrements in isometric and slow dynamic muscle strength were inde-

Conflict of interest

The Authors declare no conflicts of interest in relation to the content of this article.

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