The Journal of Physiology

http://jp.msubmit.net

JP-RP-2015-271712

Title: The effect of age and unilateral leg immobilization for 2 weeks on substrate utilization during moderate intensity exercise in human skeletal muscle

Authors: Andreas Vigelsø Hansen
Martin Gram
Rie Dybboe
Anja Birk Kuhlman
Clara Prats
Former Editor Paul Greenhaff
Tim Constantin
Jesper Birk
Jørgen Wojtaszewski
Flemming Dela
Joern Wulff Helge

Author Conflict: No competing interests declared

Author Contribution: Andreas Vigelsø Hansen: Conception and design; Financial Support; Administrative support; Provision of study materials or patients; Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Martin Gram: Conception and design; Provision of study materials or patients; Collection and assembly of data; Data analysis and interpretation; Final approval of manuscript (required) Rie Dybboe: Collection and assembly of data; Data analysis and interpretation; Final approval of manuscript (required) Anja Birk Kuhlman: Collection and assembly of data; Data analysis and interpretation; Final approval of manuscript (required) Clara Prats: Conception and design; Financial Support; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Former Editor Paul Greenhaff: Conception and

design; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Tim Constantin: Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Jesper Birk: Data analysis and interpretation; Final approval of manuscript (required) Jørgen Wojtaszewski: Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Flemming Dela: Conception and design; Financial Support; Administrative support; Provision of study materials or patients; Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Joern Wulff Helge: Conception and design; Financial Support; Administrative support; Provision of study materials or patients; Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required)

Running Title: Fat utilization during exercise in young and older men

Dual Publication: The manuscript represents original work which has not been published be-fore or is under consideration elsewhere. However, this research paper origi-nates from a large study from where we have previously reported other data in a different physiological context (1-4). Furthermore, one manuscript is re-cently accepted with minor comments for publication in Journal of Physiol-ogy with manuscript # JP-RP-2015-270211 (5) References 1. Gram M, Vigelso A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M, and Dela F. Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. Experimental gerontology 58C: 269-278, 2014. 2. Nørregaard J, Gram M, Vigelsø A, Wiuff C, Kuhlman AB, Helge JW, and Dela F. The effect of reduced physical activity and retraining on blood lipids and body composition in young and older adult men. Journal of Aging and Physical Activity epub ahead of print: 2014. 3. Reihmane D, Hansen AV, Gram M, Kuhlman AB, Norregaard J, Pedersen HP, Lund MT, Helge JW, and Dela F. Immobilization increases interleukin-6, but not tumour necrosis factor-alpha, release from the leg during exercise in humans. ExpPhysiol 98: 778-783, 2013. 4. Vigelsø A, Gram M, Wiuff C, Andersen JL, Helge JW, and Dela F. 6 weeks' aerobic retraining after 2 weeks' immobilization restores leg lean mass, and aerobic capacity but does not fully rehabilitate leg strength in young and older men. Journal of Rehabilitation Medicine [EPUB AHEAD OF PRINT]: 2015. 5. Gram M, Vigelsø A, Yokota T, Helge JW, Dela F, Hey-Mogensen M. Skeletal muscle mitochondrial H2O2 emission increases with immobilization and decreases after aerobic training in young and older men. Accepted, journal of physiology.

Funding: This work was supported by the UNIK research program: Food, Fitness & Pharma for Health and Disease (Danish Ministry of Science, Technology and Innovation), The Nordea Foundation, A.P. Møller and Hustru Chastine Mc-Kinney Møllers Foundation.

Title page

The effect of age and unilateral leg immobilization for 2 weeks on substrate utilization during moderate intensity exercise in human skeletal muscle

Running title: Fat utilization during exercise in young and older men

Key words: Age, immobilization, substrate utilization

[1]*A Vigelsø, [1]M Gram, [12]R Dybboe, AB Kuhlman, [1]C Prats, [3]PL Greenhaff, [3]D Constantin-Teodosiu[2], JB Birk, [2]JFP Wojtaszewski, [1]F Dela, and [1]JW Helge**.**

[1]XLAB, Center for Healthy Aging, Dept. of Biomedical Sciences, University of Copenhagen [2]Section of Molecular Physiology, The August Krogh Centre, Department of Nutrition, Exercise and Sports, University of Copenhagen. [3]MRC/Arthritis Research UK Centre for Musculoskeletal Ageing Research, Arthritis Research UK Centre for Sport, Exercise and Osteoarthritis, School of Life Sciences, The Medical School, University of Nottingham

*Address for correspondence:

Andreas Vigelsø

XLAB - Center for Healthy Aging

Department of Biomedical Sciences

Blegdamsvej 3

2200 N

Denmark

E-mail: avhansen@Sund.ku.dk

Ph: + 45 35 32 60 02

Fax: + 45 35 32 74 20

Key point's summary

- This study aimed to provide molecular insight into the differential effects of age and physical inactivity on the regulation of substrate metabolism during moderate intensity exercise.
- Using the A-V balance technique, we studied the effect of one-leg immobilization for 2 weeks, on leg substrate utilization in young and older men during two-legged dynamic knee-extensor moderate intensity exercise, as well as changes in key proteins in muscle substrate metabolism before and after exercise.
- Age and immobilization did not affect relative carbohydrate and fat utilization during exercise, but the older men had higher uptake of exogenous fatty acids (FA), while the young men relied more on endogenous FA during exercise.
- Having used a combined whole leg and molecular approach we provide evidence to suggest that both age and physical inactivity result in metabolic inflexibility, but that this only partially occurs through the same mechanisms.

Abstract

Age and inactivity have been associated with metabolic inflexibility. Here, we attempt to disentangle these factors by studying the effect of 2 weeks' unilateral leg immobilization on substrate utilization across the legs during moderate intensity exercise in young (n=17; 23±1 years) and older (n=15; 68±1 years) men, while the contralateral leg served as control. After immobilization, the participants performed two-legged isolated knee-extensor exercise with each leg kicking in a separate ergometer at 20±1 Watt (~50% $Watt_{max}$) for 45 min with catheters inserted in the brachial artery and both femoral veins. Biopsy samples obtained from *vastus lateralis* muscles of both legs before and after exercise were used for analysis of protein content and enzyme activities. During exercise, leg substrate utilization (RQ) did not differ between groups or legs. Leg fatty acid (FA) uptake was greater in older than in young men, and while young men demonstrated net leg glycerol release during exercise, older men showed net glycerol uptake. At baseline, muscle pyruvate dehydrogenase complex activity, protein content of adipose triglyceride lipase (ATGL), acetyl-CoA carboxylase 2, AMP-activated protein kinase (AMPK)γ3 were higher in young than in older men. Furthermore, ATGL, plasma membrane-associated FA binding protein, and AMPKγ3 subunit protein content were lower in the immobilized than the contralateral leg in young and older men. Despite no change in RQ, there were several changes in muscle with immobilization, thus suggesting a deranged regulation of substrate utilization, which could lead to metabolic inflexibility. Furthermore, the young and older men preferentially mobilized FA for oxidation from different sources during moderate intensity exercise.

Abbreviations list: ACC2, Acetyl-CoA carboxylase 2; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; AUC, area under the curve; CON, control leg; CS, citrate synthase activity; DANHES, Danish Health Examination Survey; DXA, dual energy X-ray absorptiometry; FA, fatty acid; FABPpm, plasma membrane-associated fatty acid binding protein; HAD, β-hydroxyacyl-CoA dehydrogenase; HSL, hormone-sensitive lipase; IM, immobilized leg; IMTG, intramuscular triglyceride; LPL, lipoprotein lipase; PDC, pyruvate dehydrogenase complex; RER, respiratory exchange ratio, RQ, respiratory quotient; VDAC, voltage dependent ion channel.

Introduction

Metabolic inflexibility is defined as the inability to adjust substrate oxidation and storage to substrate availability (Bonadonna *et al.*, 1994; Ritz *et al.*, 1998; Kelley & Mandarino, 2000; Kelley *et al.*, 2002; Storlien *et al.*, 2004). Both age and inactivity are associated with muscle metabolic inflexibility although it is currently not known whether one is secondary to the other or if the effects are additive. Thus, there is a gap in our knowledge as to how age and inactivity affect muscle substrate uptake, storage and oxidation both at rest and during exercise and how this may be coupled to metabolic inflexibility and subsequent lipid accumulation. This potentially has major health implications because metabolic inflexibility promotes lipid accumulation that may lead to impaired glucose metabolism (Kelley & Mandarino, 2000).

The relative rate of fuel oxidation is influenced by training status and exercise intensity/duration (Henriksson, 1977; Hurley *et al.*, 1986; Martin *et al.*, 1993; Friedlander *et al.*, 2007; Helge *et al.*, 2007), as well as plasma FA availability (O'Neill *et al.*, 2004; Watt *et al.*, 2004). Since whole-body aerobic capacity is decreased with age and inactivity (Lexell *et al.*, 1988; Fielding *et al.*, 2011) the latter two may be associated with a decline in muscle fat oxidation (Meredith *et al.*, 1989; Lonnqvist *et al.*, 1990; Sial *et al.*, 1996; Levadoux *et al.*, 2001; Solomon *et al.*, 2008). Although this might be secondary to a decrease in physical activity level with age. In line with this contention, the decline in skeletal muscle respiratory capacity (Sial *et al.*, 1996; Conley *et al.*, 2000) and the observed lipid accumulation in skeletal muscle (Petersen *et al.*, 2003; Cree *et al.*, 2004; Wall *et al.*, 2015) may account for the age-related changes in whole body composition (Levadoux *et al.*, 2001).

Several intramusclular steps in metabolism are likely to play a role in age and inactivity mediated lipid accumulation (e.g. fatty acid (FA) uptake, lipid uptake and storage and regulation of mitochondrial substrate uptake). Indeed, while expression of muscle FABPpm, although not CD36

and FATP, is higher in trained than untrained individuals (Kiens *et al.*, 2004) and higher after one-leg training for 3 weeks (≈50%) (Kiens *et al.*, 1997), it is still unclear whether aging and inactivity affect muscle FABPpm and CD36.

The regulation of lipolysis is probably another pivotal factor in lipid accumulation. At rest ~50 % of the FA taken up by the skeletal muscle is stored in intramuscular lipid droplets and there is a complete turnover of the lipid pool in ~29 hours (Sacchetti *et al.*, 2004). Therefore, the influence of acute, as well as chronic activity and inactivity on the regulation storage and release of FA from the lipid droplets for oxidation, has recieved considerable attention. In young men endurance training increases protein levels of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) activity (increased Ser^{660} and decreased Ser^{565} phosphorylation, but not HSL protein level; (Alsted *et al.*, 2009), whereas conversely bed rest decreases HSL activity (increased Ser^{565} phosphorylation; (Alibegovic *et al.*, 2010). However, the effect of age and inactivity on ATGL and HSL is not well described.

AMPK is considered a master switch in muscle metabolism and key in the regulation of transport of fuel into the mitochondria for oxidation. During exercise increased AMPK activity stimulates FA utilization (Fentz *et al.*, 2015) and inhibits other energy-consuming processes (Jorgensen *et al.*, 2004; Jensen *et al.*, 2009; Richter & Ruderman, 2009; Hardie, 2011). Furthermore, AMPK may partly favor FA oxidation through inhibition of Acetyl-CoA carboxylase 2 (ACC2) (Stephens *et al.*, 2002), although this may not be a limiting factor (Dzamko *et al.*, 2008). Additionally, older individuals seem to have augmented AMPK activation in response to acute exercise (Drummond *et al.*, 2008; Mortensen *et al.*, 2009). As AMPK also inhibits pyruvate dehydrogenase complex (PDC) activity (Klein *et al.*, 2007) that controls the rate of pyruvate transport into the mitochondria (Constantin-Teodosiu *et al.*, 1992), this may partly account for the lower rate of glucose disposal in older compared to young individuals during exercise. Immobilization for 2 weeks did not affect

AMPKα1, α2, β2 or α-subunit level (Eijnde *et al.*, 2005) in young men whereas bed rest for seven days was associated with a lower AMPKαThr172 phosphorylation after 45 min one-legged knee-extensor exercise at ~60 % of $Watt_{max}$ compared to before bed rest (Ringholm *et al.*, 2011). Overall, the effect of inactivity and age on AMPK, ACC2 and PDC activity and how these mechanisms affects muscle metabolic inflexibility remains to be fully elucidated.

To date, most studies have investigated the effect of endurance training on metabolic inflexibility in young and older men, but information on the effect of inactivity in these groups is lacking. Furthermore, there is a gap in our knowledge whether age and inactivity affect the same or different mechanisms that control fuel mobilization and oxidation. To address this question, we immobilized one leg in both young and older men for 2 weeks while the other leg served as control. After immobilization, the participants performed a bout of 45 min isolated one-leg kicking exercise at moderate intensity with both legs. The AV-balance technique was used to assess indirect calorimetry and source of substrate mobilized for oxidation in the legs. Our hypothesis was that both age-difference and inactivity would be associated with changes that could be related to IMTG accumulation and hence metabolic inflexibility. We hypothesized that this would be associated with an increased relative glucose utilization (i.e. the opposite adaptation of endurance training) and a derangement of several key proteins regulating substrate metabolism in skeletal muscle. This would lead to more FA being mobilized from the circulation without being oxidized, which would lead to IMTG accumulation and in turn metabolic inflexibility.

Methods

Subjects

Seventeen young and 15 older men were included. Age inclusion criteria were 20-27 and 60-75 yrs, respectively. The participants were selected to have average VO_{2max} (42-49 and 25-35 ml O_2/min/kg), BMI (22-27 and 20-29 kg/m^2) and whole-body fat percent (15-25 % and 20-30 %, for young and older men, respectively) for their age-group according to the Danish Health Examination Survey (DANHES) (Eriksen *et al.*, 2011) (Table I). The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of Copenhagen (H-4-2010-85). All subjects were carefully informed (verbal and written material) about the possible risks and discomfort involved before written consent to participate was obtained. The subjects received remuneration for participation and all transportation costs during the immobilization and to/from meetings at the department were reimbursed.

Experimental protocol

The experimental protocol has been previously described in detail (Nørregaard *et al.*, 2014). Both young and older men were screened prior to recruitment to exclude individuals with diabetes (measured by glycated hemoglobin (HbA1c > 6.5 mmmol/mol)), musculoskeletal disease, cardiovascular disease (resting ECG in the older men) or known predisposition to deep venous thrombosis. None of the young men took medication, but some of the older men were in medical treatment for hypertension (n = 2; thiazide diuretic + angiotensin II inhibitor; angiotensin II receptor antagonist), prostate enlargement (n = 2; α-blocker), mild asthma (n = 1; anticholinergic pro re nata), mild depression (n = 1; Selective serotonin reuptake inhibitor) and attention deficit hyperactive disorder (n = 1; modafinil). None of the participants were smokers.

The participants were instructed to eat a weight-maintaining diet throughout the study following the national guidelines for macro-nutrient composition (Fogelholm, 2013). In the three days prior to each biopsy sampling, the subjects were instructed to abstain from alcohol intake. In addition, the participants were instructed to avoid strenuous exercise three days before the test days.

The present study is a part of a larger study on the effect of immobilization and aerobic retraining in young and older men. The previous studies have investigated IL-6 and TNFα release during exercise in the young men (Reihmane *et al.*, 2013); and changes in mitochondrial respiration and H_2O_2 production (Gram *et al.*, 2014; Gram *et al.*, 2015), leg function (e.g. leg lean mass, strength and muscle fiber type composition) (Vigelsø *et al.*, 2015b), and plasma lipid profile (Nørregaard *et al.*, 2014) with immobilization and retraining. It follows that most of the descriptive data on these subjects have been reported previously (Reihmane *et al.*, 2013; Gram *et al.*, 2014; Nørregaard *et al.*, 2014; Vigelso *et al.*, 2015) and this will be clearly referenced in the present paper.

Anthropometric measurements

Body composition was determined by dual energy X-ray absorptiometry (DXA) scanning (Lunar iDXA, GE Medical Systems, Madison, US) at inclusion and after immobilization. EnCORE software (encore software version 14.10.022, GE Medical Systems, Madison, US) automatically determined the regions of interest (e.g. the legs).

At inclusion and after immobilization, a graded VO_{2max} test and a test of the maximal work capacity of each leg ($Watt_{max}$) was performed. VO_{2max} was achieved, when two criteria were met: plateau in oxygen consumption in spite of increasing workload and respiratory exchange ratio above 1.15 for the young and 1.05 for the elderly on average over 20 s (Howley *et al.*, 1995). Polar RS400 heart rate monitors (Polar Electro Oy, Kempele, Finland) were used to measure heart rate. At the $Watt_{max}$ test prior to the experiment the participants were accustomed to exercise in the knee extension

ergometer. The maximal work capacity ($Watt_{max}$) of each leg was then determined (Andersen et al. 1985). In brief, a graded test (starting at 10 watts + 2 min at 20 watt and then 5 watt/2 min increments) was performed, and pulmonary VO_2, VCO_2, and heart rate were measured. The workload at which exercise could not be performed without involving additional muscle was defined as one-leg $Watt_{max}$.

Immobilization

The immobilized leg was chosen by randomization. The chosen leg was immobilized (IM) with a DonJoy® knee brace (DJO Nordic, Malmö, Sweden) locked at 60° for 2 weeks. The other leg served as control (CON). The DonJoy® was secured with plastic strips that, if broken, would reveal that the brace had been removed. The subjects were given a pair of crutches and were repeatedly instructed not to engage in any weight-bearing activity with the immobilized leg. However, they ambulated freely during the entire 2 weeks. The subjects reported to the laboratory at least once during the 2-week immobilization to control and adjust the DonJoy® brace. All subjects received 75 mg acetylsalicylic acid per day in the first 10 days to reduce the risk of deep venous thrombosis. This treatment was withdrawn the last 4 days before the experimental days to remove potential interference with the measurements.

Acute exercise

After 2 weeks' immobilization of one leg, the subjects reported to the laboratory in the morning after an overnight fast (12 hours). A DXA scan was performed to determine the impact of immobilization on leg muscle mass. A muscle biopsy was obtained from both legs. The procedure was done after local anesthesia (lidocaine; 5 mg/ml, Amgros I/S, Copenhagen, Denmark) of the skin and the superficial muscle fascia using the Bergström needle modified with suction (Bergstrom, 1979). The biopsy was immediately frozen in liquid nitrogen and stored at -80 °C for

further analysis. Thereafter, catheters were placed in the brachial artery (20G arterial cannula, Becton Dickinson A/S, Albertslund, Denmark) and both femoral veins (14G catheter, Arrow International, ViCare Medical, Birkeroed Denmark) under local anesthesia. The catheters were inserted into the femoral vein distal to the inguinal ligament and in the antegrade direction. The catheters were kept patent by a slow drip of isotonic sodium chloride infusate. On one occasion, a subject was unable to perform acute exercise due to a vasovagal syncope and on five occasions we were unable to insert a catheter in one of the legs. Hence, the data presented here is for 14 and 13 control legs and 16 and 14 immobilized legs in the young and older men, respectively. After one hour of rest, the subject were positioned in a custom-made isolated one-leg knee extension ergometer in a semi-supine position. Blood was sampled simultaneously from the brachial artery and femoral veins -15 min and just before exercise at time 0 min. Subsequently, the subjects performed 45 min isolated dynamic knee-extensor exercise with both legs with each leg in a separate one-legged ergometer. The absolute leg workload was set to 50 % of $Watt_{max}$, which was determined before the immobilization, i.e. both legs performed the same absolute amount of work. During exercise, blood was sampled at 15, 30 and 45 min. Femoral arterial blood flow was measured at all time points in both legs by Doppler ultrasound (ACUSON S2000, Siemens Healthcare, Ballerup, Denmark). Heart rate was recorded continuously before and during the exercise. Furthermore, subjects were requested to report perceived work load on a scale from 1-10 (1 was: “Can go on forever” and 10 was “I have to stop within seconds”) after 5, 25 and 40 min of exercise. Whole-body oxygen consumption was measured from 20 to 27 min of exercise using an Oxycon Pro (Jaeger, CareFusion GmbH, Hoechberg, Germany). Throughout the experiment participants had free access to water and exercise was performed at an ambient temperature of 20°C. Another muscle biopsy was obtained from both legs immediately (5-10 min) after the acute exercise. The participants reported to the laboratory the day after in order to determine changes in

$Watt_{max}$ after immobilization. The test was performed the day after the acute exercise so it could not interfere with the effects of immobilization.

Analytical procedures

Blood was sampled anaerobically and distributed into tubes containing heparin or Trasylol/EDTA. The heparinized samples were immediately analyzed for hematocrit (ABL800 Flex, Radiometer, Copenhagen, Denmark). Plasma for determination of glucose, glycerol and FA was cooled down and separated by centrifugation at 2000 g at 4°C for 10 min, frozen on dry ice, and stored at -80°C until further analysis. Plasma glucose, FFA, and glycerol were analyzed on a Cobas 6000 c 501 (Roche, Glostrup, Denmark).

Calculations

The plasma concentration of O_2 and CO_2 were calculated as previously described (Siggaard-Andersen *et al.*, 1988; Peronnet & Massicotte, 1991). The Fick principle was used to calculate the leg uptake or release of O_2, CO_2, glucose, lactate, FA and glycerol across the legs at rest and during exercise (i.e. the brachial arterial and femoral venous plasma concentration differences multiplied by plasma flow (blood flow x (1-Hct)). Indirect calorimetry was used to calculate total energy contribution of glucose and FA oxidation (Peronnet & Massicotte, 1991). The energy contribution from FA uptake and glycerol release was calculated by converting the rate of oxidation (μmol/min) to its molar mass equivalent (272.4 and 860 g/mole, respectively; (Jeukendrup & Wallis, 2005) and by assuming that oxidation of 1 gram of triglycerides yields 9.75 calories (Jeukendrup & Wallis, 2005). The area under the curve (AUC) was calculated by the trapezoid method with x-axis as a baseline. However, AUC for leg glycerol release is presented with the resting value as a baseline because the young men had a net release, and the older men had a net uptake (Figure 2C).

Western blotting

Two laboratories performed the Western blotting analysis of protein content and, therefore, two Western blotting procedures are described. The protein content was analyzed in the biopsies obtained before the acute exercise (i.e. and phosphorylation of proteins were analyzed in the biopsies obtained both before and after the exercise (i.e. ACC2 and pACC Ser221).

Lipid metabolism related proteins

The analysis was performed as previously described in detail (Larsen *et al.*, 2014; Vigelsø *et al.*, 2015a). In brief: 4.0-4.5 mg (dry weight) of skeletal muscle were homogenized in cold RIPA buffer enriched with protease and phosphatase inhibitors (50 mM Tris pH 8.0, 150 mM NaCl, 1 % NP-40, 0.5 % Na-Deoxycholate, 0.1 % SDS, 2.5 mM PMSF, 20 mM β-glycerophosphate, 10 mM Pyrophosphate, 2 mM Sodium Ortovanadate, including mini EDTA-free protease inhibitor tablet according to the instructions of the manufacturer (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured by bicinchoninic acid assay (Pierce, Rockford, IL, USA) in triplicate, and a maximal coefficient of variation of 5 % between replicates was accepted.

Twenty µg of protein lysate in sample buffer was heated to 95 ˚C for 10 min and separated on 12 % Criterion TGX Stain-Free polyacrylamide sodium dodecyl sulfate pre-casted gels (Criterion, BioRad, Copenhagen, Denmark). After SDS-electrophoresis, the gels were activated with UV light for 5 min followed by a 1 s image in an LAS 4000 image analyzer (GE Healthcare, Little Chalfont, UK). The activated gel was transferred to a polyvinylidene fluoride (PVDF) membrane (0.2 µm pores, BioRad, Copenhagen, Denmark) using the Trans-Blot Turbo Transfer System (BioRad, Copenhagen, Denmark) with Trans-Blot Turbo Midi Transfer Packs. After the transfer, another 1 s image of the membrane and gel with UV light to visualize protein transfer was taken. The membranes were blocked for 1½ hr at room temperature with either skimmed milk or bovine serum albumin (BSA) diluted in Tris-buffered saline (10 mM Tris Base, 150 mM NaCl, pH 7.4) + 0.05 %

Tween 20. The membranes were then probed with primary antibodies: anti-ATGL (Ab109251, Abcam, Cambridge, UK), anti-FABPpm (GOT2, Ab93928, Abcam, Cambridge, UK), anti-HSL (G7, sc-74489, Santa Cruz biotechnology, Inc., Heidelberg, Germany), and anti-LPL (H53, sc-32885, Santa Cruz biotechnology, Inc., Heidelberg, Germany) overnight at 4°C. Thereafter, a horseradish peroxidase-conjugated secondary goat antibody against rabbit were added (Dako, Glostrup, Denmark). After primary and secondary antibody incubations, the membranes were washed 3 x 10 min in Tris-buffered saline +/- 0.05 % Tween 20. Blots were developed in ECL detection reagents (GE Healthcare), and the chemiluminescence emitted from immune-complexes was visualized with an LAS 4000 image analyzer (GE Healthcare). The images of the membranes and stain-free gels were quantified by ImageQuant TL software version 7.0 (GE Healthcare). Because we previously have observed that GAPDH, β-actin and α-tubulin not are suitable as loading control in this dataset (Vigelsø *et al.*, 2015a), the intensities of the bands of interest were normalized to the total Stain-Free fluorescence (total protein).

Muscle lysate preparation for AMPK analysis

Lysates were prepared from 20 mg freeze-dried muscle, dissected free of visible connective tissue, blood and fat and homogenized in 50 mM HEPES (pH 7.5), 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 2 mM Na_3VO_4, 3 mM benzamidine. Homogenates rotated end over end at 4 °C for one hour. Lysates were prepared by centrifugation of the homogenates for 20 min at 16,000 g. Total lysate protein content was analyzed by the bicinchoninic acid method (Pierce Biotechnology, USA).

SDS-PAGE and Western blotting

AMPK subunit isoform protein levels, AMPK (Thr^{172}) and pACC2 Ser221 phosphorylation were measured on lysate mixed in sample buffer (350 mM Tris-HCl, pH 6.8, 30 % Glycerol, 350 mM SDS, 600 mM DTT, 0.2 mM Bromophenol blue) and heated 5 min at 96 °C. Fifteen μg of protein were separated using 7.5 and 10 % Criterion TGX Stain-Free Precast Gels (BioRad, CA) and transferred (semi-dry) at 20 V for 30 min (BioRad Trans-Blot Turbo Transfer System) to PVDF membranes (Immobilon Transfer Membrane; Millipore, Denmark). After blocking (in Tris-buffered saline + 0.05 % Tween-20 (TBST) + 2 % skim milk) for 45 min at room temperature, the membranes were incubated with primary antibodies (in TBST + 2 % skimmed milk) overnight. The antibodies used were: α_1AMPK (Abcam ab32047), β_1AMPK (Arexis, (Mahlapuu *et al.*, 2004), β_2AMPK (donated by Grahame Hardie, College of Life Sciences, The University of Dundee), AMPKγ_1 (Abcam, ab32508), AMPKα_2 (SC19131), AMPKγ_3 (Santa Cruz biotechnology, Inc., Heidelberg, Germany, sc-20166), pACCSer221 (Millipore, 07-303), pAMPKThr172 (Cell Signaling, #2531). Membranes were incubated with the appropriate horseradish peroxidase-conjugated (HRP) secondary antibody for 45 min at RT (TBST + 2 % skimmed milk) (DAKO, Denmark or Jackson ImmunoResearch, PA). Detection of ACC was performed by incubating the blocked (3% BSA) membrane with HRP-conjugated streptavidin (Dako, P0397) (in 3 % BSA) overnight. Blots were developed in ECL detection reagent (ECL, Millipore ECL Forte) and visualized by a charge-coupled device camera (ChemiDoc^{TM} MP System BioRad, CA). Band densitometry was performed using BioRad ImageLab (version 4.0). The protein content was expressed in arbitrary units subtracted background and related to the mean of a human skeletal muscle standard sample loaded twice on the corresponding gel in order to minimize assay variation.

By loading a control-sample in different amounts, it was ensured that quantification was within the linear response range for each particular protein probed for.

One membrane (25-50 kDa) was sequentially probed for γ_1 (38 kDa) and then β_2 (30 kDa) since they have distinguishable molecular weights and bands. Two membranes were reprobed with an alternate antibody (pACC -> ACC and α_1 -> α_2) after being incubated for 60 min at 50^0C in stripping buffer (62.3 mM Tris-HCl, 69.4 mM SDS, ddH_2O and 0.8 % β-Mercaptoethanol) and tested for signal from the first antibody.

AMPK activity assay

AMPK complex specific activities were measured on heterotrimeric complexes isolated by a sequential immunoprecipitation. A mixture of 300 µg of muscle lysate protein, a γ_3 isoform-specific antibody (Dr. Hardie, Dundee) and protein G-agarose beads (Milipore, # 16-266)) in IP-buffer (50 mM NaCl, 1 % Triton-X 100, 50 mM NaF, 5 mM Na-pyrophosphate, 20 mM Trizma Base pH 7.5, 500 µM PMSF, 2 mM DTT, 4 µg/ml Leupeptin, 50 µg/ml Soybean Trypsin Inhibitor T9128, 6 mM Benzamidine, 250 mM Sucrose) rotated end over end overnight at 4 °C. The samples were centrifuged for 2x60 s at 520 g at 4 ^{0}C. The immunoprecipitate (IP) with bound $\alpha_2\beta_2\gamma_3$ was washed once in IP-buffer, once in 480 mM HEPES (pH 7.0) and 240 mM NaCl, and twice in 240 mM HEPES (pH 7.0) and 120 mM NaCl leaving only the agarose after last wash. The kinase reaction ran for 30 min at 30 °C in a total volume of 30 µl containing 833 µM DTT, 200 µM AMP, 100 µM AMARA-peptide, 5 mM $MgCl_2$, 200 µM ATP and 2 µCi of ATP[γ-^{33}P] (Perkin Elmer, DK). The reaction was stopped by adding 10 µl of 1 % phosphoric acid to the reaction, after which 20 µl was spotted onto P81 filter paper (Whatman, GE Healthcare, DK), which was then washed 4x15 min in 1 % phosphoric acid. The dried filter paper was analyzed for activity using a Storm 840 PhosphoImager (Molecular Dynamics). The $\alpha_2\beta_2\gamma_1$ activity was analyzed on a α_2 (Dr. Hardie, Dundie) IP on supernatant immunodepleted for $\alpha_2\beta_2\gamma_3$. $\alpha_1\beta_2\gamma_1$ activity was measured an α_1 (Dr.

Hardie, Dundie) IP on supernatant immunodepleted for both $\alpha_2\beta_2\gamma_3$ and $\alpha_2\beta_2\gamma_1$- as performed previously (Birk & Wojtaszewski, 2006).

HAD enzyme activity

β-hydroxyacyl-CoA dehydrogenase (HAD) was measured using spectrophotometry. Approximately 2 mg of the dissected tissue were homogenized in 600 µl 0.3 M K_2 HPO_4, 0.05 % BSA, pH 7.7 for 2 min on a Tissuelyzer (Qiagen, Venlo, Limburg, Netherlands). Six µl of 10 % triton was added and the samples were left on ice for 15 min before they were stored at -80 °C for later analysis. The homogenate was diluted 70 times in a solution containing 0.33mM acetoacetyl-CoA, 180 µM reduced nicotinamide adenine dinucleotide (NADH), 41.7 µM ethylenediaminetetraacetic acid (EDTA), 27.1 mM imidazole (pH 7.0). The changes in NADH at 37°C were measured spectrophotometrically at 340 nm (Bergmeyer, 1974) on an automatic analyzer, Cobas 6000, C 501 (Roche Diagnostics). Enzyme activities are expressed as micromoles substrate per minute per gram dry weight of muscle tissue.

Muscle PDC enzyme activity assay

A small portion of frozen 'wet' muscle was used to determine PDC activity as previously described (Constantin-Teodosiu *et al.*, 1991). Briefly, the activity of PDC in its dephosphorylated active form (PDCa) was assayed in a buffer containing NaF and DCA, and was expressed as a rate of acetyl-CoA formation (mmol/min/kg wet muscle) at 37°C.

Statistics

To investigate the effects of group (young and older men), leg (immobilized and control) and acute exercise (rest (-15 and 0 min), 15, 30 and 45 min) and possible interactions (group x leg x acute exercise), a mixed model ANOVA was performed with least squares post hoc tests followed by a

Tukey-Kramer adjustment. Systematic effects in the model were group, leg and acute exercise with random levels for leg nested within subject. When interactions were non-significant, the statistical model was reduced accordingly. Data that were not normally distributed or had unequal variance were log-transformed before statistical analysis. In the case of randomly missing values, the Satterthwaite approximation was used. Outliers were systematically removed from the dataset if the data point was > mean ± 2 SD. The level of significance was set at $P < 0.05$. Statistical analysis was conducted in SAS Enterprise Guide 4.3 (SAS Institutes, Cary, NC, USA). All data are presented as mean ± SEM.

Results

Anthropometric data

The participants were included to be representative for their age-group. Hence, the older men had higher BMI and body fat percent and the young men had higher VO_2max (Table 1) (Reihmane *et al.*, 2013; Gram *et al.*, 2014; Nørregaard *et al.*, 2014).

$Watt_{max}$ and workload during acute exercise

During the acute exercise, there was no difference in workload (watt), heart rate relative to maximal heart rate, whole-body respiratory exchange ratio (RER) between the groups (Table 2). At inclusion, there was no difference in $Watt_{max}$ between the groups or the legs (Vigelsø *et al.*, 2015b). With immobilization $Watt_{max}$ decreased by -14 ± 5 % ($P<0.05$) and - 9 ± 4 % ($P<0.05$) in the young and older men's immobilized leg, respectively. $Watt_{max}$ did not change in the control leg in either group (Vigelsø *et al.*, 2015b) (Table 2). Hence, the immobilized leg worked at a relatively higher workload compared to the control leg in both young and older volunteers (Table 2).

Reported perceived exertion (1-10) for the individual legs increased ($P<0.05$) in both groups (main effect) and legs (main effect) from the beginning (5 min) compared to after 25 and 40 min of exercise. Throughout the exercise, the participants reported greater perceived exertion in the immobilized leg compared to the control leg ($P<0.05$; main effect, data not shown). Hence the average perceived exertion was reported to be higher (main effect, $P<0.05$) in the immobilized leg compared to the control leg (corresponding to 4: "I can continue for several hours", 5: "I have to stop within an hour", Table 2).

Acute exercise, blood flow, RQ

Average blood flow during exercise was 41 % greater ($P<0.05$) in the young compared to the older men with no difference between the legs, but oxygen extraction was 14 % higher ($P<0.05$) in the older compared with the young men. Nevertheless, the young men had 32 % greater ($P<0.05$) absolute leg VO_2 during the acute exercise, but this difference disappeared when VO_{2max} was normalized to leg lean mass (Figure 1A). However, the young men had higher leg O_2 uptake over the complete exercise bout (area under the curve) compared to the older men (Figure 1B). In the older men, the RQ was higher ($P<0.05$) after 15 and 30 min of exercise compared to 0 (rest) and 45 min (Figure 1C). During exercise the RQ for both legs in the older men increased ($P<0.05$) to the level of the young men (Figure 1C).

Exogenous substrate utilization

Leg glucose uptake increased ($P<0.05$) with the onset of exercise with no difference between groups or legs during exercise (Figure 2A). At rest leg FA uptake and leg glycerol release did not differ between the legs in either young or older men (Figure 2B and C). From rest to exercise, leg FA uptake increased ($P<0.05$) in both groups and legs. Moreover the older men (both legs) had a greater FA uptake ($P<0.05$) compared to the young men (Figure 2 B and C). However, leg glycerol

release only increased (P<0.05) from rest to exercise in the young men (main effect) and not in the older men. Hence, the young men had a net leg glycerol release and the older men had a net glycerol uptake (Figure 2C).

Arterial delivery of FA per leg lean mass (young: control leg: 2.0 ± 0.2; immobilized leg: 2.2 ± 0.3 μmol/kg LLM/45 min and older men: control leg:2.1 ± 0.3; immobilized leg: 2.0 ± 0.2 mmol/kg LLM/45 min) and glycerol (young: control leg: 281 ± 81; immobilized leg:319 ± 35 μmol/kg LLM/45 min, and older men: control leg: 252 ± 40; immobilized leg:299 ± 56 μmol/kg LLM/45 min) did not differ between the groups or the legs, respectively.

Lactate release increased (P<0.05) in both groups and legs (main effect) from rest to exercise (Figure 2D). Furthermore, the lactate release was greater (main effect) in the immobilized leg compared to the control leg in both groups (Figure 2D).

Endogenous substrate utilization

After immobilization, but before the acute exercise, the older men had 69 and 89 % greater (P<0.05) IMTG than the young men in the control and immobilized leg, respectively (Table 2). Furthermore, IMTG was 50 ± 23 and 45 ± 20 % higher (main effect, P>0.05) in the immobilized leg compared to the control leg after immobilization in the young and older men, respectively (Table 2). IMTG content did not change with acute exercise in either groups or legs (Table 2).

Muscle glycogen content was greater (main effect, P>0.05, 17 %) in the older men compared to the young men (Table 2). The muscle glycogen content decreased (main effect, P>0.05) with acute exercise (Table 2).

Enzyme activity

Muscle HAD activity at rest did not differ between the groups and was significantly greater in the control leg than in the immobilized leg in both age-groups (12 ± 6 % and 10 ± 6 %, respectively; $P<0.05$, Table 2).

Proteins in lipid metabolism

Protein content of FABPpm tended (main effect, $P=0.08$) to be 20 % higher in the young compared to the older men. Additionally, there was 25% more ($P<0.05$) FAPBpm protein in the control leg (main effect) compared to the immobilized leg (Figure 3A). ATGL protein levels were 30 % higher ($P<0.05$) in the young compared to the older men. Likewise, the ATGL protein content was 20 % higher (main effect, $P<0.05$) in the control leg compared to the immobilized leg (Figure 3B). Furthermore, there was a trend (main effect, $P=0.06$) of 32 % higher LPL content in the older men (Table 3). There was no difference between the legs or groups in HSL protein content (Table 3).

AMPK and ACC2 protein content

AMPKα1 and α2 protein content did not differ between age-groups. The protein content of AMPKβ2 was higher ($P<0.05$) in the young compared to the older men (Table 3). AMPKβ1 protein content was lower (main effect, $P<0.05$) in the control leg compared to the immobilized leg in both groups (Table 3). AMPKγ1 protein content did not differ between age-groups or the legs (Table 3). AMPKγ3 protein content was higher in young compared to older men and in the control leg than in the immobilized leg in both age-groups ($P<0.05$; Table 3).

ACC2 protein content in the young was higher than in the older men (Figure 4A). Furthermore, phosphorylation of ACCSer221 increased (main effect, $P<0.05$) with acute exercise in both groups and legs (Figure 4B). Moreover, the acute exercise-induced ACCSer221 phosphorylation was

higher ($P<0.05$) in the immobilized compared to the control leg (Figure 4B). Collectively, the ACCSer221 phosphorylation to ACC2 protein content-ratio increased (main effect, $P<0.05$) with acute exercise and was higher in the immobilized leg (main effect, $P<0.05$). Additionally, there was a trend (main effect, $P=0.07$) towards higher ACCSer221 phosphorylation to ACC2 protein content-ratio in the immobilized leg (Figure 4C).

AMPK complex specific activity and PDC activity

The activity of AMPK$\alpha_1\beta_2\gamma_1$ in the young men was lower (main effect, $P<0.05$) than in the older men. Moreover, in response to acute exercise AMPK$\alpha_1\beta_2\gamma_1$ activity decreased (main effect, $P<0.05$) in the immobilized leg (Figure 5A). There was no difference between the age-groups, between legs or in response to acute exercise in AMPK$\alpha2\beta2\gamma1$-activity (Figure 5B) or AMPKThr172 phosphorylation, representing total AMPK activity (Table 3). There was an increase across age and treatment groups (main effect) in AMPK$\alpha2\beta2\gamma3$ activity with acute exercise (Figure 5C).

Muscle PDC activity in the young was higher (main effect, $P<0.05$) than in the older men (Figure 6A). Furthermore, there was a negative correlation ($R^2 = 0.40$, $P<0.05$) between the AUC for lactate release (Figure 2D) and ΔPDC activity pre- and post-exercise (Figure 6B).

Calculations of energy consumption: indirect calorimetry and substrate utilization

The relative glucose and lipid oxidation calculated by indirect calorimetry did not differ between groups or legs (young men (glucose/lipid): 72 ± 5 % / 28 ± 5 % and 75 ± 5 % / 25 ± 5 %; Older men: 70 ± 6 % / 30 ± 6 % and 72 ± 6 % / 28 ± 6 % for the control and immobilized leg, respectively). The young men had an equal contribution of FA derived exogenously (control leg: 44 ± 7 % and immobilized leg: 33 ± 6 %) and endogenously in the leg (control leg: 56 ± 7 % and immobilized leg: 67 ± 6 %). Whereas, the older men that had a larger exogenous contribution

(control leg: 85 ± 6 % and immobilized leg: 79 ± 4 %) compared to the endogenous contribution (control leg: 15 ± 6 % and immobilized leg: 21 ± 4 %).

Discussion

In this study, our main finding was that older men have greater uptake of exogenous FA, while the young men have greater use of endogenous fat stores during an acute bout of exercise performed with both legs each in a separate one-legged ergometer. This finding is based on the difference in net leg glycerol release between groups. The proposed difference in fat mobilization between young and older men may have been accounted for by the higher ATGL protein content recorded in the young than in the older men, which could explain greater skeletal muscle lipolysis rates in the young men. In addition, higher AMPKβ2 and AMPKγ3 subunit protein content was observed in young compared to older men, which support the contention of augmented AMPK expression with age (Drummond *et al.*, 2008; Mortensen *et al.*, 2009). In contrast to our hypothesis and despite the muscle adaptations to the leg immobilization (i.e. decreased HAD activity, ATGL, AMPKβ1 and AMPKγ3 protein content) and the age-difference (i.e. greater ATGL and ACC2 content and PDC activity in the young than the older men) did not change the relative carbohydrate and fat utilization. Overall, we demonstrate that both inactivity and age are associated with metabolic inflexibility seen as an IMTG accumulation, but that this only partially occurs through the same mechanisms.

Respiratory quotient and relative substrate utilization

We hypothesized that age and immobilization would increase the glucose uptake and hence lead to an increased RQ during a subsequent exercise bout at moderate intensity. However, we found no differences in RQ between the immobilized and control leg during acute one-legged exercise in young or older men. The immobilization protocol was sufficent to induce changes in muscle metabolism (e.g. increased relative work load, decreased CS and mitochondrial respiration (Gram *et al.*, 2014) and HAD activity, a lower AMPKβ_2 (in the young men only), AMPKγ_3, ATGL and FABPpm protein content and a higher ACCSer221 phosphorylation after acute exercise). Hence,

the metabolic changes induced by immobilization were either insufficient to induce a change in RQ, and/or the workload was not sufficiently high to elicit a difference. We cannot rule out that a difference in substrate utilization for young and older men may become detectable at a higher exercise intensity. However, based on pilot studies we found that higher intensities were not feasible for the participants. Moreover, the relevance of using the present exercise intensity may bear a clinical and physiological significance because it elucidates limitations in substrate mobilization during an intensity that mimics an everyday work-intensity.

Lipid mobilization during exercise

We hypothesized that immobilization and age would change the source of FA mobilized during exercise to rely more on exogenous FA. Leg FA uptake did indeed increase during exercise, although this was greater in older men than in young men. In addition, in response to exercise, young men skeletal muscle showed net leg glycerol release, whereas in contrast, exercise induced net leg glycerol uptake in older men.

Suppression of adipose tissue lipolysis with nicotinic acid has been shown to be associated to increases in IMTG utilization in young men (Watt *et al.*, 2004), leading the authors to suggest that plasma FA availability is a regulator of IMTG utilization (O'Neill *et al.*, 2004; Watt *et al.*, 2004). However, presently there was no difference in the delivery of FA and glycerol throughout the exercise and the observed difference can thus not be explained by substrate availability during exercise. Nevertheless, we have previously reported that older men have higher fasting plasma FA and visceral adipose tissue (Nørregaard *et al.*, 2014). Hence, increased exogenous FA utilization may be an age-related adaptation to chronic high resting FA availability. FA infusion has been shown to lead to IMTG accumulation (Schenk *et al.*, 2005), and high plasma FA are known to inhibit IMTG utilization (O'Neill *et al.*, 2004; Watt *et al.*, 2004). Hence, the higher resting fasting plasma FA in the older men may have contributed to induce the observed higher IMTG. IMTG

accumulation is related to insulin resistance, although not necessarily causual, and it is thus likely that the IMTG accumulation in the older men at some point will lead to insulin resistance (Unger, 2002; Moro *et al.*, 2008). We have previously reported that young and older men did not differ in capillarization, muscle fibre type distribution, or muscle fiber size (Vigelsø *et al.*, 2015b), and therefore the muscle morphology cannot explain the higher FA uptake and ATGL protein content in the young men. Interestingly, ATGL has been proposed as the major lipase in skeletal muscle during contraction (Alsted *et al.*, 2013) and this indicates that young men have a higher intramuscular lipolytic capacity, and thus a higher endogenous FA supply capacity, which may explain the higher leg glycerol release during exercise.

Lipid uptake and lipolytic proteins

FABPpm (Kiens *et al.*, 1997; Kiens *et al.*, 2004) and ATGL (Alsted *et al.*, 2009; Louche *et al.*, 2013) have previously been shown to increase with endurance training. This is the first study to show that these proteins decrease with immobilization for 2 weeks. Furthermore, it is also novel that the protein content of ATGL was higher in young than in older men.

AMPK and regulation of β-oxidation

It is a new finding that protein content of ACC2 and AMPKβ2 subunit were higher in young than in older men. However, our observation of decreased AMPKγ3 protein in the immobilized legs of both the young and older men is in contrast to two studies which employed different models of inactivity. Mortensen et al. observed increased AMPKγ3 protein content after 9 days bedrest (Mortensen *et al.*, 2014), while Kostovski et al. observed increased AMPKγ3 in long term compared to recent spinal injuried individuals (Kostovski *et al.*, 2013).

In skeletal muscle, AMPK may partly regulate fatty acid β-oxidation through the inhibition of ACC2 and thereby decreasing CPT1 mediated FA uptake in the mitochondria (Stephens *et al.*,

2002). Little is known about this pathway in relation to immobilization and age. In this study, acute exercise increased the phosphorylation of ACC2Ser221 in the immobilized legs of both groups. Paradoxically, this implies that the acute exercise in the immobilized leg increased mitochondrial FA supply and oxidation, which was not the case. We speculate that this may be related to the higher relative workload in the immobilized leg or that the ACC2 pathway possibly compensates for a decreased contribution of other pathways affected by the immobilization. The latter contention is supported by the observation that muscle fatty oxidation can occur without changes in malonyl-CoA concentrations (Odland *et al.*, 1996; Odland *et al.*, 1998; Dean *et al.*, 2000). If the concentration of malonyl-CoA is not essential for switching between glucose and fat oxidation in skeletal muscle, it seems unlikely that AMPK mediated ACC2 inhibition plays a vital role in regulation of FA uptake by the mitochondria. Support for this notion is provided in a recent study in AMPKα KO-mice where Fentz et al. suggested that the AMPKα subunit exerts additional indirect effects on FA utilization during exercise through regulation of FABPpm content (Fentz *et al.*, 2015).

PDC activity and lactate release

To our knowledge, this is the first evidence that muscle mitochondrial PDC activation status at rest is lower in older men than in young and this remained unchanged after exercise. Although these observations would be intuitively expected given that PDC activation status is related to the aerobic/mitochondrial capacity (Constantin-Teodosiu, 2013), this is in contrast to the recent report by Wall *et al.* where muscle PDC activation is reported to increase in older men, but not in young, following 5 days of leg immobilization (Wall *et al.*, 2015). Additionally, another important finding of the current study was that leg lactate release (AUC) during exercise was negatively associated with the change in PDC activation during exercise (Figure 6A and B).

Indeed, PDC activity is the rate-limiting step in glucose oxidation and therefore, at least partly, controls muscle glucose and FA oxidation (Constantin-Teodosiu *et al.*, 1992; van Loon *et al.*, 2001). The lower PDC activity in the older men therefore implies a lower capacity of muscle to oxidize glucose. However, this was not detected as a decreased glucose utilization during exercise. Moreover, the difference in PDC activity could not be related to differences in the mitochondrial content or function, as previously suggested (Constantin-Teodosiu, 2013). We have previously published that mitochondrial content (measured as CS activity, voltage dependent ion channel (VDAC), mitochondrial complex protein content and mitochondrial respiratory capacity) did not differ between the groups (Gram *et al.*, 2014). Since IMTG accumulation has been proposed to be an inhibitor of PDC activity (Gurd *et al.*, 2008) the lower PDC activity in the older men could be potentially accounted for by the greater levels of IMTG.

AMPKα1 and protein synthesis

Although it was not our primary hypothesis, it is a noteworthy finding that AMPKα1 specific activity was higher in older men than in young (Fig. 5A), despite no difference in AMPKα1 protein content. High specific activity of AMPKα1 has been suggested to inhibit the mTORC1 pathway, thereby inhibiting protein synthesis in skeletal muscle (Mounier *et al.*, 2009; Mounier *et al.*, 2011), and to be important for lipid metabolism (Fentz *et al.*, 2015). Hence, high AMPKα1 activity in older men could contribute to a lower contraction induced protein synthesis by mTORC1, which have been linked, albeit in rodents, to sarcopenia (Parkington *et al.*, 2004; Thomson & Gordon, 2006).

Implications for metabolic inflexibility

In agreement with others, we observed that age was associated with metabolic inflexibility seen as IMTG accumulation (Petersen *et al.*, 2003; Cree *et al.*, 2004; Wall *et al.*, 2015). However, in

contrast to another recent study using 5 days' one-leg immobilization (Wall *et al.*, 2015), we observed IMTG accumulation with our immobilization intervention. It is likely that over time the IMTG accumulation observed in older men and in the immobilized leg will lead to insulin resistance (Unger, 2002; Moro *et al.*, 2008). The IMTG accumulation may have arisen from the age-related shift in source of FA for oxidation and an inactivity related decline in ATGL and FA oxidative capacity.

Limitations

It is a limitation that we did not use tracers, such that we could have distinguished between sources of glycerol release, i.e. glycerol coming from blood lipoproteins (VLDL), plasma TG or IMTG. However, the primary aim was to investigate the overall difference in substrate utilization measured by indirect calorimetry and to elucidate the source of FA mobilization (exogenous or endogenous) during a bout of moderate intesity exercise undertaken by young and older men. Equally, it is important to note that glycerol is both released and taken up by tissues (van Hall *et al.*, 2002; Stallknecht *et al.*, 2004; Helge *et al.*, 2007) and the net glycerol release is probably underestimating total glycerol release. On the other hand, the catheters were inserted in the anterograde direction and therefore the measurements of glycerol release may be slightly overestimated and FA uptake slightly underestimated due to contamination of venous blood from *v. circumflexea ilium superficialis* (van Hall *et al.*, 1999). However, this contribution is minor (van Hall *et al.*, 1999) and since this was done systematically the contamination is likely to have equally affected both groups and legs. Finally, the protein content obtained by Western blotting does not provide information on intramuscular compartmentalization, functionality or activity (Prats *et al.*, 2011), and this should be a topic for future research.

Conclusions

Using an efficient immobilization protocol, we found that immobilization and age-difference did not affect relative substrate metabolism during a bout of exercise of moderate intensity. This occurred despite impairments in muscle metabolism (lower PDC activity, AMPKβ2 and AMPKγ3 protein content) with age and despite an clear effect of the immobilization (i.e. lower muscle HAD activity; lower ATGL protein; lower AMPKα1, β1 and γ3 protein; higher ACCser221 phosphorylation and higher lactate release during exercise in the young and older men with immobilization). Thus, our data support the idea that AMPK is impaired with both immobilization and age. Furthermore, the higher lipolytic capacity in the young men, as suggested by their higher ATGL protein content than in the older men, probably contributed to the increased endogenous FA utilization and higher glycerol release during exercise. In agreement with others, we report that age and inactivity are associated with metabolic inflexibility seen as IMTG accumulation. However, our findings indicate that age and immobilization only partly lead to IMTG accumulation through the same mechanisms. Thus, the age-related metabolic inflexibility may be related to a shift in the source of mobilized FA (i.e. primarily reliance on exogenous FA recruited from the circulation), whereas the immobilization-related metabolic inflexibility was only seen at the muscle protein level.

References

Alibegovic AC, Hojbjerre L, Sonne MP, van Hall G, Alsted TJ, Kiens B, Stallknecht B, Dela F & Vaag A. (2010). Increased rate of whole body lipolysis before and after 9 days of bed rest in healthy young men born with low birth weight. *Am J Physiol Endocrinol Metab* **298,** E555-564.

Alsted TJ, Nybo L, Schweiger M, Fledelius C, Jacobsen P, Zimmermann R, Zechner R & Kiens B. (2009). Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training. *Am J Physiol Endocrinol Metab* **296,** E445-453.

Alsted TJ, Ploug T, Prats C, Serup AK, Hoeg L, Schjerling P, Holm C, Zimmermann R, Fledelius C, Galbo H & Kiens B. (2013). Contraction-induced lipolysis is not impaired by inhibition of hormone-sensitive lipase in skeletal muscle. *J Physiol* **591,** 5141-5155.

Bergmeyer HU. (1974). *Methods of enzymatic analysis*. Academic Press, NewYork.

Bergstrom J. (1979). Muscle-biopsy needles. *Lancet* **1,** 153.

Birk JB & Wojtaszewski JF. (2006). Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol* **577,** 1021-1032.

Bonadonna RC, Groop LC, Simonson DC & DeFronzo RA. (1994). Free fatty acid and glucose metabolism in human aging: evidence for operation of the Randle cycle. *Am J Physiol* **266,** E501-509.

Conley KE, Jubrias SA & Esselman PC. (2000). Oxidative capacity and ageing in human muscle. *J Physiol* **526 Pt 1,** 203-210.

Constantin-Teodosiu D. (2013). Regulation of muscle pyruvate dehydrogenase complex in insulin resistance: effects of exercise and dichloroacetate. *Diabetes Metab J* **37,** 301-314.

Constantin-Teodosiu D, Cederblad G & Hultman E. (1991). A sensitive radioisotopic assay of pyruvate dehydrogenase complex in human muscle tissue. *Anal Biochem* **198,** 347-351.

Constantin-Teodosiu D, Cederblad G & Hultman E. (1992). PDC activity and acetyl group accumulation in skeletal muscle during prolonged exercise. *J Appl Physiol (1985)* **73,** 2403-2407.

Cree MG, Newcomer BR, Katsanos CS, Sheffield-Moore M, Chinkes D, Aarsland A, Urban R & Wolfe RR. (2004). Intramuscular and liver triglycerides are increased in the elderly. *J Clin Endocrinol Metab* **89,** 3864-3871.

Dean D, Daugaard JR, Young ME, Saha A, Vavvas D, Asp S, Kiens B, Kim KH, Witters L, Richter EA & Ruderman N. (2000). Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. *Diabetes* **49,** 1295-1300.

Drummond MJ, Dreyer HC, Pennings B, Fry CS, Dhanani S, Dillon EL, Sheffield-Moore M, Volpi E & Rasmussen BB. (2008). Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging. *Journal of applied physiology (Bethesda, Md : 1985)* **104,** 1452-1461.

Dzamko N, Schertzer JD, Ryall JG, Steel R, Macaulay SL, Wee S, Chen ZP, Michell BJ, Oakhill JS, Watt MJ, Jorgensen SB, Lynch GS, Kemp BE & Steinberg GR. (2008). AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. *J Physiol* **586,** 5819-5831.

Eijnde BO, Derave W, Wojtaszewski JF, Richter EA & Hespel P. (2005). AMP kinase expression and activity in human skeletal muscle: effects of immobilization, retraining, and creatine supplementation. *Journal of applied physiology (Bethesda, Md : 1985)* **98,** 1228-1233.

Eriksen L, Gronbaek M, Helge JW, Tolstrup JS & Curtis T. (2011). The Danish Health Examination Survey 2007-2008 (DANHES 2007-2008). *Scand J Public Health* **39,** 203-211.

Fentz J, Kjobsted R, Birk JB, Jordy AB, Jeppesen J, Thorsen K, Schjerling P, Kiens B, Jessen N, Viollet B & Wojtaszewski JF. (2015). AMPKalpha is critical for enhancing skeletal muscle fatty acid utilization during in vivo exercise in mice. *FASEB J*.

Fielding RA, Vellas B, Evans WJ, Bhasin S, Morley JE, Newman AB, Abellan van Kan G, Andrieu S, Bauer J, Breuille D, Cederholm T, Chandler J, De Meynard C, Donini L, Harris T, Kannt A, Keime Guibert F, Onder G, Papanicolaou D, Rolland Y, Rooks D, Sieber C, Souhami E, Verlaan S & Zamboni M. (2011). Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. *J Am Med Dir Assoc* **12,** 249-256.

Fogelholm M. (2013). New Nordic Nutrition Recommendations are here. *Food Nutr Res* **57**.

Friedlander AL, Jacobs KA, Fattor JA, Horning MA, Hagobian TA, Bauer TA, Wolfel EE & Brooks GA. (2007). Contributions of working muscle to whole body lipid metabolism are altered by exercise intensity and training. *Am J Physiol Endocrinol Metab* **292,** E107-116.

Gram M, Vigelso A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M & Dela F. (2014). Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. *Experimental gerontology* **58,** 269-278.

Gram M, Vigelso A, Yokota T, Helge JW, Dela F & Hey-Mogensen M. (2015). Skeletal muscle mitochondrial H2 O2 emission increases with immobilization and decreases after aerobic training in young and older men. *J Physiol* **593,** 4011-4027.

Gurd BJ, Peters SJ, Heigenhauser GJ, LeBlanc PJ, Doherty TJ, Paterson DH & Kowalchuk JM. (2008). O2 uptake kinetics, pyruvate dehydrogenase activity, and muscle deoxygenation in young and older adults during the transition to moderate-intensity exercise. *Am J Physiol Regul Integr Comp Physiol* **294,** R577-584.

Hardie DG. (2011). Energy sensing by the AMP-activated protein kinase and its effects on muscle metabolism. *The Proceedings of the Nutrition Society* **70,** 92-99.

Helge JW, Stallknecht B, Richter EA, Galbo H & Kiens B. (2007). Muscle metabolism during graded quadriceps exercise in man. *JPhysiol* **581,** 1247-1258.

Henriksson J. (1977). Training induced adaptation of skeletal muscle and metabolism during submaximal exercise. *J Physiol* **270,** 661-675.

Howley ET, Bassett DR, Jr. & Welch HG. (1995). Criteria for maximal oxygen uptake: review and commentary. *Med Sci Sports Exerc* **27,** 1292-1301.

Hurley BF, Nemeth PM, Martin WH, III, Hagberg JM, Dalsky GP & Holloszy JO. (1986). Muscle triglyceride utilization during exercise: effect of training. *JApplPhysiol (1985)* **60,** 562-567.

Jensen TE, Wojtaszewski JF & Richter EA. (2009). AMP-activated protein kinase in contraction regulation of skeletal muscle metabolism: necessary and/or sufficient? *Acta physiologica (Oxford, England)* **196,** 155-174.

Jeukendrup AE & Wallis GA. (2005). Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* **26 Suppl 1,** S28-37.

Jorgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, Richter EA & Wojtaszewski JF. (2004). The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53,** 3074-3081.

Kelley DE, He J, Menshikova EV & Ritov VB. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* **51,** 2944-2950.

Kelley DE & Mandarino LJ. (2000). Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* **49,** 677-683.

Kiens B, Kristiansen S, Jensen P, Richter EA & Turcotte LP. (1997). Membrane associated fatty acid binding protein (FABPpm) in human skeletal muscle is increased by endurance training. *BiochemBiophysResCommun* **231,** 463-465.

Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J & Nielsen JN. (2004). Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *JApplPhysiol* **97,** 1209-1218.

Klein DK, Pilegaard H, Treebak JT, Jensen TE, Viollet B, Schjerling P & Wojtaszewski JF. (2007). Lack of AMPKalpha2 enhances pyruvate dehydrogenase activity during exercise. *Am J Physiol Endocrinol Metab* **293,** E1242-1249.

Kostovski E, Boon H, Hjeltnes N, Lundell LS, Ahlsen M, Chibalin AV, Krook A, Iversen PO & Widegren U. (2013). Altered content of AMP-activated protein kinase isoforms in skeletal muscle from spinal cord injured subjects. *Am J Physiol Endocrinol Metab* **305,** E1071-1080.

Larsen S, Danielsen JH, Sondergard SD, Sogaard D, Vigelsoe A, Dybboe R, Skaaby S, Dela F & Helge JW. (2014). The effect of high-intensity training on mitochondrial fat oxidation in skeletal muscle and subcutaneous adipose tissue. *Scandinavian journal of medicine & science in sports*.

Levadoux E, Morio B, Montaurier C, Puissant V, Boirie Y, Fellmann N, Picard B, Rousset P, Beaufrere B & Ritz P. (2001). Reduced whole-body fat oxidation in women and in the elderly. *Int J Obes Relat Metab Disord* **25,** 39-44.

Lexell J, Taylor CC & Sjostrom M. (1988). What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men. *J Neurol Sci* **84,** 275-294.

Lonnqvist F, Nyberg B, Wahrenberg H & Arner P. (1990). Catecholamine-induced lipolysis in adipose tissue of the elderly. *J Clin Invest* **85,** 1614-1621.

Louche K, Badin PM, Montastier E, Laurens C, Bourlier V, de Glisezinski I, Thalamas C, Viguerie N, Langin D & Moro C. (2013). Endurance exercise training up-regulates lipolytic proteins and reduces triglyceride content in skeletal muscle of obese subjects. *J Clin Endocrinol Metab* **98,** 4863-4871.

Mahlapuu M, Johansson C, Lindgren K, Hjalm G, Barnes BR, Krook A, Zierath JR, Andersson L & Marklund S. (2004). Expression profiling of the gamma-subunit isoforms of AMP-activated protein kinase suggests a major role for gamma3 in white skeletal muscle. *Am J Physiol Endocrinol Metab* **286,** E194-200.

Martin WH, III, Dalsky GP, Hurley BF, Matthews DE, Bier DM, Hagberg JM, Rogers MA, King DS & Holloszy JO. (1993). Effect of endurance training on plasma free fatty acid turnover and oxidation during exercise. *AmJPhysiol* **265,** E708-E714.

Meredith CN, Frontera WR, Fisher EC, Hughes VA, Herland JC, Edwards J & Evans WJ. (1989). Peripheral effects of endurance training in young and old subjects. *J Appl Physiol (1985)* **66,** 2844-2849.

Moro C, Bajpeyi S & Smith SR. (2008). Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity. *Am J Physiol Endocrinol Metab* **294,** E203-213.

Mortensen B, Friedrichsen M, Andersen NR, Alibegovic AC, Hojbjerre L, Sonne MP, Stallknecht B, Dela F, Wojtaszewski JF & Vaag A. (2014). Physical inactivity affects skeletal muscle insulin signaling in a birth weight-dependent manner. *J Diabetes Complications* **28,** 71-78.

Mortensen B, Poulsen P, Wegner L, Stender-Petersen KL, Ribel-Madsen R, Friedrichsen M, Birk JB, Vaag A & Wojtaszewski JF. (2009). Genetic and metabolic effects on skeletal muscle AMPK in young and older twins. *American journal of physiology Endocrinology and metabolism* **297,** E956-964.

Mounier R, Lantier L, Leclerc J, Sotiropoulos A, Foretz M & Viollet B. (2011). Antagonistic control of muscle cell size by AMPK and mTORC1. *Cell cycle (Georgetown, Tex)* **10,** 2640-2646.

Mounier R, Lantier L, Leclerc J, Sotiropoulos A, Pende M, Daegelen D, Sakamoto K, Foretz M & Viollet B. (2009). Important role for AMPKalpha1 in limiting skeletal muscle cell hypertrophy. *FASEB J* **23,** 2264-2273.

Nørregaard J, Gram M, Vigelsø A, Wiuff C, Kuhlman AB, Helge JW & Dela F. (2014). The Effect of Reduced Physical Activity and Retraining on Blood Lipids and Body Composition in Young and Older Adult Men. *J Aging Phys Act* **epub ahead of print**.

O'Neill M, Watt MJ, Heigenhauser GJ & Spriet LL. (2004). Effects of reduced free fatty acid availability on hormone-sensitive lipase activity in human skeletal muscle during aerobic exercise. *J Appl Physiol (1985)* **97,** 1938-1945.

Odland LM, Heigenhauser GJ, Lopaschuk GD & Spriet LL. (1996). Human skeletal muscle malonyl-CoA at rest and during prolonged submaximal exercise. *The American journal of physiology* **270,** E541-544.

Odland LM, Howlett RA, Heigenhauser GJ, Hultman E & Spriet LL. (1998). Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. *The American journal of physiology* **274,** E1080-1085.

Parkington JD, LeBrasseur NK, Siebert AP & Fielding RA. (2004). Contraction-mediated mTOR, p70S6k, and ERK1/2 phosphorylation in aged skeletal muscle. *J Appl Physiol (1985)* **97,** 243-248.

Peronnet F & Massicotte D. (1991). Table of nonprotein respiratory quotient: an update. *Can J Sport Sci* **16,** 23-29.

Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW & Shulman GI. (2003). Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* **300,** 1140-1142.

Prats C, Gomez-Cabello A & Hansen AV. (2011). Intracellular compartmentalization of skeletal muscle glycogen metabolism and insulin signalling. *Exp Physiol* **96,** 385-390.

Reihmane D, Hansen AV, Gram M, Kuhlman AB, Norregaard J, Pedersen HP, Lund MT, Helge JW & Dela F. (2013). Immobilization increases interleukin-6, but not tumour necrosis factor-alpha, release from the leg during exercise in humans. *ExpPhysiol* **98,** 778-783.

Richter EA & Ruderman NB. (2009). AMPK and the biochemistry of exercise: implications for human health and disease. *The Biochemical journal* **418,** 261-275.

Ringholm S, Bienso RS, Kiilerich K, Guadalupe-Grau A, Aachmann-Andersen NJ, Saltin B, Plomgaard P, Lundby C, Wojtaszewski JF, Calbet JA & Pilegaard H. (2011). Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle. *Am J Physiol Endocrinol Metab* **301,** E649-658.

Ritz P, Acheson KJ, Gachon P, Vico L, Bernard JJ, Alexandre C & Beaufrere B. (1998). Energy and substrate metabolism during a 42-day bed-rest in a head-down tilt position in humans. *Eur J Appl Physiol Occup Physiol* **78,** 308-314.

Sacchetti M, Saltin B, Olsen DB & van Hall G. (2004). High triacylglycerol turnover rate in human skeletal muscle. *J Physiol* **561,** 883-891.

Schenk S, Cook JN, Kaufman AE & Horowitz JF. (2005). Postexercise insulin sensitivity is not impaired after an overnight lipid infusion. *Am J Physiol Endocrinol Metab* **288,** E519-525.

Sial S, Coggan AR, Carroll R, Goodwin J & Klein S. (1996). Fat and carbohydrate metabolism during exercise in elderly and young subjects. *Am J Physiol* **271,** E983-989.

Siggaard-Andersen O, Wimberley PD, Fogh-Andersen N & Gothgen IH. (1988). [Blood oxygen parameters: uncompensated venous oxygen tension and cardiac compensation factors]. *Ugeskr Laeger* **150,** 2899-2901.

Solomon TP, Marchetti CM, Krishnan RK, Gonzalez F & Kirwan JP. (2008). Effects of aging on basal fat oxidation in obese humans. *Metabolism* **57,** 1141-1147.

Stallknecht B, Kiens B, Helge JW, Richter EA & Galbo H. (2004). Interstitial glycerol concentrations in human skeletal muscle and adipose tissue during graded exercise. *Acta Physiol Scand* **180,** 367-377.

Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE & McConell GK. (2002). Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. *Am J Physiol Endocrinol Metab* **282,** E688-694.

Storlien L, Oakes ND & Kelley DE. (2004). Metabolic flexibility. *Proc Nutr Soc* **63,** 363-368.

Thomson DM & Gordon SE. (2006). Impaired overload-induced muscle growth is associated with diminished translational signalling in aged rat fast-twitch skeletal muscle. *J Physiol* **574,** 291-305.

Unger RH. (2002). Lipotoxic diseases. *Annu Rev Med* **53,** 319-336.

van Hall G, Gonzalez-Alonso J, Sacchetti M & Saltin B. (1999). Skeletal muscle substrate metabolism during exercise: methodological considerations. *Proc Nutr Soc* **58,** 899-912.

van Hall G, Sacchetti M, Radegran G & Saltin B. (2002). Human skeletal muscle fatty acid and glycerol metabolism during rest, exercise and recovery. *J Physiol* **543,** 1047-1058.

van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH & Wagenmakers AJ. (2001). The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* **536,** 295-304.

Vigelso A, Gram M, Wiuff C, Andersen JL, Helge JW & Dela F. (2015). Six weeks' aerobic retraining after two weeks' immobilization restores leg lean mass and aerobic capacity but does not fully rehabilitate leg strenght in young and older men. *J Rehabil Med*.

Vigelsø A, Dybboe R, Hansen CN, Dela F, Helge JW & Guadalupe Grau A. (2015a). GAPDH and beta-actin protein decreases with aging, making Stain-Free technology a superior loading control in Western blotting of human skeletal muscle. *J Appl Physiol (1985)* **118,** 386-394.

Vigelsø A, Gram M, Wiuff C, Andersen JL, Helge JW & Dela F. (2015b). 6 weeks' aerobic retraining after 2 weeks' immobilization restores leg lean mass, and aerobic capacity but does not fully rehabilitate leg strength in young and older men. *J Rehabil Med* **[EPUB AHEAD OF PRINT]**.

Wall BT, Dirks ML, Snijders T, Stephens FB, Senden JM, Verscheijden ML & van Loon LJ. (2015). Short-term muscle disuse atrophy is not associated with increased intramuscular lipid deposition or a decline in

the maximal activity of key mitochondrial enzymes in young and older males. *Exp Gerontol* **61,** 76-83.

Watt MJ, Holmes AG, Steinberg GR, Mesa JL, Kemp BE & Febbraio MA. (2004). Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle. *Am J Physiol Endocrinol Metab* **287,** E120-127.

Additional information

Competing interests

The authors have no conflict of interest to declare

Funding

This work was supported by the UNIK research program: Food, Fitness & Pharma for Health and Disease (Danish Ministry of Science, Technology and Innovation), The Nordea Foundation, A.P. Møller and Hustru Chastine Mc-Kinney Møllers Foundation.

Acknowledgements

The technical assistance of Michael Taulo Lund, MD; Merethe Hansen, MD; Jesper Nørregaard, MD; Christina Neigaard Hansen, PhD; Regitze Kraunsøe; Katrine Qvist; and Jeppe Bach is gratefully acknowledged.

Tables

Table 1. Characteristics of the young and older men at inclusion and after immobilization for 2 weeks.

	Inclusion			After 2 weeks' immobilization		
	Young (n= 17)		Older men (n=15)	Young		Older men
Age (years)	23 ± 1	*	68 ± 1			
Weight (kg)	80 ± 2		83 ± 2	80 ± 2		83 ± 2
BMI (kg/m^2)	24 ± 1	*	27 ± 1	24 ± 1	*	27 ± 1
Fat (%)	21 ± 1	*	28 ± 1	21 ± 1	*	29 ± 1
Lean body mass (kg)	60 ± 1	(*)	57 ± 2	60 ± 1	(*)	56 ± 2
Hba1c (mmol/mol)	5.2 ± 0.1	*	5.6 ± 0.1	5.2 ± 0.1	*	5.5 ± 0.1
VO_2 (ml O_2/min/kg)	48 ± 1	*	33 ± 2	44 ± 1#	*	32 ± 2

Data are means ± SEM. * $p < 0.05$ and (*) $0.05<P>0.1$ young vs. older men same time point; # $p < 0.05$ vs. inclusion in the young men.

Table 2. Whole-body and single-leg data after immobilization for 2 weeks and during 45 min acute isolated kicking exercise at moderate intensity in young and older men.

	Young men			Older men	
Whole body					
Work load (watt)	20 ± 1			20 ± 1	
Heart rate (beats/min)	113 ± 4		*	95 ± 5	
$\%Hr_{max}$ (%)	59 ± 2			61 ± 4	
$ssVO_2$ (ml/min) (20-30min)	1066 ± 38		(*)	874 ± 35	
$ssVCO_2$ (ml/min) (20-30 min)	931 ± 35		(*)	738 ± 35	
Respiratory exchange rate (RER)	0.87 ± 0.01			0.86 ± 0.01	
	Control leg	Immobilized leg		Control leg	Immobilized leg
Isolated leg					
§Leg lean mass (kg)	10.7 ± 0.3&	10.1 ± 0.3	*	9.3 ± 0.3	9.2 ± 0.3
§$Watt_{max}$ (W)	42 ± 3&	37 ± 3		45 ± 3&	40 ± 3
Relative work load (% of $Watt_{max}$)	50 ± 2&	57 ± 3		47 ± 4&	52 ± 3
$ssVO_2$ (ml/min) (30-45 min)	212 ± 9	220 ± 12	*	148 ± 10	154 ± 14
§IMTG at rest (µmol/g d.w.)	68 ± 9&	92 ± 11	*	114 ± 13&	174 ± 39
IMTG utilization (µmol/g d.w.)	20 ± 17	20 ± 13		18 ± 18	48 ± 21
§Glycogen at rest (µmol/g d.w.)	221 ± 24	214 ± 23	*	257 ± 27	265 ± 37
Glycogen utilization (µmol/g d.w.)	-36 ± 11	-12 ± 23	*	-41 ± 13	0 ± 16
§HAD activity (µmol/min/g d.w.)	102 ± 4&	87 ± 5		101 ± 7&	88 ± 5
Reported perceived exertion (1-10)	4.3 ± 0.4&	4.9 ± 0.4		4.2 ± 0.4&	4.3 ± 0.4

Data are means ± SEM. * $p < 0.05$ and (*) $0.1 > P < 0.05$ young vs. older men (main effect); & $p < 0.05$ control leg vs. immobilized leg same group (main effect). § previously published data (Reihmane *et al.*, 2013; Gram *et al.*, 2014; Nørregaard *et al.*, 2014). $SsVO_2$: steady state oxygen uptake during exercise i.e. the average of 30-45 min.

Table 3. Protein content measured by Western blotting.

	Young men		Older men		
	Control leg	Immobilized leg	Control leg	Immobilized leg	Main effect
Lipoprotein lipase	0.95 ± 0.18	1.07 ± 0.21	1.30 ± 0.19	1.53 ± 0.22	(*)
Hormone-sensitive lipase	0.60 ± 0.10	0.48 ± 0.07	0.53 ± 0.06	0.64 ± 0.14	
pAMPKthr172	1.08 ± 0.09	1.15 ± 0.09	1.00 ± 0.12	1.12 ± 0.13	
AMPKα1	0.95 ± 0.09	1.03 ± 0.06	0.91 ± 0.11	1.02 ± 0.10	&
AMPKα2	0.97 ± 0.03	0.95 ± 0.02	0.93 ± 0.03	0.96 ± 0.04	
AMPKβ1	0.90 ± 0.09	1.14 ± 0.09	0.97 ± 0.12	1.24 ± 0.11	&
AMPKβ2	1.24 ± 0.08 &*	1.11 ± 0.05	0.98 ± 0.06	0.99 ± 0.05	
AMPKγ1	1.35 ± 0.07	1.44 ± 0.09	1.21 ± 0.11	1.34 ± 0.13	
AMPKγ3	1.90 ± 0.17	1.64 ± 0.16	1.37 ± 0.13	1.21 ± 0.10	&*

Data are means ± SEM. * $p < 0.05$ and (*) $0.1 > P < 0.05$ young vs. older men; & $p < 0.05$ immobilized leg vs. control. It is a main effect of either group or leg if marked in "main effect" or an interaction between group and leg if marked in the table (AMPKβ2). See representative Western blots in Figure 7.

Figures and legends

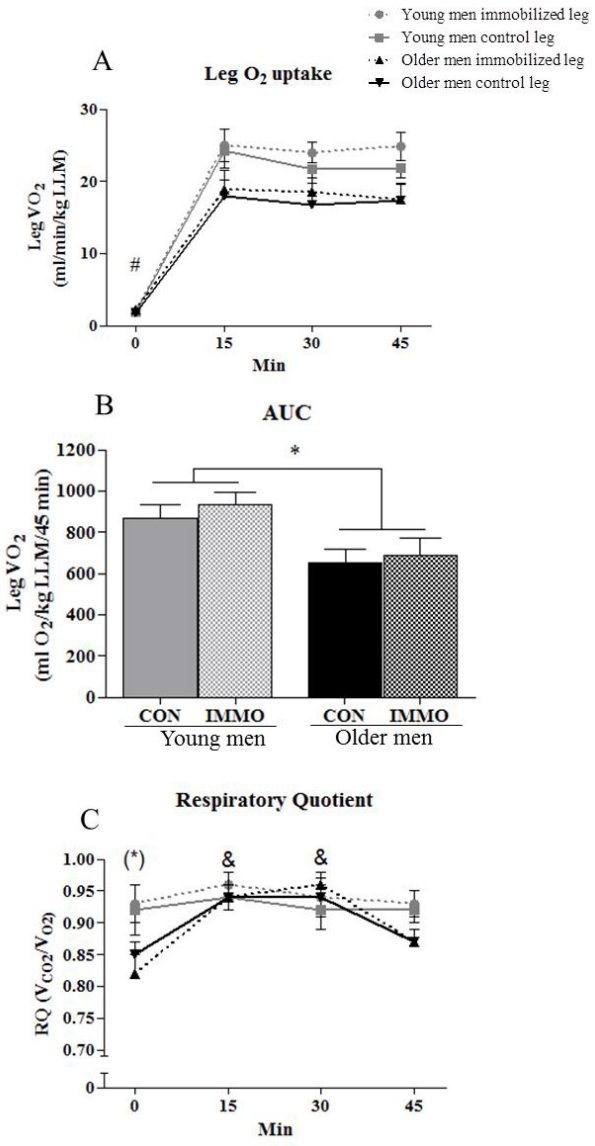

Figure 1. Indirect calorimetry. A) Leg O_2-uptake, B) area under curve (AUC) for leg O_2-uptake and C) RQ in young and older men after 2 weeks' unilateral immobilization (IMMO) with the other leg serving as control (CON) during 45 min moderate intensity exercise. * $P<0.05$ and (*) $0.05>P<0.1$ young vs. older men; $^{\#}$ $P<0.05$ rest (0 min) vs. exercise (15, 30 and 45 min); $^{\&}$ $P<0.05$ 15 and 30 min vs. 0 and 45 min in older men. Data are means ± SEM.

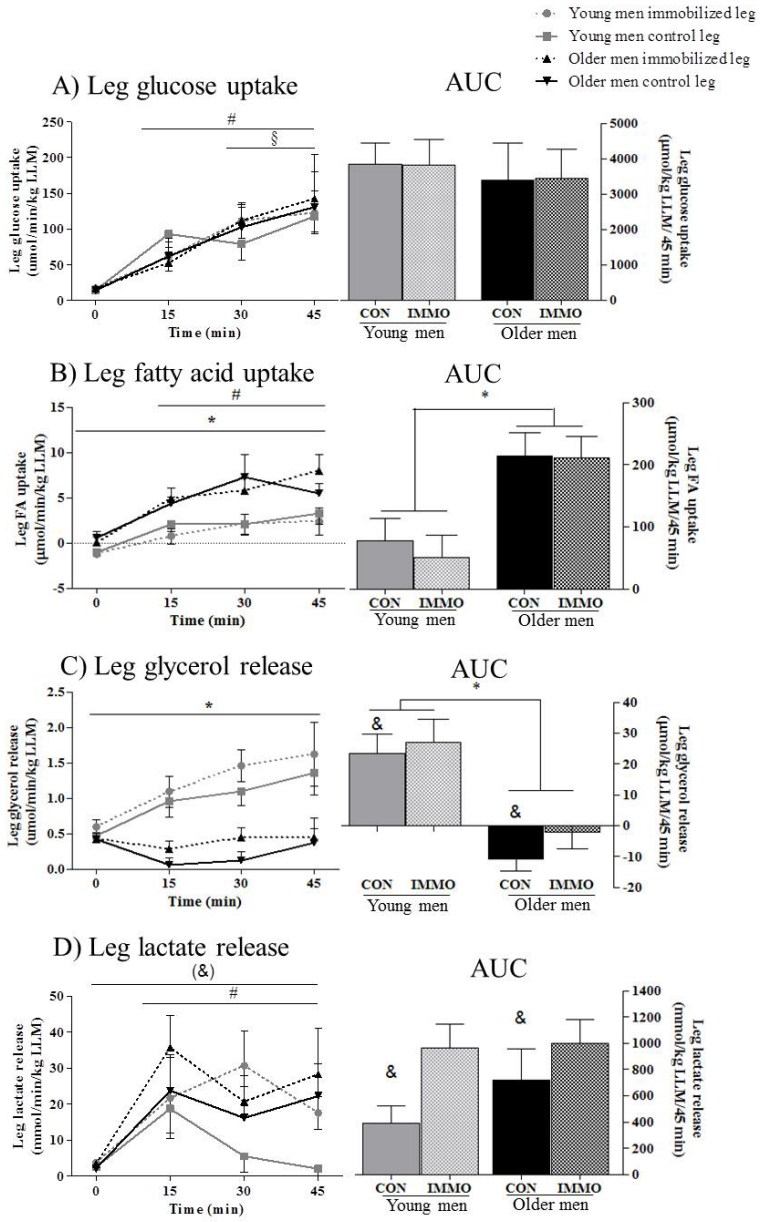

Figure 2. Utilization of exogenous substrates. Leg A) glucose uptake, B) fatty acid uptake and C) glycerol release in young and older men after 2 weeks' unilateral immobilization (IMMO) with the other leg serving as control (CON) during 45 min moderate intensity exercise with both legs, with the corresponding area under curve (AUC). * P<0.05 young vs. older men (main effect); $^{\&}$ P < 0.05 and $^{(\&)}$ 0.05 <P>0.1 immobilized leg vs. control leg (main effect); $^{\#}$ P<0.05 0 min vs. 15, 30 and 45 min; § P<0.05 15 min vs. 30 and 45 min. Data are mean ± SEM.

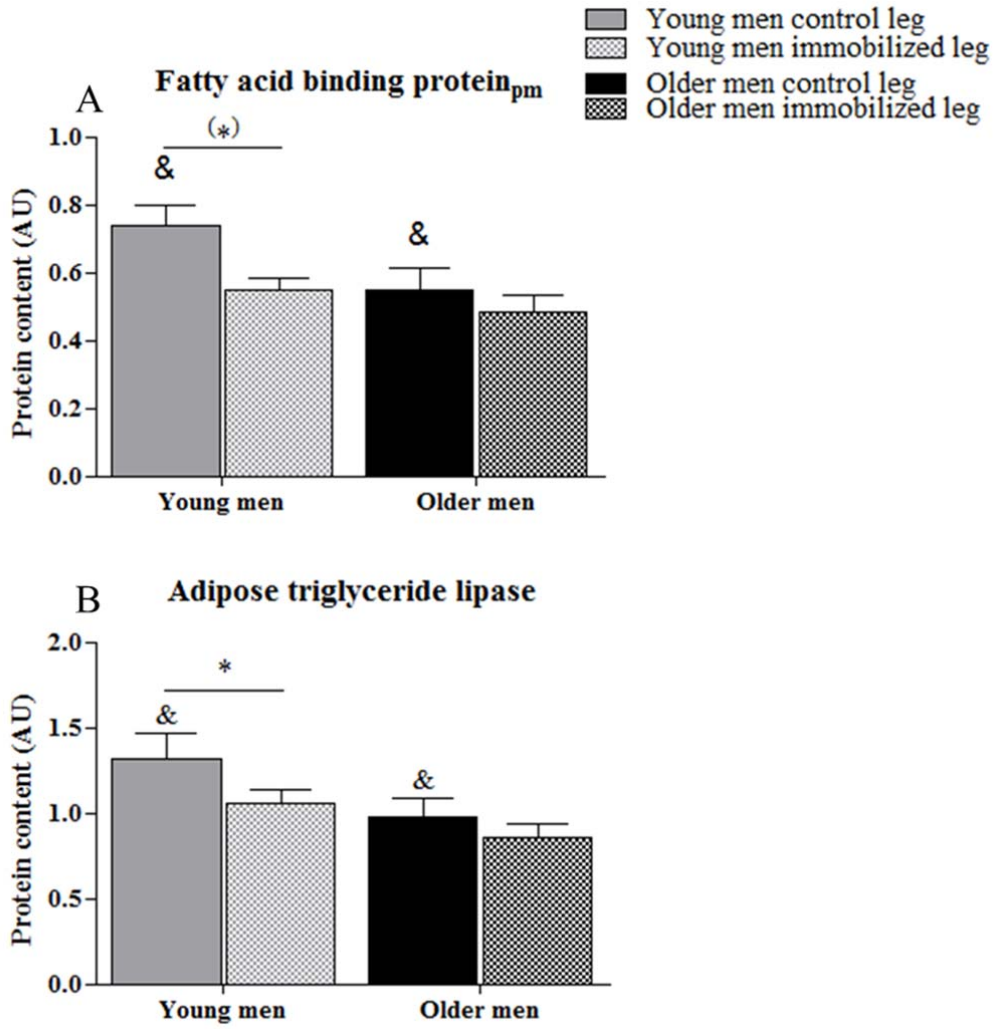

Figure 3. Protein content of A) plasma membrane (pm) bound fatty acid binding protein and B) adipose triglyceride lipase (ATGL) in young and older men after 2 weeks' unilateral immobilization with the other leg serving as a control. $^{\&}P < 0.05$ immobilized leg vs. control leg same group. *P<0.05 and (*) 0.05>P<0.1 young vs. older men (main effect). Data are means ± SEM. IM: immobilized and CON control leg. See representative Western blots in Figure 7.

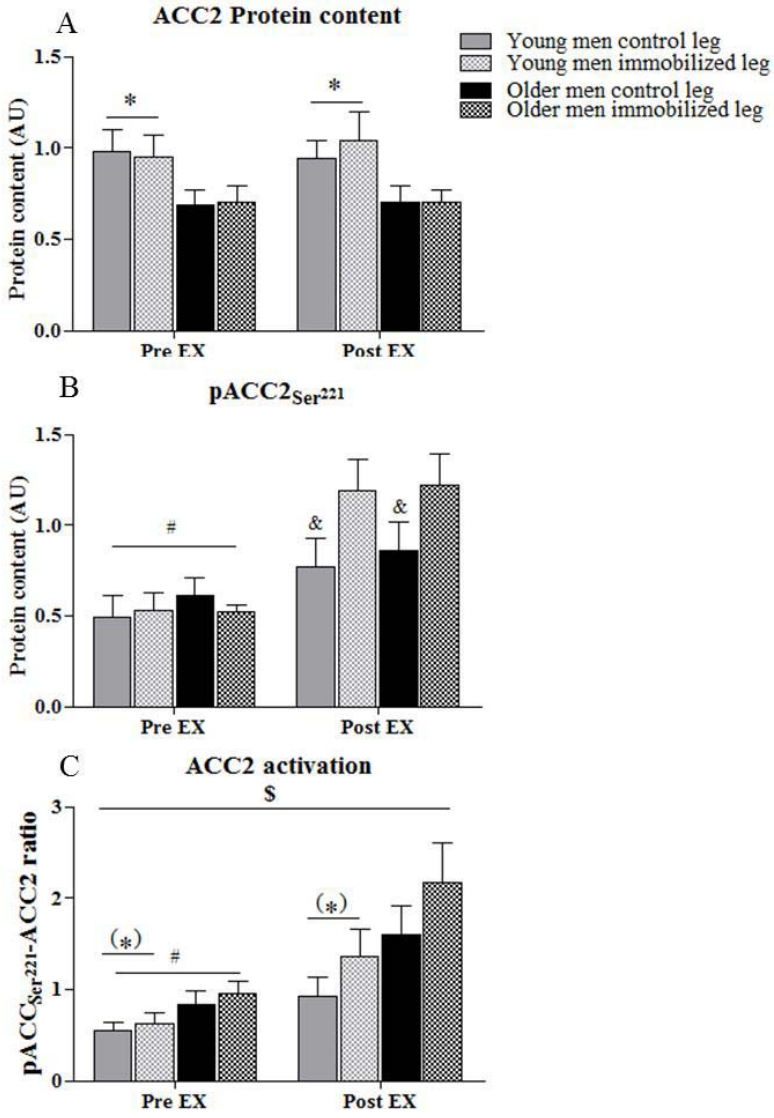

Figure 4. ACC2 activation. Protein content of A) ACC2 B) pACC2Ser^{221} and C) relative ACC2 activation (pACC2Ser^{221}/ACC2 ratio) in young and older men after 2 weeks' unilateral immobilization with the other leg serving as a control. Pre and post 45 min isolated kicking exercise (EX) at moderate intensity with both legs.*P<0.05 and (*) 0.05>P<0.1 young vs. older men (main effect), $^{\&}$P < 0.05 immobilized leg vs. control leg same group, $^{\#}$P<0.05 Pre vs. Post EX (main effect). Data are means ± SEM. See representative Western blots in Figure 7.

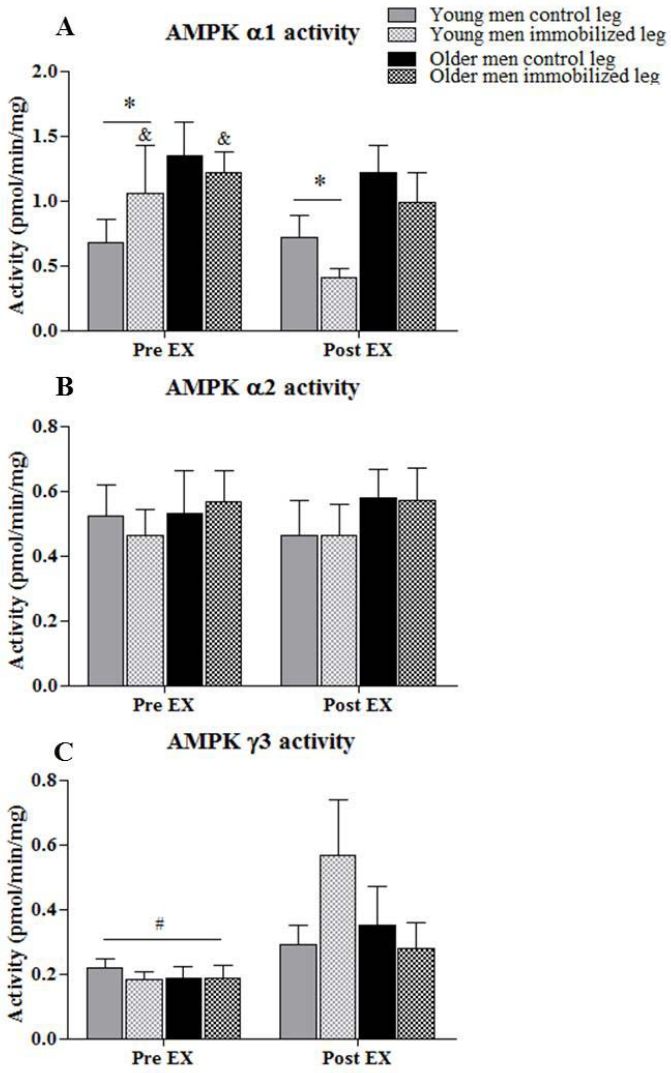

Figure 5. AMPK complex specific activity of A) AMPK$\alpha_2\beta_2\gamma_3$ B) AMPK$\alpha_2\beta_2\gamma_1$ C) AMPK$\alpha_1\beta_2\gamma_1$ in young and older men after 2 weeks' unilateral immobilization with the other leg serving as a control. Pre and post 45 min isolated kicking exercise (EX) at moderate intensity with both legs. * $P < 0.05$ young vs. older men. $^{\#}P < 0.05$ Pre vs. post EX. $^{\&}P < 0.05$ pre vs. post EX same leg. Data are means ± SEM.

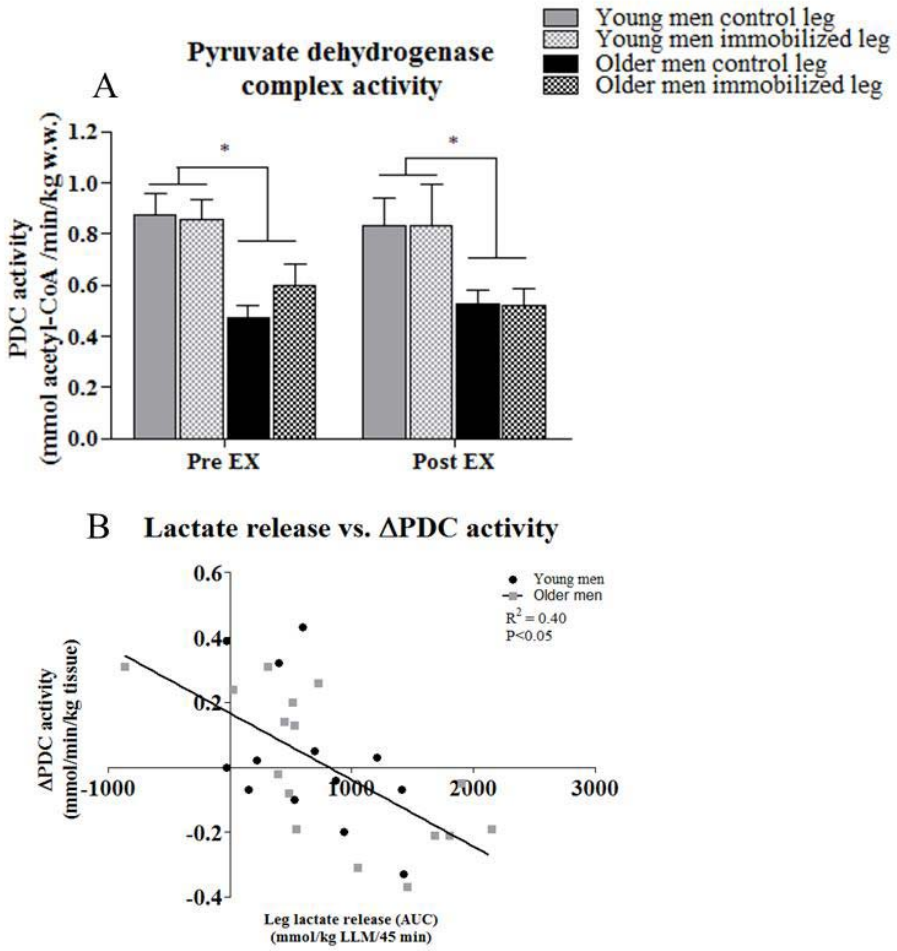

Figure 6. A) Pyruvate dehydrogenase complex (PDC) specific activity and B) correlation between ΔPDC activity and area under the curve for leg lactate release (Figure 2D) in young and older men after 2 weeks' unilateral immobilization with the other leg serving as a control. Pre and post 45 min isolated kicking exercise (EX) at moderate intensity with both legs. * $P < 0.05$ young vs. older men. Data are means ± SEM.

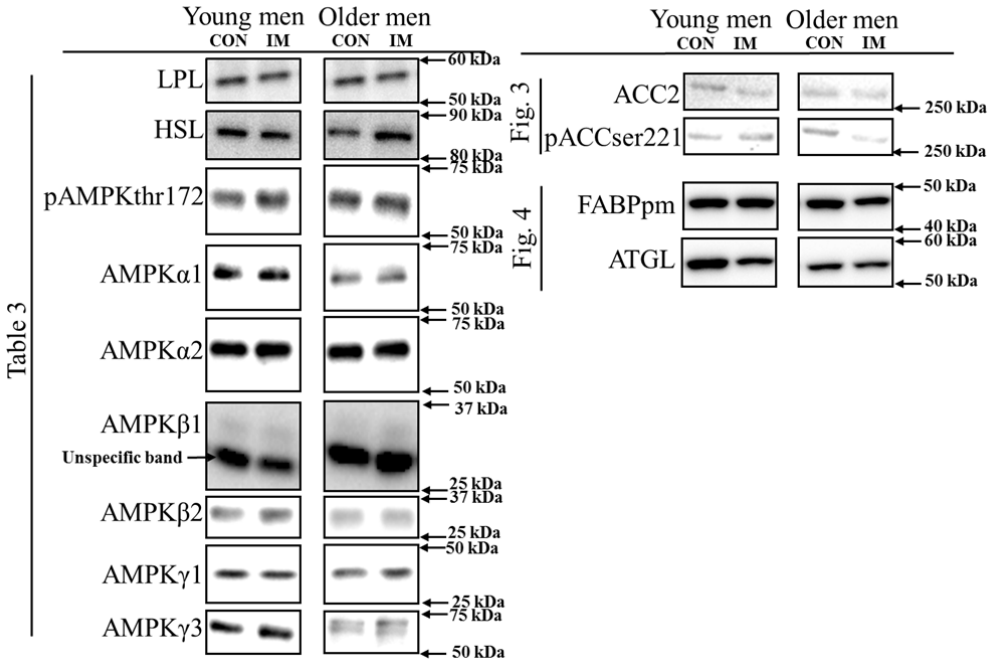

Figure 7. Representative Western blots for Table 3, Figure 2 and 3. ACC2, Acetyl-CoA carboxylase 2 (and phosphorylation (p) at serine 221 (ser221); AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; CON, control leg; FABPpm, plasma membrane-associated fatty acid binding protein; HSL, hormone-sensitive lipase; IM, immobilized leg; LPL, lipoprotein lipase;