

# Life-long endurance running is associated with reduced glycation and mechanical stress in connective tissue

Christian Couppé · René B. Svensson · Jean-Francois Grosset ·  
Vuokko Kovanen · Rie H. Nielsen · Morten R. Olsen · Jytte O. Larsen ·  
Stephan F. E. Praet · Dorthe Skovgaard · Mette Hansen · Per Aagaard ·  
Michael Kjaer · S. Peter Magnusson

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**Abstract** Life-long regular endurance exercise is known to counteract the deterioration of cardiovascular and metabolic function and overall mortality. Yet it remains unknown if life-long regular endurance exercise can influence the connective tissue accumulation of advanced glycation endproducts (AGEs) that is associated with aging and lifestyle-related diseases. We

therefore examined two groups of healthy elderly men: 15 master athletes ( $64 \pm 4$  years) who had been engaged in life-long endurance running and 12 old untrained ( $66 \pm 4$  years) together with two groups of healthy young men; ten young athletes matched for running distance ( $26 \pm 4$  years), and 12 young untrained ( $24 \pm 3$  years). AGE cross-links (pentosidine) of the patellar tendon

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C. Couppé · R. B. Svensson · R. H. Nielsen · M. R. Olsen ·  
D. Skovgaard · M. Hansen · M. Kjaer · S. P. Magnusson  
Institute of Sports Medicine, Department of Orthopaedic  
Surgery M, Bispebjerg Hospital and Center for Healthy  
Aging, Faculty of Health Sciences, University of  
Copenhagen,  
Bldg. 8, Bispebjerg Bakke 23, DK-2400 Copenhagen, NV,  
Denmark  
e-mail: ccouppe@gmail.com

C. Couppé (✉) · S. P. Magnusson  
Department of Physical Therapy, Musculoskeletal  
Rehabilitation Research Unit, Bispebjerg Hospital,  
Copenhagen, Denmark  
e-mail: ccouppe@gmail.com

J.-F. Grosset  
CNRS UMR 7338, Biomécanique et Bioingénierie,  
Université de Technologie de Compiègne,  
60205 Compiègne cedex, France

J.-F. Grosset  
UFR de Santé, Médecine et Biologie Humaine, Université  
Paris 13, Sorbonne Paris Cité,  
93017 Bobigny, France

V. Kovanen  
Department of Health Sciences, University of Jyväskylä,  
Jyväskylä, Finland

J. O. Larsen  
Department of Neuroscience and Pharmacology, Faculty of  
Health Sciences, University of Copenhagen,  
Copenhagen, Denmark

S. F. E. Praet  
Department of Rehabilitation Medicine, MOVEFIT- Sports  
Medicine, Erasmus University Medical Center,  
Rotterdam, The Netherlands

D. Skovgaard  
Cluster for Molecular Imaging, Faculty of Health Sciences,  
University of Copenhagen,  
Copenhagen, Denmark

M. Hansen  
Section for Sports Science, Department of Public Health,  
Aarhus University,  
Aarhus, Denmark

P. Aagaard  
Institute of Sports Science and Clinical Biomechanics, SDU  
Muscle Research Cluster, University of Southern Denmark,  
Odense, Denmark

were measured biochemically, and in the skin, it was assessed by a fluorometric method. In addition, we determined mechanical properties and microstructure of the patellar tendon. Life-long regular endurance runners (master athletes) had a 21 % lower AGE cross-link density compared to old untrained. Furthermore, both master athletes and young athletes displayed a thicker patellar tendon. These cross-sectional data suggest that life-long regular endurance running can partly counteract the aging process in connective tissue by reducing age-related accumulation of AGEs. This may not only benefit skin and tendon but also other long-lived protein tissues in the body. Furthermore, it appears that endurance running yields tendon tissue hypertrophy that may serve to lower the stress on the tendon and thereby reduce the risk of injury.

**Keywords** Master athletes · Life-long · Endurance training · AGE · Aging

## Introduction

The extracellular matrix in collagen-rich connective tissues such as skin, cornea, cartilage, lung, arteries and tendon undergoes progressive, irreversible, and detrimental changes as part of the normal aging process (Monnier et al. 2005). The connective tissue of tendon has traditionally been considered relatively inert, but several reports demonstrate that young human tendons respond to physical activity by increased metabolic activity (Bojsen-Moller et al. 2006; Kalliokoski et al. 2005), and collagen synthesis (Langberg et al. 1999, 2001), increased size and altered mechanical properties (Kongsgaard et al. 2007; Seynnes et al. 2009; Arampatzis et al. 2007). In fact, human studies have shown that endurance runners have larger patellar tendon and Achilles tendon cross-sectional area (CSA; hypertrophy), which lowers the tendon stress (Westh et al. 2007; Magnusson et al. 2007; Rosager et al. 2002). Collectively, these data demonstrate that tendons readily respond and adapt to loading. However, it has been shown that fibroblast cell number decreases with age and it is therefore uncertain if tendons maintain this adaptive capacity throughout life (Nagy et al. 1969).

The collagen fibril is the fundamental tensile bearing structure of tendon connective tissue and it is made up of collagen molecules linked together by cross-links. These bonds provide structural integrity to the fibrils

and ensure appropriate force transmission (Avery and Bailey 2005; Eyre et al. 1984). There are basically two different types of cross-links: one enzymatically controlled by lysyl-oxidase during maturation (Eyre et al. 1984; Fujimoto et al. 1978), and one formed via nonenzymatic glycation (Monnier et al. 2005). The latter results in the formation of irreversible covalent advanced glycation endproducts (AGE), i.e., Maillard reaction, which accompanies the aging process (Monnier and Cerami 1981; Maillard 1912).

AGE cross-links accumulate with age in long-lived proteins, such as collagen, resulting in markedly higher concentrations in older than in younger individuals (Chen et al. 2000; DeGroot et al. 2001; Haus et al. 2007; Bank et al. 1999; Suzuki et al. 2008). The accumulation appears to be accelerated with age-related chronic diseases such as diabetes (Dyer et al. 1993; Vogt et al. 1982) and is believed to influence the function of several tissues. This aging process may be the reason for the higher incidence of connective tissue injuries by age (Murrell and Walton 2001; Englund et al. 2012). In line with this, we recently demonstrated that both enzymatic hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) cross-links and pentosidine, which is a marker of AGE, were more abundant in the patellar tendon of old men compared with that of young men (Couppe et al. 2009). It is believed that greater AGE cross-link density increases tendon stiffness (Reddy et al. 2002; Galeski et al. 1977; Andreassen et al. 1988; Bai et al. 1992), however, mechanical properties of both animal and human tendon has been reported both to increase (Nielsen et al. 1998; Shadwick 1990; Kubo et al. 2007a; Wood et al. 2011), decrease (Stenroth et al. 2012; Karamanidis and Arampatzis 2006; Dressler et al. 2002; Onambele et al. 2006; Mian et al. 2007) or remain unaltered with aging (Couppe et al. 2009; Haut et al. 1992; Hubbard and Soutas-Little 1984; Johnson et al. 1994; Carroll et al. 2008). Thus, to what extent aging influence mechanical properties of tendon tissue remains largely unknown.

A modest reduction in AGE cross-link density in tendon has been shown after 12 weeks of physical resistance training (Kongsgaard et al. 2009), and 12 weeks of moderate walking exercise appears to lower serum levels of AGE (Yoshikawa et al. 2009). However, if long-term physical activity can modify the accumulation of enzymatic and AGE cross-links in human tendon with aging has never been investigated. It appears that endurance training in aging animals yield a stronger and

more compliant tendon (Gosselin et al. 1998; Viidik et al. 1996; Simonsen et al. 1995; LaCroix et al. 2013; Nielsen et al. 1998), but this remains to be confirmed in a human model. Furthermore, at the microstructural level, the size and/or density of tendon collagen fibrils seems to be reduced with aging (Curwin et al. 1994; Nakagawa et al. 1994; Partington and Wood 1963; Patterson-Kane et al. 1997), but if these age-related adaptations are influenced by physical activity remains unknown (Edwards et al. 2005).

Collectively, it remains unknown if life-long regular endurance running in humans influence 1) accumulation of AGE cross-links in tendon connective tissue 2) tendon mechanical properties, and 3) tendon fibril morphology. We therefore sought to investigate the effect of aging and physical activity on collagen cross-linking and mechanical properties of the human patellar tendon, in vivo. We hypothesized that life-long regular endurance runners display lower AGE cross-link density and mechanical properties/fibril structure of their patellar tendon that are more similar to that observed in young

individuals compared to age-matched sedentary controls.

## Methods

### Subjects

A total of 49 men were recruited for this study, 15 endurance runners [master athletes; running distance of  $49 \pm 3$  km week<sup>-1</sup> (4–6 running days week<sup>-1</sup>) over the last  $28 \pm 2$  years (mean  $\pm$  SE)], 12 old untrained weight-matched healthy controls (old untrained), and ten young men matched for current running distance to the old trained (young athletes;  $43 \pm 5$  km week<sup>-1</sup> (4–6 running days week<sup>-1</sup>), endurance runners during the last  $6 \pm 1$  years), and 12 young untrained weight-matched controls (young untrained; Table 1). The trained subjects were moderately to well-trained runners (Saltin and Grimby 1968). The untrained subjects had been sedentary for at least 5 years and, currently, did not perform

**Table 1** Subject characteristics and physical activity

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
<i>Subject characteristics</i>						
Number of subjects	10	12	15	12		
Age (years)	26 $\pm$ 4*	24 $\pm$ 3 <sup>§</sup>	64 $\pm$ 4	66 $\pm$ 4		<i>p</i> <0.001, K–W
Height (cm)	179 $\pm$ 4	178 $\pm$ 6	176 $\pm$ 5	175 $\pm$ 4		
Weight (kg)	73 $\pm$ 6	70 $\pm$ 8	71 $\pm$ 6	75 $\pm$ 4		
BMI (kg m <sup>-2</sup> )	23 $\pm$ 2	22 $\pm$ 2 <sup>§</sup>	23 $\pm$ 2	25 $\pm$ 2		<i>p</i> <0.05
Waist circumference (cm)	79 $\pm$ 5*	80 $\pm$ 5 <sup>§</sup>	83 $\pm$ 5	90 $\pm$ 4*		<i>p</i> <0.001
<i>Physical activity</i>						
Running distance per week (km)	43 $\pm$ 5		49 $\pm$ 3			
Running distance last week (km)	48 $\pm$ 7		43 $\pm$ 5			
Running years	6 $\pm$ 1		28 $\pm$ 2			
IPAQ (MET min week <sup>-1</sup> )	3,724 $\pm$ 579	919 $\pm$ 199 <sup>#</sup>	8,881 $\pm$ 1,791	1,277 $\pm$ 575*		<i>p</i> <0.001, K–W
VO <sub>2</sub> peak (ml O <sub>2</sub> min <sup>-1</sup> kg <sup>-1</sup> )	59 $\pm$ 1	45 $\pm$ 1	48 $\pm$ 2	30 $\pm$ 2	-13, 17	<i>A</i> <0.001, <i>T</i> <0.001
Knee extensor moment (Nm)	202 $\pm$ 11, (56, YU),	147 $\pm$ 10 <sup>#</sup>	152 $\pm$ 9 <sup>#</sup>	145 $\pm$ 10		<i>A</i> <0.05, <i>T</i> <0.01, <i>A</i> × <i>T</i> <0.05
	(50, MA)					

Height, weight, BMI and waist circumference are given as mean  $\pm$  SD. Other data are given as mean  $\pm$  SE. Single *p* values are shown for one-way ANOVA, and *p* values for effects are shown for two-way ANOVA (*A*, *T*, *A* × *T*). For further details, see text. Main effect sizes are given as old–young and athlete–untrained. Effect size is shown in parenthesis under the relevant group for significant interactions

K–W Kruskal–Wallis test, *A* age, *T* training, *A* × *T* interaction

\* Different from master athletes (*p*<0.05)

# Different from young athletes (*p*<0.05)

§ Different from old untrained (*p*<0.05)

any type of regular physical activity. The trained individuals were all endurance runners. Moreover, neither the trained nor the untrained had any physically demanding occupational jobs. Thus, their physical activity level was primarily determined by their leisure time activities. All subjects were healthy normotensive (<140/90 mmHg) and nonobese (BMI<28), did not take any prescription medication, had no overt signs or symptoms of diabetes, prediabetes, or atherosclerosis, and had no known joint, muscle, or tendon pathology. The groups had similar diet as assessed by a brief questionnaire. The questionnaire represents diet habits of typical food intake for the last 3 months. The questionnaire has been used in an investigation of the Danish population food habits ( $n=1007$ ) by the Danish Nutrition Council ([http://sundhedsstyrelsen.dk/publ/mer/2002/Befolkningens\\_foedevareforbrug\\_1996\\_2\\_udg\\_2002.pdf](http://sundhedsstyrelsen.dk/publ/mer/2002/Befolkningens_foedevareforbrug_1996_2_udg_2002.pdf)). Before the test days, subjects abstained from caffeine intake and fasted overnight to be studied in the postabsorptive state. Subjects were studied at least 24 h after their last running session to avoid the immediate acute effects of exercise, but they were still considered in their regular training state. The Ethics Committee of the Capital Region of Denmark approved this study (journal number 25543), and all procedures conformed to the Declaration of Helsinki. Written, informed consent was obtained from all subjects before the study.

### Study design

On the first test day, subjects arrived fasted in the morning and blood samples were obtained. An oral glucose tolerance test (OGTT) was performed, anthropometrics were measured, and questionnaires were filled up. Finally magnetic resonance image (MRI) scanning of the thigh was performed to determine the cross-sectional area (CSA) of the quadriceps muscle and patellar tendon, respectively. On the second test day, mechanical properties of the patellar tendon and muscle strength (knee extensor moment) was measured. An hour after consuming a light sandwich, fruit, and drinks, maximal oxygen consumption was measured. Test days 1 and 2 were performed on consecutive days. The anthropometric measures, blood samples, and physical activity data shown below are already reported elsewhere (Mikkelsen et al. 2013).

### Anthropometric measures

Waist circumference was measured as the smallest circumference between anterior superior iliac spine and the lower ribs to the nearest millimeter. Height was measured to the nearest millimeter and weight to the nearest 100 g, wearing light clothes and without shoes. The latter was used to calculate body mass index, BMI ( $\text{kg m}^{-2}$ ).

### Blood samples

Fasted plasma samples were drawn from an antecubital vein. For analyses of insulin, plasma was drawn into EDTA tubes and cooled on ice for 10 min, serum was allowed to clot at room temperature for 30 min followed by centrifugation of both plasma and serum (10 min at  $3,060 \times g$  at  $4^\circ\text{C}$ ), and the plasma or serum phase was stored at  $-80^\circ\text{C}$  until analysis.

A glucose tolerance test was performed by oral administration of 75 g glucose dissolved in water and glucose and insulin analysis of blood sampled before and after 2 h. In addition, blood lipid profile was determined by measuring triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density-lipoprotein cholesterol (LDL), and leucocytes. Blood samples were analyzed at the Clinical Biochemistry Department, Bispebjerg Hospital, Copenhagen. The blood samples were transported to the laboratory and analyzed no more than 2 h from the time of collection according to the laboratory standard operating procedures (Nordin et al. 2004). Authorized lab technicians handled the analyses. Cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, and blood glucose concentrations were measured by a colorimetric slide test in a Vitros 5.1 FS (Ortho Clinical Diagnostics, Raritan, NJ, USA; Sennels et al. 2011, 2012). Leucocyte concentrations were measured by optical spread light in a Sysmex XE (Sysmex, Kobe, Japan; Sennels et al. 2011). HbA1c was measured using an ion-exchange high-performance liquid chromatography (HPLC) method (Osoh G8 - Tosoh Bioscience, Yamaguchi, Japan; Dunn et al. 1979; Mosca et al. 1986).

Plasma insulin concentration was measured using a standard insulin ELISA kit, K6219 (Dako Denmark A/S, Glostrup, Denmark) at the Institute of Sports Medicine, Department of Orthopaedic Surgery M, Bispebjerg Hospital and the Center for Healthy Aging, Faculty of Health Sciences, University of Copenhagen,

Denmark. The homeostatic model assessment (HOMA) used to quantify insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin as follows:  $\text{HOMA-IR} = (\text{glucose in mmol/l} \times \text{insulin in mU/l}) / 22.5$ . The quantitative insulin sensitivity check index (QUICKI) was derived from fasting insulin and fasting glucose as follows:  $\text{QUICKI} = 1 / [\log (\text{insulin } \mu\text{U ml}^{-1}) + \log (\text{glucose mg dl}^{-1})]$ .

#### Physical activity level

To quantify the physical activity level of the subjects, the International Physical Activity Questionnaire (IPAQ) was used (Craig et al. 2003). The IPAQ questionnaire quantifies the physical activity level of the subjects as the average weekly metabolic equivalent of task (MET) score,  $\text{MET min week}^{-1}$ . One MET =  $1 \text{ kcal} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  is equivalent to the resting metabolic rate (RMR) obtained during quiet sitting (Ainsworth et al. 2011). The World Health Organization (WHO) has defined a weekly MET score below 600 as low physical activity level and above 3,000 as a high physical activity level.

Peak oxygen consumption was measured on a Monark electronically braked ergometer bike (Monark 839 Ergomedic, Proterapi, Brøndby, Denmark) using an incremental protocol with a 25 W increase in load every minute starting at 50 W (old untrained) or 75 W (all other groups) until exhaustion which was reached within 4–12 min in all subjects. Subjects were instructed to aim at a pedalling frequency around 80–90 rounds  $\text{min}^{-1}$  (RPM) and were verbally encouraged to perform their max. Oxygen consumption ( $\text{VO}_2$ ),  $\text{CO}_2$  excretion, respiratory exchange ratio (RER), heart rate (HR), minute ventilation (VE), and cadence (RPM) were continuously recorded (breath-by-breath) using Masterscreen CPX equipment (Care Fusion, San Diego, CA, USA). All subjects reached RER values above 1.09 (range 1.09–1.34), indicating that high exertion levels were obtained. Peak oxygen uptake ( $\text{VO}_2$  peak) was determined as the highest mean  $\text{VO}_2$  value reached during a 15-s period (ignoring the highest and lowest value within that interval), and given as fitness level ( $\text{ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ ).

#### Muscle and tendon dimensions

The protocol for measuring anatomical cross-sectional area of the quadriceps femoris muscle (Q-CSA) and patellar tendon CSA/length has been described

elsewhere (Couppe et al. 2008; Mikkelsen et al. 2013). In brief, Q-CSA of the mid-thigh level and tendon CSA/length was measured using magnetic resonance imaging (MRI; General Electric, Sigma Horizon LX 1.5 T, T1-weighted SE, Millwaukee, WI, USA). For the Q-CSA, T1-weighted (T1w) turbo spin echo (TSE) images were performed using a lower extremity coil and parameters as previously described. The lean muscle mass of the Q-CSA was measured (subcutaneous and intermuscular noncontractile tissue were excluded). The mean value of three measurements of the same image was used for further analysis. The typical error percentage was 2 %.

Patellar tendon CSA and length was determined by T1w TSE para axial and sagittal plane MRI using a knee coil and parameters as described previously (Couppe et al. 2008). A plastic tube containing 1.0 %  $\text{CuSO}_4$  was placed in the FOV for normalization of tendon signal intensity. The axial scans were performed perpendicular to the patellar tendon. As described in detail elsewhere, the tendon CSA was measured region-specific (proximal, mid, and distal; Couppe et al. 2008). Average tendon CSA was calculated from the three sites and used for further analysis. Tendon length was obtained as previously described. The color range of each image was adjusted to set the minimum (black) at zero signal and the maximum (white) at the peak signal of the 1 %  $\text{CuSO}_4$  solution. Tendon CSA and length were measured using the grayscale image display. The typical error percent of repeated measures of site-specific tendon CSA was 3.1–5 %. Moreover, from the axial scans, tendon signal intensity [mean gray value (MGV)] were determined after measuring tendon CSA by using MATLAB (Carroll et al. 2008). The value of all slices from proximal to distal was averaged to determine mean tendon signal intensity. Both Q-CSA and patellar tendon CSA/length were manually outlined using the DICOM file viewer and associated measurement software Osirix 2.7.5 (OsiriX medical imaging software, Geneva, Switzerland). The MRI investigator (JFG) was blinded with regard to the subjects.

#### Mechanical properties of tendon

Details of the measurement, including the reliability of the method in our laboratory, has been reported previously (Hansen et al. 2006). The subjects were asked to refrain from strenuous exercise 48 h prior to the experiments. The subjects had 5 min warm-up on a stationary bike before the test, and all measurements were

performed on the nondominant leg. In brief, subjects were seated in a rigid chair with 90° knee and hip angle. Knee extension ramps over 10 s were performed with a strain gauge attached to the lower leg measuring force production, while synchronized B-mode ultrasound of the patellar tendon was recorded. Displacement of the patella and tibial tuberosity was used to determine tendon deformation. Tendon force was calculated from the measured extensor moment, using the distance from the lateral epicondyle of the knee to the attachment point of the strain gauge as the external moment arm and estimating the tendon moment arm from the femur length (Visser et al. 1990). Each force–deformation curve was fitted to a second-order or third-order polynomial ( $r^2 > 0.97$ ), which has previously been shown to describe the data curves well. Force and deformation was normalized to stress and strain using the CSA and resting length determined by MRI. Stiffness and elastic modulus was determined in the final 20 % of the curve. To improve the comparability between subjects achieving different maximal tendon loads, the data was also analyzed at the greatest common force level across all subjects. One master athlete subject did not contribute to mechanical data due to technical error during mechanical testing.

#### Tendon biopsies

Tendon biopsies were obtained from the nondominant extremity (Couppe et al. 2008) according to procedures described in detail elsewhere (Kongsgaard et al. 2009). In brief, after sterilization of the insertion site, the skin was injected with local anesthetic (1 % lidocaine), and a 3- to 5-mm-long incision was made distally to the patella apex. Tendon biopsies (~8 mg) were obtained by using a 16 G Monopty biopsy instrument (Bard Inc, Covington, GA, USA) with a disposable core biopsy needle (14 gauge). The biopsy was taken at an angle of ~60° relative to the patella. Biopsies were cleared of external adipose tissue and blood. The piece used for analysis of collagen cross-links was snap-frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis. Part of the tendon biopsy was used for transmission electron microscopy (TEM). The biopsy specimens for TEM were fixed in a 2 % glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) and stored at 4 °C until subsequent analyses. The procedure for TEM and the following measurements of collagen fibril diameter have previously been described in details (Magnusson et al. 2002).

#### Biochemical analysis of collagen cross-links

Freeze-dried tendon samples were hydrolyzed in 6° M HCl (+108 °C, 24 h), evaporated into dryness and dissolved in H<sub>2</sub>O. Hydroxyproline, the collagen-specific amino acid, was measured spectrophotometrically to quantify collagen content assuming 13.7 % hydroxyproline by mass (Creemers et al. 1997). HP, LP, and pentosidine cross-link concentrations were determined in a single reversed-phase high-performance liquid chromatography (HPLC) run and detected on the basis of their natural fluorescence (Bank et al. 1997). At 0–16 min, the wavelength for HP and LP fluorescence was 400 nm for emission and 295 nm for excitation. The wavelengths were changed at 16–60 min to 378/328 nm to measure pentosidine. For the elution of the cross-links, a gradient was built up to contain 17 % eluent B (75 % acetonitrile with 0.13 % heptafluorobutyric anhydride) at 0 min and 25 % eluent B at 30 min. Eluent A was 0.13 % heptafluorobutyric anhydride. Flow rate was 1 ml min<sup>-1</sup>. HP was eluted at 12 min, LP at 13.5 min, and pentosidine at 23 min. The HPLC system used included Quaternary Gradient Pump unit, PU-2089 Plus, Intelligent Autosampler AS-2057 Plus, and Intelligent Fluorescence Detector FP-2020 by Jasco. Data processing software was Jasco Chrompass. The LiChroCART 125-4 column was from Merck Hitachi. The results for HP, LP, and pentosidine are given compared with the standards injected at four different concentrations in each HPLC run. The intra-assay CV % based on duplicates within a run was 2.6, 3.7, and 3.9 % for HP, LP, and pentosidine, respectively. The detection limit for HP and LP is 0.4 and 0.05 pmol for pentosidine. Two subjects were not included in the final analysis due to an error in the original sample.

#### Stereology

A random sample of ten digitized electron microscopy images were obtained from each biopsy cross-section. The stereological analyses of collagen fibrils were carried out on a computer monitor onto which the digitized electron microscopy image was merged with a graphic representation of the stereological test systems (C.A.S.T.-grid software, The International Stereology Center at Olympus). Each TEM image was examined with 16 uniformly positioned points and 16 uniformly positioned unbiased counting frames (Gundersen et al. 1988), each of area 0.0426 μm<sup>2</sup>, in a fixed position

relative to the image. The counting frames covered 3 % of the area of the TEM images. On average,  $430 \pm 66$  fibrils (range 334–569) were analyzed per biopsy cross-section in the master athletes specimens, and  $394 \pm 88$  from each of the old untrained specimens (range 276–584). Furthermore,  $490 \pm 186$  fibrils (range 280–857) were analyzed per biopsy cross-section in the young athletes specimens, and  $452 \pm 104$  from each of the young untrained specimens (range 320–678). All measurements were performed in a blinded fashion.

#### AGE-reader measurements

AGE levels on the volar side in the forearm skin were measured with the AGE reader, a noninvasive, portable device, which has been comprehensively validated with AGEs measured in skin biopsies (Meerwaldt et al. 2004), DiagnOptics BV, Groningen, the Netherlands. The AGE reader measurements were made after the main study due to the availability of the AGE reader equipment. All measurements were always performed by the same observer (RBS). The AGE reader illuminates a skin surface of  $1 \text{ cm}^2$ , with an excitation light source using wavelengths between 300 and 420 nm. Only light from the skin is measured with a spectrometer in the 300–600 nm range, using 200  $\mu\text{m}$  glass fiber. Autofluorescence (AF) was calculated as follows: ratio between average light intensity, measured in the range 420–600 nm and the average light intensity in the range 300–420 nm (emission/excitation), and this ratio is expressed as arbitrary units. Three consecutive measurements, from each patient, were obtained, of which the mean was calculated. Within-day test–retest reproducibility was assessed in eight subjects before and after 1 h in our lab. Typical error% was 4.8 %. No systematic difference was found between the first and second session. The coefficient of determination for multiple within day assessments averaged  $r^2=0.81$ .

#### Statistical analysis

Effects of age ( $A$ ), training ( $T$ ), and age  $\times$  training ( $A \times T$ ) were tested using two-way analysis of variance (ANOVA), with Holm–Sidak multiple comparison post hoc testing, if nothing else is stated. When a significant interaction was observed, the post hoc test determined effects of training within the old (master athletes vs. old untrained), training within the young (young athletes vs. young untrained), age within the trained (master athletes

vs. young athletes), and age within the untrained (old untrained vs. young untrained). Nonparametric one-way ANOVA (Kruskal–Wallis) test, with Dunn’s multiple comparison post hoc test was used for Age and IPAQ (Table 1). Height, weight, BMI, and waist circumference were analysed using an ordinary one-way ANOVA with Sidak multiple comparison post hoc test to compare selected groups, as listed above. Data are given as mean (SD) or mean (SE), as stated in the “Results” section. SigmaPlot version 11 (Systat Software Inc) and Graph Pad Prism version 4 (GraphPad Software Inc., La Jolla, CA, USA) were used for statistical analyses.

## Results

### Subject characteristics

Subject characteristics are shown in Table 1. Old participants tended to be shorter than young participants ( $p=0.052$ ), and they had slightly higher BMI ( $p=0.012$ ). Waist circumference was higher in old than young groups ( $p<0.001$ ) and in the untrained than trained groups ( $p=0.04$ ).

### Physical activity data

Data are shown in Table 1. Regular running distance per week was similar in old and young runners (master athletes  $49 \pm 3$ ; young athletes  $43 \pm 5 \text{ km week}^{-1}$ ). Likewise, running distance during the last week prior to the study was similar between these groups (master athletes  $43 \pm 5$ ; young athletes  $48 \pm 7 \text{ km week}^{-1}$ ). The master athletes had been running for  $28 \pm 2$  years, and the young athletes for  $6 \pm 1$  years. Physical activity level assessed by IPAQ was significantly higher in master athletes compared to that in old untrained ( $p<0.001$ ) and in young athletes than in young untrained ( $p<0.05$ ), but similar between master athletes vs. young athletes and old untrained vs. young untrained.

### Muscle performance

In the following  $A$ ,  $T$ , and  $A \times T$  refer to effects of aging, training, and an interaction between aging and training, respectively. Data on muscle performance are shown in Table 1. Quadriceps muscle strength was greater in young than in old men ( $A$ ,  $p<0.05$ ) and in athletes vs.

untrained ( $T, p < 0.01$ ). A training by age interaction was observed ( $A \times T, p < 0.05$ ) with young athletes being stronger than young untrained and master athletes. Likewise,  $\text{VO}_2$  peak ( $\text{ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$ ) was higher in young than old men ( $A, p < 0.001$ ) and in athletes vs. untrained individuals ( $T, p < 0.001$ ), while no interaction between age and training was observed.

### Blood lipid profile

Data are shown in Table 2. Athletes demonstrated lower triglyceride, total cholesterol, and LDL cholesterol levels ( $T, p < 0.01$ ). Old men had higher total, LDL, and HDL cholesterol than their young counterparts ( $A, p < 0.01$ ). The difference in HDL was largely explained by a higher HDL level in master athletes. No interaction of age by training was observed for the blood lipids.

### Glucose homeostasis

Data are shown in Table 3. Fasting insulin was lower in the athletes than in the untrained ( $T, p < 0.001$ ), but was unaffected by age. Fasting glucose was lower in young vs. old men ( $A, p < 0.001$ ). A training by age interaction was observed for fasting glucose ( $A \times T, p < 0.05$ ), where old untrained had higher values than master athletes and young untrained. Likewise, blood glucose 2 h after glucose ingestion was lower in young vs. old men ( $A, p < 0.01$ ). Glycosylated hemoglobin (HbA1c), which identifies the average plasma glucose concentration over prolonged periods of time, was also reduced in young compared to old men ( $A, p < 0.001$ ) and was unaffected by training. Insulin resistance assessed by HOMA-IR was lower in athletes vs. untrained ( $T, p < 0.001$ ) and was not affected by aging. Insulin sensitivity assessed by

QUICKI was higher in athletes vs. untrained participants ( $T, p < 0.001$ ) and was not affected by age.

### Collagen concentration and cross-link density

Data are shown in Table 4. Pentosidine density was higher with age ( $A, p < 0.001$ ). There was an interaction between age and training ( $A \times T, p < 0.05$ ) with master athletes showing lower tendon pentosidine density than old untrained ( $p < 0.01$  (Fig. 1)). There was significant interaction for HP ( $A \times T, p < 0.01$ ), where HP was elevated in young athletes compared to young untrained ( $p < 0.05$ ) and master athletes ( $p < 0.05$ ). Effects of age ( $A, p < 0.001$ ) and training ( $T, p < 0.05$ ) were observed on skin AGE levels (autofluorescence; Fig. 2).

### Muscle and tendon dimensions

Data are shown in Table 5. Quadriceps muscle cross-sectional area (Q-CSA) as well as Q-CSA normalized with respect to body weight were larger in young than old men ( $A, p < 0.001$ ) and in athletes vs. untrained ( $T, p < 0.001$ ). In addition, an interaction between age and training was observed for Q-CSA ( $A \times T, p < 0.05$ ), where master athletes had a greater Q-CSA than old untrained ( $p < 0.001$ ). Patellar tendon CSA demonstrated an interaction between age and training ( $A \times T, p < 0.05$ ), master athletes had larger tendons than any of the other groups ( $p < 0.01$ ). Moreover, after normalizing patellar tendon CSA to body weight, a higher CSA was found with age ( $A, p < 0.0001$ ) and training ( $T, p < 0.0001$ ). Again, an interaction between age and training was demonstrated ( $A \times T, p < 0.05$ ), master athletes had larger tendons than any of the other individuals ( $p < 0.001$ ; Fig. 3). Furthermore, young athletes tended to have

**Table 2** Blood lipid profile

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	$N=10$	$N=12$	$N=15$	$N=12$		
Triglycerides (mmol/l)	$0.73 \pm 0.06$	$1.21 \pm 0.20$	$0.70 \pm 0.04$	$1.24 \pm 0.15$	-0.52	$T < 0.01$
Total cholesterol (mmol/l)	$4.44 \pm 0.19$	$5.18 \pm 0.42$	$5.76 \pm 0.24$	$6.67 \pm 0.27$	1.40, -0.83	$A < 0.001, T < 0.01$
HDL cholesterol (mmol/l)	$1.49 \pm 0.08$	$1.44 \pm 0.07$	$1.93 \pm 0.09$	$1.60 \pm 0.11$	0.30, 0.19	$A < 0.01 (T=0.051)$
LDL cholesterol (mmol/l)	$2.61 \pm 0.15$	$3.20 \pm 0.39$	$3.51 \pm 0.18$	$4.51 \pm 0.22$	1.11, -0.79	$A < 0.001, T < 0.01$

Data are given as mean  $\pm$  SE. *p* Values for significant main effects of age (*A*) and training (*T*) are given. Main effect sizes are given as old-young and athlete-untrained

*A* age, *T* training,  $A \times T$  interaction



**Table 3** Glucose homeostasis

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	<i>N</i> =10	<i>N</i> =12	<i>N</i> =15	<i>N</i> =12		
Insulin (fasted, pmol/l)	14.9±1.9	22.8±2.8	13.9±1.1	26.0±2.4	-10	<i>T</i> <0.001
Glucose (fasted, mmol/l)	4.66±0.11	4.58±0.10 <sup>§</sup>	4.91±0.09	5.26±0.10*, (0.35, MA), (0.68, YU)		<i>A</i> <0.001, <i>A</i> × <i>T</i> <0.05
Glucose (2 h, mmol/l)	4.49±0.34	4.70±0.15	5.05±0.27	5.53±0.20	0.69	<i>A</i> <0.01
HbA1c (mean mmol/l)	5.58±0.16	5.51±0.15	6.03±0.16	6.45±0.11	0.70	<i>A</i> <0.001
HOMA-IR	0.53±0.09	0.79±0.11	0.51±0.04	1.02±0.10	-0.39	<i>T</i> <0.001
QUICKI	0.44±0.01	0.41±0.01	0.44±0.01	0.39±0.01	0.04	<i>T</i> <0.001

Data are given as mean ± SE. Main effect sizes are given as old–young) and athlete–untrained. Effect size is shown in parenthesis under the relevant group for significant interactions

*A* age, *T* training, *A* × *T* interaction\*\*\*

\* Different from master athletes (*p*<0.05)

§ Different from old untrained (*p*<0.05)

greater patellar tendon CSA than young untrained (*p*=0.103). Finally, an interaction between age and training (*A* × *T*, *p*<0.01) was observed for tendon MRI signal intensity; master athletes had higher MRI signal intensity than old untrained men and young athletes (*p*<0.05; Fig. 4).

#### Mechanical tendon properties

Mechanical properties of the tendon obtained at maximum force are shown in Table 6. Maximum patellar

tendon force was greater in athletes vs. untrained men (*T*, *p*<0.01). Furthermore, an interaction was found between age and training (*A* × *T*, *p*<0.05), where young athletes had higher maximum force than master athletes (*p*<0.01) and young untrained (*p*<0.001). Tendon stress was significantly lower with age (*A*, *p*<0.001). Likewise, there was a significant interaction between age and training for tendon stress (*A* × *T*, *p*<0.05), with young athletes showing a greater stress than young untrained. However, no other effects were observed with respect to tendon deformation, stiffness, strain or Young's

**Table 4** Tendon collagen cross-links

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	<i>N</i> =10	<i>N</i> =12	<i>N</i> =13	<i>N</i> =12		
Collagen (mg/mg dry wt)	0.84±0.04	0.85±0.03	0.80±0.03	0.79±0.03		
Hydroxylslyl pyridinoline (HP, mmol/mol collagen)	657±48*, (142, YU), (197, MA)	515±44 <sup>§</sup>	460±42	563±44		<i>A</i> × <i>T</i> <0.01
Lysyl pyridinoline (LP, mmol/mol collagen)	31±4	24±2	32±3	31±3		
Pentosidine, (mmol/mol collagen)	11±2	9±1	38±4, (27, YA)	48±3**, (10, MA), (39, YU)		<i>A</i> <0.001, <i>A</i> × <i>T</i> <0.05

Data are given as mean ± SE. Effect size is shown in parenthesis under the relevant group for significant interactions

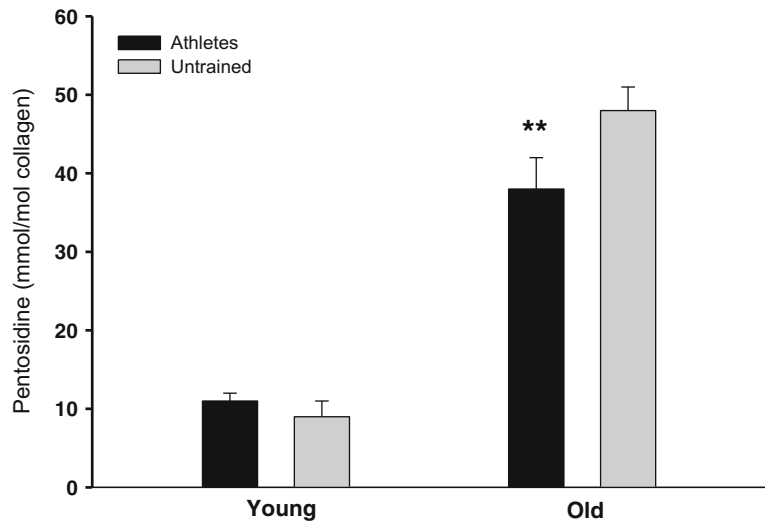
*A* age, *T* training, *A* × *T* interaction

\* Different from master athletes (*p*<0.05)

\*\* Different from master athletes (*p*<0.01)

§ Different from young athletes (*p*<0.05)

**Fig. 1** Pentosidine cross-link density (mmol/mol collagen). Pentosidine cross-link density was higher with age ( $p<0.001$ ). There was an interaction between age and training ( $p<0.05$ ), with **\*\*** master athletes having a lower tendon pentosidine cross-link density than old untrained ( $p<0.01$ ). Data are given as mean  $\pm$  SE



modulus determined at maximal force. Mechanical tendon properties at a common force are shown in Table 7. For patellar tendon stress a main effect of both aging ( $A$ ,  $p<0.01$ ) and training ( $T$ ,  $p<0.01$ ) was detected, so that patellar tendon stress at common force levels was lower with age and training.

#### Collagen fibril characteristics

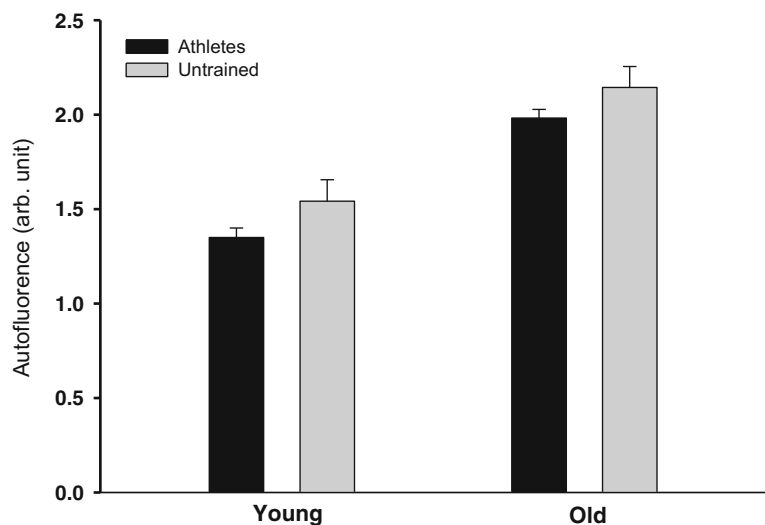
Data are shown in Table 8. Tendon fibril density tended to be reduced with age ( $A$ ,  $p=0.084$ ). An interaction between age and training was observed for fibril volume fraction ( $A \times T$ ,  $p<0.05$ ). Fibril volume fraction in old

untrained strongly tended to be lower than in young untrained ( $p=0.053$ ). No other effects were observed.

#### Discussion

The benefits of life-long vigorous regular exercise on cardiovascular, metabolic parameters, and overall mortality are well documented (Chakravarty et al. 2008; Yataco et al. 1997; Tanaka and Seals 2008). These previous findings were confirmed by several of the variables obtained in the present study, where the master athletes had similar cardiovascular and metabolic profiles to that of the young sedentary controls. In both our

**Fig. 2** Skin autofluorescence (AGE levels). Data are given as mean  $\pm$  SE. A significant effect of age ( $p<0.001$ ) and training ( $p<0.05$ ) was observed on skin autofluorescence. Data are given as mean  $\pm$  SE



**Table 5** Tendon and muscle dimensions

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	<i>N</i> =10	<i>N</i> =12	<i>N</i> =15	<i>N</i> =12		
Tendon length (mm)	41±2	42±2	44±2	42±2		
Tendon CSA (mm <sup>2</sup> )	84±5**	76±5	110±4, (26, YA), (24, OU)	86±5**		<i>A</i> <0.001, <i>T</i> <0.001, <i>A</i> × <i>T</i> <0.05
Tendon CSA (mm <sup>2</sup> kg <sup>-2/3</sup> )	5.0±0.3**	4.6±0.3	6.7±0.3, (1.7, YA), (1.7, OU)	5.0±0.3**		<i>A</i> <0.001, <i>T</i> <0.001, <i>A</i> × <i>T</i> <0.05
Quadriceps CSA (mm <sup>2</sup> )	7,859±201	6,792±201	6,481±200, (977, OU)	5,504±210*		<i>A</i> <0.001, <i>T</i> <0.001, <i>A</i> × <i>T</i> <0.05
Quadriceps CSA (mm <sup>2</sup> kg <sup>-2/3</sup> )	45.1±1.1	39.9±1.0	38.3±0.7, (7.2, OU)	31.1±1.3*		<i>A</i> <0.001, <i>T</i> <0.001, <i>A</i> × <i>T</i> <0.05

Data are given as mean ± SE. Effect size is shown in parenthesis under the relevant group for significant interactions

*A* age, *T* training, *A* × *T* interaction

\* Different from master athletes (*p*<0.05)

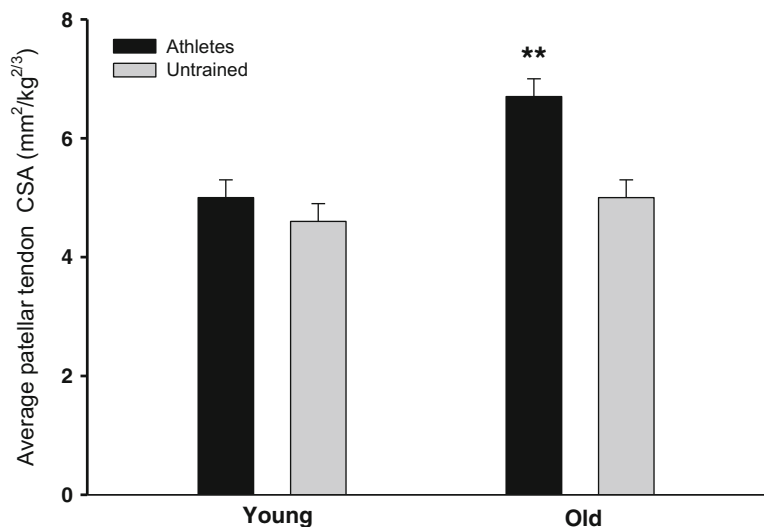
\*\* Different from master athletes (*p*<0.01)

old and young nonobese healthy men, regular long-term endurance running was associated with improved insulin sensitivity and lower triglyceride levels. Further, age-related changes in waist circumference, VO<sub>2</sub> peak, cholesterol, LDL, and leg muscle size were counteracted by the effects of chronic physical activity.

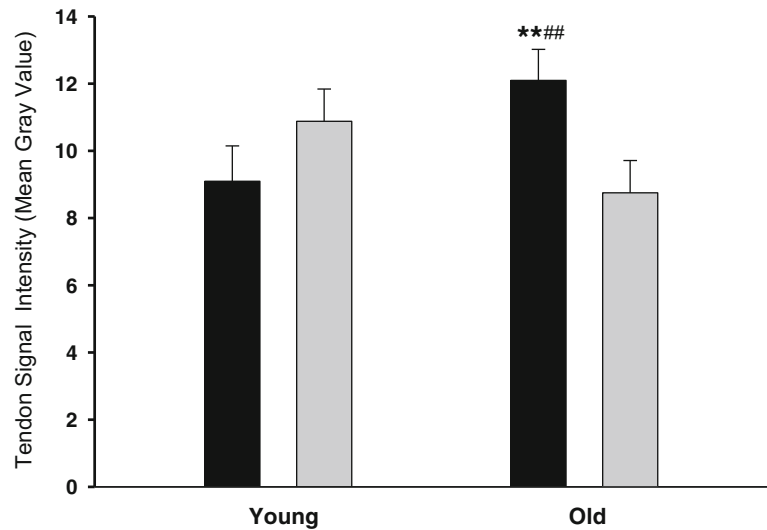
To the best of our knowledge, this is the first study that has examined if aging and life-long endurance exercise in humans influence the accumulation of non-enzymatic (AGE) and enzymatic cross-links in connective tissue and fibril structure morphology. The main findings were that the master athletes had a 21 % lower AGE cross-link density (pentosidine) in their patellar

tendons compared to old untrained men, and skin AGE levels (autofluorescence) were 11 % lower in athletes. Furthermore, both master athletes and young athletes had a higher cross-sectional area of the patellar tendon, which lowered the tendon stress for any given mechanical load. Master athletes also had a 30 % greater MRI signal intensity of their patellar tendon, in vivo. The present data suggest that life-long regular endurance exercise, such as running, can counteract the aging process in connective tissue of tendon and skin by reducing the age-related rise in AGE cross-link density, which may also affect other long-lived protein tissues. Furthermore, it appears that endurance running yields

**Fig. 3** Average patellar tendon CSA normalized to body weight (mm<sup>2</sup> kg<sup>-2/3</sup>) A greater effect of age (*p*<0.0001) and training (*p*<0.0001) was observed on weight-normalized tendon CSA. There was a significant interaction between age and training (*p*<0.05), namely, the \*\*master athletes had a greater tendon CSA than any of the other groups (*p*<0.01). Data are given as mean ± SE



**Fig. 4** Tendon MRI signal intensity—mean gray value. A significant interaction between age and training ( $p<0.01$ ) was found for tendon MRI signal intensity—mean gray value. Master athletes had a significantly higher MRI signal intensity than \*\* old untrained and ### young athletes ( $p<0.01$ ). Data are given as mean  $\pm$  SE



patellar tendon hypertrophy that reduces the stress exerted on the tendon and thereby lowers the risk of overuse injury.

#### Collagen cross-linking

##### AGE cross-links

AGE cross-links, including pentosidine, are formed when amino acid residues in the long-lived protein, such as the collagen triple helix, come into contact with

glucose and thereby modify the structure of the collagen connective tissue (Monnier and Cerami 1981). These cross-links accumulate with aging (Monnier and Cerami 1981; Avery and Bailey 2005) and in chronic diseases such as diabetes (Brownlee 1995; Monnier et al. 2005), and this process may be one of the underlying reasons for the age-related higher incidence of connective tissue injury (Murrell and Walton 2001; Englund et al. 2012). Life-long endurance exercise has been shown to have several beneficial effects that can retard the normal age-related decline in physical, cardiovascular, and metabolic

**Table 6** Tendon mechanical properties based on maximum force

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	<i>N</i> =10	<i>N</i> =12	<i>N</i> =14	<i>N</i> =12		
Max force (kN)	6.1 $\pm$ 0.4**, (1.2, MA)	4.4 $\pm$ 0.3 <sup>§</sup>	4.9 $\pm$ 0.3	4.6 $\pm$ 0.3		<i>T</i> <0.001, <i>A</i> × <i>T</i> <0.05
Deformation (mm)	2.9 $\pm$ 0.2	2.8 $\pm$ 0.2	2.7 $\pm$ 0.2	2.5 $\pm$ 0.2		
Stiffness (kN mm <sup>-1</sup> )	5.5 $\pm$ 0.5	4.2 $\pm$ 0.5	4.2 $\pm$ 0.4	4.1 $\pm$ 0.5		
Stress (MPa)	74 $\pm$ 5**, (27, MA)	61 $\pm$ 5	47 $\pm$ 4	55 $\pm$ 5		<i>A</i> <0.001, <i>A</i> × <i>T</i> <0.05
Strain (%)	7.4 $\pm$ 0.6	6.4 $\pm$ 0.5	6.3 $\pm$ 0.5	6.1 $\pm$ 0.5		
Modulus (GPa)	2.7 $\pm$ 0.3	2.4 $\pm$ 0.3	2.0 $\pm$ 0.3	1.8 $\pm$ 0.2	-0.7	<i>A</i> <0.01

Data are given as mean  $\pm$  SE. Main effect sizes are given as old–young and athlete–untrained. Effect size is shown in parenthesis under the relevant group for significant interactions

*A* age, *T* training, *A* × *T* interaction

\* Different from master athletes ( $p<0.05$ )

\*\* Different from master athletes ( $p<0.01$ )

<sup>§</sup> Different from young athletes ( $p<0.001$ )

**Table 7** Tendon mechanical properties determined based on common force

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	<i>N</i> =10	<i>N</i> =12	<i>N</i> =14	<i>N</i> =12		
Deformation (mm)	2.1±0.2	2.3±0.2	2.1±0.1	2.0±0.2		
Stiffness (kN mm <sup>-1</sup> )	3.2±0.3	3.0±0.3	3.2±0.3	3.4±0.3		
Stress (MPa)	33±2	38±2	27±2	33±2	6, 6	<i>A</i> <0.01, <i>T</i> <0.01
Strain (%)	5.2±0.5	5.0±0.4	4.8±0.4	5.0±0.4		
Modulus (GPa)	1.6±0.2	1.8±0.2	1.4±0.2	1.6±0.2		

Data are given as mean ± SE. Main effect sizes are given as old–young and athlete–untrained  
*A* age, *T* training, *A* × *T* interaction

parameters (Tanaka and Seals 2008; Yataco et al. 1997; Chakravarty et al. 2008). However, to what extent life-long endurance exercise can decelerate this age-related accumulation of irreversible AGE cross-linking and the deterioration of tendon has, to our best knowledge, not been investigated. In the present study, we demonstrate that AGE cross-link density (pentosidine) in the patellar tendon was 21 % lower in master athletes compared to old untrained subjects and fourfold higher in old men compared to activity-matched young men.

We also determined the AGE accumulation in skin using a noninvasive method based on autofluorescence (AGE reader; Meerwaldt et al. 2004). It has been shown that the difference between young and older individuals in AGE cross-link density in collagen is twofold in skin (Verzijl et al. 2000) and skeletal muscle (Haus et al. 2007), fivefold in bone (Saito et al. 1997), sevenfold in tendon (Couppe et al. 2009), ninefold in ligaments (Chen et al. 2000), and 33-fold in cartilage (Verzijl et al. 2000) demonstrating marked age-related

differences in tissue-specific turnover. The present data showed 45 % higher values in skin with aging, but also 11 % lower values by training, indicating that routine-running reduced the age-related AGE accumulation in skin. In contrast to AGE in the load-bearing tendon, the elevated AGE levels in the skin of the forearm of the athletes are likely the result of a systemic effect, which may also affect AGE accumulation in other tissues (Baynes 2000; Momma et al. 2012).

In the present study, master athletes demonstrated markedly lower fasting insulin and glucose levels, which is likely an effect of the life-long exposure to regular endurance running (Rogers et al. 1990; Seals et al. 1984). Elevated levels of blood glucose and reduced glucose tolerance could explain the higher AGE cross-link density in tendons of old untrained men in the present study. Likewise, improved glycemic control for many years may be the reason for lower AGE cross-link density in the tendons of the present master athletes (Lyons et al. 1991; Rogers et al. 1990). In individuals

**Table 8** Tendon fibril characteristics

Variable	Young athletes (YA)	Young untrained (YU)	Master athletes (MA)	Old untrained (OU)	Main effect size	<i>p</i> Value
Number of subjects	<i>N</i> =10	<i>N</i> =12	<i>N</i> =11	<i>N</i> =12		
Volume fraction (%)	53±2	57±2 <sup>§</sup> , (6, OU)	56±2	51±2		<i>A</i> × <i>T</i> < 0.05
Density (fibril μm <sup>2</sup> )	92.8±7.1	85.6±6.2	81.1±7.8	72.6±6.2	12	( <i>A</i> =0.084)
Mean fibril diameter (nm)	78±2	83±3	81±4	83±3		
Mean fibril area (nm <sup>2</sup> )	6172±624	6572±541	6433±678	6542±541		

Data are given as mean ± SE. Main effect sizes are given as old–young and athlete–untrained. Effect size is shown in parenthesis under the relevant group for significant interactions

*A* age, *T* training, *A* × *T* interaction

<sup>§</sup> Different from old untrained (*p*=0.053)

with normal glucose tolerance, it has been demonstrated that regular exercise can protect against the development of insulin resistance and the decline in glucose tolerance normally observed with sedentary aging. In addition, it has been shown that regular exercise can normalize glucose tolerance by means of short-term effects in individuals with abnormal glucose tolerance (Rogers et al. 1990). It has been proposed that glycation of collagen results in increased brittleness of connective tissue, which makes the connective tissue more prone to injury, while regular exercise may slow the glycation process (Avery and Bailey 2005). The mechanism of this process remains unclear but may relate to increased turnover of collagen with chronic exercise thereby attenuating AGE accumulation (Sell and Monnier 2012; Avery and Bailey 2005). In the present study, we assessed pentosidine, which only constitute a small fraction of the AGE cross-links, but there is strong evidence that it correlates with more prominent AGEs, such as glucosepane and carboxymethyl-lysine (CML; Dammann et al. 2012; Meerwaldt et al. 2004; Dyer et al. 1991). Altogether, the present data demonstrate that life-long regular physical activity influence the accumulation of nonenzymatic AGE cross-links tendon and skin and thereby partly counteract the aging process of the tissue.

The present study also raises questions as to the level of exercise that is needed in later life in order to reduce the magnitude of AGE cross-linking. There are prospective data indicating that AGE cross-link (pentosidine) density is reduced by 23 % with 12 weeks of heavy resistance training and that tendon mechanical properties can improve by short-term physical activity in elderly subjects, which indicates some kind of rejuvenation of tendon tissue (Kongsgaard et al. 2009). Future studies should explore whether short-term endurance running or other types of exercise of less or more vigorous intensity may reduce AGE cross-linking density in long-lived proteins and also whether AGE cross-linking in connective tissue of elderly can be reduced by short-term physical training.

#### *Enzymatic cross-links*

The density of mature lysyl oxidase-derived intermolecular covalent cross-links HP and LP gradually increase during tendon tissue maturation and are believed to be the chief contributors to the function and mechanical properties of tendons (Eyre et al. 1984; Fujimoto et al.

1978). Data on mature cross-link density of human tendons, and how these are influenced by aging, are sparse (Bank et al. 1999; Coupe et al. 2009). A small age-related increase of LP in the supraspinatus tendon has been reported in human cadavers (Bank et al. 1999), and we have previously shown that old men have greater HP (40 %) and LP (200 %) density in the patellar tendon compared to young men (Coupe et al. 2009). This is in contrast to the present findings where no age-related effect was observed for HP and LP cross-links. However, interestingly, the young athletes had a higher HP density compared to young untrained, suggesting a training effect. Up to a 37-fold increase (Heinemeier et al. 2007) in the expression of lysyl oxidase (LOX) mRNA in tendon tissue in response to acute training has been reported, which could indicate a loading-induced upregulation in enzymatic cross-linking (HP, LP) of collagen in tendon. This mechanism could explain the increased HP density in the young athletes. It is uncertain why this effect would be absent in aging master athletes, although it is known that age may blunt the LOX response possibly due to lack of tissue renewal associated to decline in tendon cell number and function with age (Nagy et al. 1969; Heinemeier et al. 2013; Thorpe et al. 2010).

#### *Tendon collagen content*

Collagen content of the patellar tendon (Haut et al. 1992) and tail-tendon (Vogel 1983) in animals has been shown to decrease or increase (Vailas et al. 1985) with age. In humans, we recently demonstrated that collagen concentration of the patellar tendon was 34 % lower in old men compared to that in young men (Coupe et al. 2009). In the present study, the reduction in collagen content with age was not significant, and there was no effect of training either. The reason for this difference is not clear, but may be related to a stricter matching between athletes and control subjects in the present study.

#### *Structural and mechanical properties of tendon*

##### *Tendon dimensions*

There is evidence that the size and mechanical properties of human tendons can be influenced by both the level of physical activity and the aging process. Recent reports demonstrate that young human tendons respond

to physical activity by increasing tendon size, which is accompanied by altered functional mechanical properties (Kongsgaard et al. 2007; Seynnes et al. 2009). In animal models, aging is associated with an increase in tendon cross-sectional area (Nakagawa et al. 1996; Birch et al. 1999), and in humans, the Achilles tendon, but not the patellar tendon, appears to undergo hypertrophy with aging (Magnusson et al. 2003; Carroll et al. 2008). In the present study, we found a greater cross-sectional area of the patellar tendon in endurance athletes, which by definition resulted in a lower tendon stress for a given load. The master athletes had 30 % larger patellar tendon CSA than their sedentary healthy counterparts. Consequently, life-long regular endurance running appears to induce a hypertrophy response that lowers the stress on the tendon and potentially reduces the risk of injury. Moreover, the master athletes also had greater patellar tendon MRI signal intensity than old untrained and young athletes. It has been shown that MRI signal intensity correlate with histological findings such as tendon tissue hydration and collagen structure (Carroll et al. 2008; Harber et al. 2009). The present data collectively indicate that life-long endurance running increases tendon size and may change the internal milieu of tendon tissue.

#### *Collagen fibril morphology*

It has been previously shown that heavy slow resistance training is associated with changes in fibril morphology (Kongsgaard et al. 2010), and in addition, aging has been observed to reduce the size and/or density of collagen fibrils both in animals (Curwin et al. 1994; Nakagawa et al. 1994; Parry et al. 1978; Patterson-Kane et al. 1997) and humans (Sargon et al. 2005; Strocchi et al. 1991). In the present study, we did not find any significant effects on fibril morphology with age or training, although there was a tendency for lower collagen fibril density with age ( $p=0.084$ ).

#### *Tendon mechanical properties*

Stiffness of both animal and human tendons have been reported to increase (Shadwick 1990; Kubo et al. 2007a), decrease (Stenroth et al. 2012), or remain unchanged (Couppe et al. 2009) with aging. Viidik et al. (Viidik et al. 1996) showed that life-long endurance training may have counteracting systemic effects on the aging process as reflected by less stiff tail tendon

tissue. Simonsen et al. (1995) studied the effect of 38 weeks of strength training and swimming on Achilles tendon properties in rats and showed that ultimate failure decreased with aging, but that this age-related decline was counteracted only by swim training and not in rats subjected to strength training. However, this may be different in humans since improvement (change toward a young phenotype) of the mechanical properties of tendon has been observed with strength training in elderly persons (Reeves et al. 2003; Carroll et al. 2011; Seynnes et al. 2011). In the present study, the effect of chronic (life-long) endurance running appeared to yield a tendon hypertrophy response, which, however, was not accompanied by greater tendon modulus or stiffness at common force levels as may have been expected. The lack of a significant mechanical difference is likely due to different effects pulling in different directions, i.e., lower AGE cross-link density and greater tendon CSA.

#### *Limitations*

The present investigation is a cross-sectional case control study and, therefore, has inherent limitations. Natural selection cannot be excluded such that our runners have lower pentosidine levels and larger patellar tendon before loading their tendons. Furthermore, it must also be mentioned that there are considerable variations in tendon mechanics between subjects, and consequently, there is a risk that relevant differences were not found because a type 2 error was committed (Magnusson et al. 2001). Moreover, to avoid an underestimation of real PT force, the co-activation of the hamstrings should preferably be taken into account, and therefore we have previously measured the EMG (Electromyographic) activity from the biceps femoris during the ramped isometric extension (Hansen et al. 2006). Due to an unknown noise source during previous studies, which we were not able to filter, we chose to omit the biceps femoris EMG activity in the calculations. From our previous studies we calculated that this error would underestimate the tendon force and tendon stress by 5–20 %. However, studies have demonstrated that co-contraction does not seem to differ between master athletes and controls (Pearson et al. 2002) and, furthermore, does not change during maximal isometric contractions after 12 weeks of strength training (Kubo et al. 2007b; Reeves et al. 2003) or immobilization

(Kubo et al. 2000, 2004). Therefore, it was assumed that this factor would remain constant.

Finally, the control subjects were carefully matched to our athletes and also had similar diets. Therefore, the sedentary old untrained males may not necessarily represent the average population of similar age with respect to BMI, waist circumference, and blood profile. This matching was necessary to ensure that these factors would not confound the age and training effects. Nevertheless, the master athlete model is likely a suitable model for the study of successful, optimal aging (Booth and Lees 2006) since changes that take place with time can be considered the results of primary (physiological) aging rather than secondary (lifestyle-related) aging (Tanaka and Seals 2008).

To our best knowledge, this study reports the first data to demonstrate that life-long regular running in male master athletes results in adaptations of tendon that occurs at different levels including lower AGE cross-link density, greater tendon MRI signal intensity, and increased tendon size. All of these changes can be considered beneficial to the mechanical integrity of the tendon, while, in particular, the increase in tendon size results in a reduced average tendon stress. As such, the present results suggest that life-long regular endurance exercise, such as running, can counteract the aging process in collagen-rich tissue (tendons, skin) and thereby possibly reduces the risk of tendon injury during running.

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