



Muscle fiber capillarization is associated with various indices of skeletal muscle mass in healthy, older men

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ABSTRACT

Introduction: Muscle fiber capillarization plays a fundamental role in the regulation of skeletal muscle mass maintenance. However, it remains unclear to what extent capillarization is related to various other skeletal muscle characteristics. In this study we determined whether muscle fiber capillarization is independently associated with measures of skeletal muscle mass, both on a whole-body and cellular level, and post-absorptive muscle protein synthesis rates in healthy older men.

Methods: Forty-six healthy older (70 ± 4 y) men participated in a trial during which basal muscle protein synthesis rates were assessed using stable isotope tracer methodology. Blood and muscle biopsy samples were collected to assess post-absorptive muscle protein synthesis rates over a 3-hour period. Immunohistochemistry was performed to determine various indices of muscle fiber capillarization, size, type distribution, and myonuclear content/domain size. Dual energy x-ray absorptiometry scans were performed to determine whole-body and appendicular lean tissue mass.

Results: Capillary-to-fiber ratio (C/Fi) and perimeter exchange (CFPE) index correlated with whole-body lean tissue mass ($r = 0.43$, $P < 0.01$ and $r = 0.25$, $P < 0.10$, respectively), appendicular lean tissue mass ($r = 0.52$, $P < 0.001$ and $r = 0.37$, $P < 0.05$, respectively) as well as appendicular lean tissue mass divided by body mass index ($r = 0.65$, $P < 0.001$ and $r = 0.62$, $P < 0.001$, respectively). Muscle fiber size correlated with C/Fi ($r = 0.45$, $P < 0.01$), but not with CFPE index. No associations were observed between different indices of muscle fiber capillarization and post-absorptive muscle protein synthesis rates in healthy, older men.

Conclusion: The present study provides further evidence that muscle fiber capillarization may be a critical factor in the regulation of skeletal muscle maintenance in healthy older men.

1. Introduction

Skeletal muscle comprises 40–50% of total body mass and plays vital roles in breathing, locomotion, posture, and overall metabolism (Frontera and Ochala, 2015). The preservation of skeletal muscle mass, strength, and endurance are fundamental for proper quality of life from young to old age (Tieland et al., 2018). For optimal muscle tissue function and health, adequate muscle tissue perfusion is of critical importance as it is responsible for the delivery of oxygen, growth factors, and nutrients and removal of waste products (Hendrickse and Degens, 2019). The diffusion and transport to the muscle fibers is ultimately limited by the surface area of the microvascular bed (i.e. capillaries) (Pittman, 1995). As such, it is becoming clear that the capillary network structure plays a pivotal role in skeletal muscle maintenance, function, and health (Joanisse et al., 2017; Nederveen et al., 2017; Parise et al.,

2019).

Muscle tissue has a dense capillary network, with each muscle fiber being surrounded by generally 3–6 capillaries, depending on muscle fiber type and training status of the individual from whom the muscle tissue sample was collected (Plyley and Groom, 1975; Klausen et al., 1981; Andersen and Henriksson, 1977). Given the architectural structure, muscle fiber capillaries have the capacity to play a central role in the coordination of blood flow during contraction and the cellular responses to which it feeds into. As such, it is not surprising that low muscle fiber capillarization is associated with impairments in cardio-respiratory fitness (Prior et al., 2016), physical function (Nicklas et al., 2008), and glucose metabolism (Snijders et al., 2017a). This is of particular relevance in older adults, as the anatomical structure of the microvascular bed has been shown to change substantially with an advancing age, with a specific decline in type II muscle fiber size and

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associated capillaries (Groen et al., 2014). The age-related muscle fiber type-specific capillary rarefaction has been associated with a reduced exercise capacity (Prior et al., 2016), lower physical function (Nicklas et al., 2008), low physical activity level (Moro et al., 2019), impaired muscle satellite cell function (Snijders et al., 2019), elevated blood pressure (Gueugneau et al., 2016), and impaired blood glucose homeostasis (Snijders et al., 2017a) in older adults. In addition, we (Snijders et al., 2017b) and others (Moro et al., 2019) have previously reported that baseline muscle fiber capillarization may be a limiting factor in the skeletal muscle adaptive response to prolonged resistance exercise training in older adults. Hence, it has been hypothesized that muscle fiber capillarization may be a key factor modulating skeletal muscle tissue function and health in older individuals (Joanisse et al., 2017; Nederveen et al., 2017; Prior et al., 2016). However, to what extent muscle fiber capillarization is related to various other resting skeletal muscle characteristics in older adults remains ill defined. In the present study we assessed whether muscle fiber capillarization is independently associated with measures of muscle mass, both on whole-body and cellular level, as well as post-absorptive muscle protein synthesis rates in a large group of healthy, older men.

2. Materials and methods

2.1. Subjects

Forty-six healthy older men were recruited to participate in this trial (see Table 1 for participants' characteristics). Volunteers aged between 65 and 80 y and with a BMI between 18.5 and 30.0 kg/m² underwent an initial screening visit, as described previously (Gorissen et al., 2016). In short, during screening the medical history was assessed by a questionnaire. A 2-h oral glucose tolerance test (OGTT) was performed to exclude individuals with type 2 diabetes mellitus (2. Classification and Diagnosis of Diabetes, 2020). Furthermore, individuals were excluded when they were smoking, had wheat/lactose intolerance, gastrointestinal diseases, arthritic conditions, neuromuscular problems, were participating in a structured exercise program, or used anticoagulants or any medication known to affect protein metabolism. Anthropometric measurements were performed including, height, bodyweight (E1200, August Sauter GmbH, Albstadt, Germany), blood pressure (HEM-907, OMRON Healthcare Europe B.V., Hoofddorp, The Netherlands) and body composition (Dual-energy X-ray absorptiometry (DEXA) scan; Discovery A; Hologic, Bedford, USA). All included participants were deemed healthy based on the medical questionnaire and screening results. All participants were informed on the nature and possible risks of the experimental procedures before their written consent was obtained. This study was approved by the Medical Ethics Committee of Maastricht University Medical Centre and performed in accordance with the guidelines set out in the Declaration of Helsinki. The trial was registered at clinicaltrials.gov (NCT01952639). The present study was part of a greater project assessing the muscle protein synthetic response to different protein sources in older men published previously (Gorissen

Table 1
Participants' characteristics.

Age (y)	70 ± 4
Height (m)	1.76 ± 0.08
Weight (kg)	78.1 ± 10.1
BMI (kg·m ⁻²)	25.1 ± 2.6
Whole body fat mass (%)	24.2 ± 3.8
Whole body lean mass (kg)	56.7 ± 7.1
Appendicular lean mass (kg)	24.6 ± 3.4
Fasting plasma glucose concentration (mmol·L ⁻¹)	6.0 ± 0.5
2-h plasma glucose concentration (mmol·L ⁻¹)	6.1 ± 1.6
OGIS (mL·min ⁻¹ ·m ⁻²)	351 ± 54
Systolic blood pressure (mm Hg)	139 ± 17
Diastolic blood pressure (mm Hg)	71 ± 10

Values are means ± SD, n = 46. OGIS, oral-glucose insulin sensitivity.

et al., 2016). For the current analysis, participants were selected based on the availability of a muscle biopsy sample for immunohistochemical analyses.

2.2. Diet and physical activity control

Participants refrained from any strenuous physical activity and kept their diet as consistent as possible for 2 days prior to the experimental trial. On the evening before the trial, all participants consumed a standardized meal (2.38 MJ, providing 16 Energy % (En%) from protein, 33 En% from carbohydrate, and 51 En% from fat).

2.3. Infusion protocol

After an overnight fast, participants arrived at 08:00 h at the laboratory by car or public transport. A catheter was inserted into an ante-cubital vein for stable isotope labeled amino acid infusion. A second catheter was inserted into a dorsal hand vein of the contralateral arm and placed in a hot box (60 °C) for arterialized blood sampling. A baseline blood sample was taken and the plasma phenylalanine pool was primed with a single dose of L-[ring-¹³C₆]-phenylalanine (2.1 mmol/kg), after which a continuous intravenous L-[ring-¹³C₆]-phenylalanine infusion (0.048 mmol·kg⁻¹·min⁻¹) was initiated (t = -90 min). After resting in a supine position for 90 min, a second arterialized blood sample was collected and a muscle biopsy sample was taken from the *M. vastus lateralis* of a randomly chosen leg (t = 0 min). A second muscle biopsy sample from the same leg was collected 180 min after the first biopsy. Arterialized blood samples were collected at t = 60, 120, and 180 min. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) containing tubes and centrifuged at 1000g for 10 min at 4 °C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80 °C. Biopsy samples were collected from the middle region of the *vastus lateralis* muscle, ~15 cm above the patella and 3 cm below entry through the fascia, by using the percutaneous needle biopsy technique (Bergstrom, 1975). Muscle samples were dissected carefully, freed from any visible non-muscle material and one part (60–80 mg) was immediately frozen in liquid nitrogen. A second part (15–20 mg) of the biopsy sample was embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands), frozen in liquid nitrogen-cooled isopentane. All samples were stored at -80 °C until further analysis.

2.4. Plasma and muscle tissue analyses

Plasma L-[ring-¹³C₆]-phenylalanine and muscle intracellular L-[ring-¹³C₆]-phenylalanine enrichments were determined by gas chromatography–mass spectrometry (GC–MS; Agilent 7890A GC/5975C MSD; Agilent Technologies) as described in our previous work (Gorissen et al., 2014). Mixed muscle proteins were extracted from separate pieces of muscle tissue (~60 mg), as described previously (Gorissen et al., 2014; Burd et al., 2014). Mixed muscle protein-bound L-[ring-¹³C₆]-phenylalanine enrichments were determined by gas chromatography-combustion-isotope ratio mass spectrometry analysis, as described in our previous work (Kramer et al., 2015).

2.5. Fractional synthetic rate calculations

Post-absorptive mixed muscle protein fractional synthetic rates (FSRs) were calculated by using the standard precursor-product equation, as follows:

$$FSR = \frac{\Delta E_p}{E_{precursor} \cdot t} \cdot 100$$

ΔE_p is the increment in mixed muscle protein-bound L-[ring-¹³C₆]-phenylalanine enrichment after the 3 h incorporation period, $E_{precursor}$ is the weighted mean plasma or intracellular L-[ring-¹³C₆]-phenylalanine enrichment during that incorporation period, and t is the incorporation

period (h). Weighted mean plasma enrichments were calculated by taking the average enrichment between all consecutive time points and correcting for the time between these sampling time points.

2.6. Immunohistochemistry

Frozen muscle biopsies were cut into 5- μm -thick cryosections using a cryostat at $-20\text{ }^{\circ}\text{C}$, and thaw-mounted onto uncoated pre cleaned glass slides. Care was taken to properly align the samples for cross-sectional orientation of the muscle fiber. Muscle biopsies were stained for muscle fiber typing, capillarization and number of myonuclei. Following 5 min fixation in acetone and subsequent 15 min air drying at room temperature, the muscle samples were incubated for 45 min with the first primary antibody CD31 (dilution 1/50; M0823; Dako, Glostrup, Denmark) in 0.05% Tween-phosphate-buffered saline (PBS). Slides were washed 3×5 min in 0.05% Tween-PBS. Next, slides were incubated for 45 min in HAM Biotine (1:500, Vector Laboratories) in 0.05% Tween/PBS. Slide were again washed 3×5 min with PBS, followed by a 45 min incubation with Avidine Texas Red (A2006, dilution 1/400; Vector Laboratories) and antibodies against MHC-I (A4.840, dilution 1/25; DSHB), and laminin (polyclonal rabbit anti-laminin, dilution 1/50; Sigma) in 0.05% Tween/PBS. Following 3×5 min washing step in PBS, samples were finally incubated for 30 min with appropriate secondary antibodies goat anti-mouse (GAM) IgM AlexaFluor488, goat anti-rabbit (GAR) IgG AlexaFluor647 (Molecular Probes), and 4',6-diamidino-2-fenylindool (DAPI, Molecular Probes). After the final washing (3×5 min PBS), slides were mounted with Mowiol (Calbiochem). The staining procedure resulted in images with laminin in white, MHC-I in green, DAPI in blue and CD31 in red (see Supplemental Fig. 1 for representative image of the staining).

Images were visualized and automatically captured at $20\times$ magnification with a fluorescent microscope equipped with an automatic stage (IX81 motorized inverted microscope, Olympus, Hamburg, Germany), an EXi Aqua CCD camera (QImaging). Micromanager 1.4 software was used for image acquisition (Edelstein et al., 2014). Quantitative analyses were performed using ImageJ version 1.46d software package (version 1.46d, National Institute of Health, MD (Strandberg et al., 2010)). On average, 315 ± 148 muscle fibers were analyzed per muscle biopsy sample collected to determined muscle fiber type distribution, size, and myonuclear content/domain size. The quantification of muscle fiber capillaries was performed on 30 type I and 30 type II muscle fibers based on the work of Hepple et al. (Hepple, 1997). Quantification was made of (i) capillary contacts (CC), (ii) the capillary-to-fiber ratio (C/Fi), (iii) capillary-to-fiber perimeter exchange (CFPE) index and (iv) capillary density, (CD) (see Supplemental Fig. 1 for example calculations). All parameters of capillarization were calculated both for fiber type specific and for mixed fiber types (i.e. not fiber type specific). Fiber type specific capillarization indices were used for correlations with other fiber type specific parameters at the muscle fiber level. Mixed fiber capillarization indices were used for correlations with measures of lean tissue mass on whole body level and muscle protein synthesis rates, as both these measures are not fiber type specific.

2.7. Statistical analyses

Data are expressed as mean \pm SD. Normal distribution of all parameters was verified by the Kolmogorov-Smirnov test. Pearson (r) correlation analyses were performed when parameters were normally distributed, while Spearman rank (ρ) correlation analyses were used when parameters were not-normally distributed. Correlation analyses were performed between different indices of muscle fiber capillarization and 1. whole body lean mass, 2. appendicular lean mass, 3. muscle fiber size, 4. fiber type distribution, 5. myonuclear content/domain size, 6. post-absorptive fractional synthetic rates. A significant r - or ρ -value between 0 and 0.19 was regarded as 'very weak', between 0.2 and 0.39 as 'weak', between 0.40 and 0.59 as 'moderate', between 0.6 and 0.79 as

'strong', and between 0.8 and 1 as 'very strong' correlation. Statistical significance was accepted as $P < 0.05$. All calculations were performed using SPSS version 22.0 (SPSS version 22.0, IBM Corp., USA).

3. Results

3.1. Participants' characteristics

Age of the 46 included participants ranged from 64 to 79 years old. BMI ranged from 20.1 to 30.2 kg/m^2 , with 52% of participants categorized as overweight ($>25\text{ kg}/\text{m}^2$) and one participant classified as obese ($>30\text{ kg}/\text{m}^2$). Systolic and diastolic blood pressure ranged from 107 to 183 and 53 to 94 mmHg, respectively. Seventy percent of the participants were categorized as hypertensive (systolic and/or diastolic blood pressure > 130 or > 90 mm Hg, respectively). Based on fasting blood glucose values, 20% of all participants had a normal and 80% an impaired fasting glucose (5.6 mmol/L vs >5.6 mmol/L, respectively). Based on the glucose concentration at the 2-h time point during the OGTT, 85% of participants showed normal (<7.8 mmol/L), while 15% showed impaired glucose tolerance (>7.8 mmol/L). No participants were categorized as diabetic based on fasting (>7 mmol/L) or 2-hour glucose concentrations (>11.1 mmol/L) (2. Classification and Diagnosis of Diabetes, 2020).

Muscle fiber capillarization quantified as CFPE index ranged from 3.2 to 7.1 capillaries-1000 μm^{-1} (Fig. 1A), while CD ranged from 157 to 422 capillaries- mm^{-2} (Fig. 1B) for the included participants. Appendicular lean mass divided by BMI ranged from 0.74 to 1.29 (Fig. 1C), with 3 participants classified as sarcopenic based on previously determined cut-off values (i.e. <0.789) (Cawthon et al., 2014). Muscle fiber size ranged from 3006 to 7842 μm^2 (Fig. 1D), while post-absorptive muscle protein synthesis, expressed as fractional synthetic rate calculated based on the plasma precursor pool, ranged from 0.013 to 0.061% h^{-1} (Fig. 1E) in this group of healthy older men.

3.2. Lean body mass vs capillarization

A significant, but weak positive correlation was observed between whole-body lean mass and CC ($P < 0.01$, Table 2). A significant moderate positive correlation was observed between whole-body lean mass and C/Fi ($P < 0.01$; Table 2). Whole-body lean mass tended ($P = 0.098$) to be positively correlated with CFPE index (Fig. 2A), while no significant correlation was found with CD (Table 2). A significant weak positive correlation was observed between appendicular lean mass and CFPE index ($r = 0.37$, $P < 0.05$; Fig. 2B), whereas this was moderately correlated with CC or C/Fi ($P < 0.01$ and $P < 0.001$, respectively; Table 2). No correlation was observed between appendicular lean mass and CD (Table 2). Strong positive correlations were observed between appendicular lean mass divided by BMI and CFPE index ($r = 0.62$, $P < 0.001$; Fig. 2C) or C/Fi ($P < 0.001$; Table 2). Finally, appendicular lean mass divided by BMI was moderately correlated with CD ($P < 0.01$; Table 2) or CC ($P < 0.001$; Table 2).

3.3. Muscle fiber size vs capillarization

For type 1 and type 2 muscle fibers specifically, significant moderate positive correlations were observed between fiber size and CC or C/Fi (all $P < 0.01$; Table 3). Both type 1 and type 2 muscle fiber size showed a moderate negative correlation with type 1 and type 2 muscle fiber type specific CD, respectively ($P < 0.01$; Table 3). No significant correlation was observed between type 1 and type 2 muscle fiber size and type 1 and type 2 muscle fiber type specific CFPE index, respectively (Table 3). When fiber size is expressed per mixed fiber type, a significant moderate correlation was observed with mixed muscle fiber CC and C/Fi ($P < 0.001$ and $P < 0.01$, respectively; Table 3). A significant moderate negative correlation was observed between mixed muscle fiber size and mixed muscle fiber CD ($P < 0.01$; Table 3). Mixed muscle fiber size was

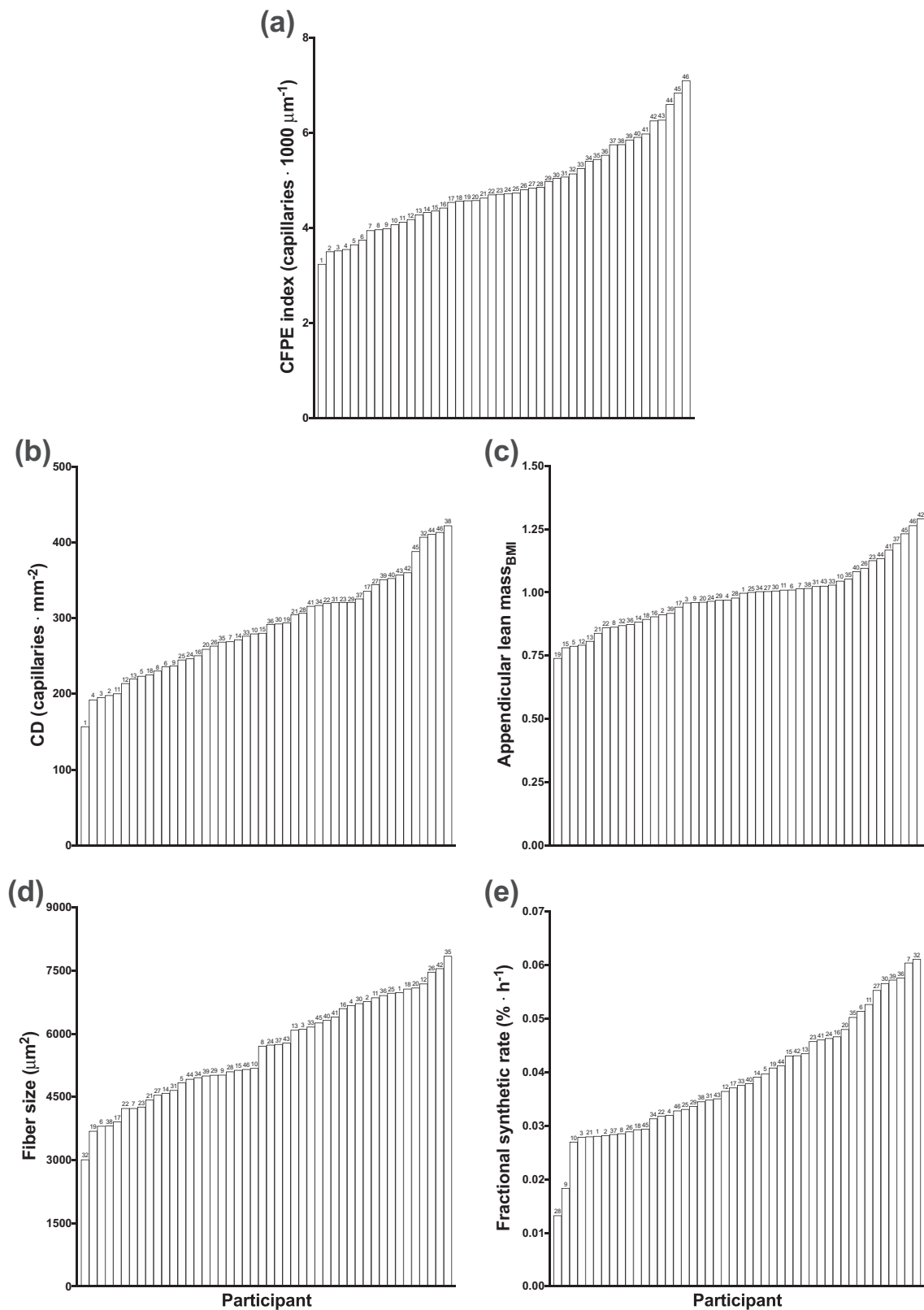


Fig. 1. Histograms of the individual values for capillary-to-fiber perimeter exchange (CFPE) index (A), capillary density (CD) (B), appendicular lean mass divided by body mass index (BMI) (C), fiber size (D), and post-prandial muscle protein synthesis, expressed as fractional synthetic rate calculated based on the plasma precursor pool (E). Numbers above the bars represent the individual participants (A) and match with the same participant number presented in panels B-E. Numbers were given to the participants based on their CFPE index value, with low numbers representing low CFPE index.

Table 2
Associations between skeletal muscle fiber capillarization and lean tissue mass, muscle fiber characteristics, and post-absorptive muscle protein synthesis rates.

	CC	C/Fi	CD
Whole body level			
Whole-body lean mass	0.39**	0.43**	-0.02
Appendicular lean mass	0.46**	0.52***	0.10
Appendicular lean mass _{BMI}	0.52***	0.65***	0.45**
Muscle protein synthesis			
FSR _{IC} precursor (%·h ⁻¹)	0.01	0.08	0.10
FSR _{plasma} precursor (%·h ⁻¹)	-0.02	0.02	0.15

Statistically significant Pearson (r) correlation: *P < 0.05, **P < 0.01, ***P < 0.001. CC, capillary contacts; C/Fi, capillary-to-fiber ratio; BMI, body mass index; FSR, fractional synthetic rate; IC, intracellular.

not significantly correlated with mixed muscle fiber type CFPE index (Table 3).

3.4. Muscle fiber type distribution vs capillarization

No significant correlation was observed between the proportion of type I muscle fibers and CC (Table 3). The proportion of type I muscle fibers was significantly, but weakly, correlated with C/Fi (P < 0.05; Table 3), CFPE index (P < 0.01; Table 3) or CD (P < 0.05; Table 3). Significant weak correlations were observed between the proportion of the cross-sectional area occupied by type 1 muscle fibers and C/Fi, CFPE index or CD (all P < 0.05; Table 3). No significant correlation was observed between the proportion of the cross-sectional area occupied by type 1 muscle fibers and CC (Table 3).

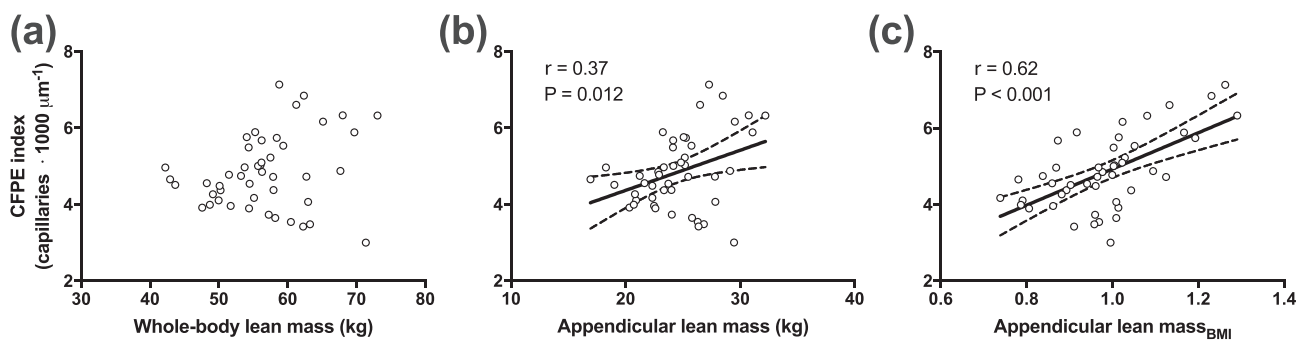


Fig. 2. Associations between muscle fiber capillary-to-fiber perimeter exchange (CFPE) index and various indices of lean body mass. Pearson correlations (r) between CFPE index and **A)** whole-body lean mass, **B)** appendicular lean mass, and **C)** appendicular lean mass divided by body mass index (BMI). Solid lines represent linear regression, dashed lines represent the 95% confidence interval.

Table 3
Associations between muscle fiber type-specific capillarization and muscle fiber characteristics.

	CC			C/Fi			CFPE index (capillaries-1000 μm ⁻¹)			CD (capillaries-mm ⁻²)		
	Type 1	Type 2	Mixed	Type 1	Type 2	Mixed	Type 1	Type 2	Mixed	Type 1	Type 2	Mixed
Proportion of type 1 fibers (%)	-	-	r = 0.12	-	-	r = 0.34*	-	-	r = 0.38**	-	-	r = 0.31*
CSA occupied by type 1 fibers (%)	-	-	r = 0.08	-	-	r = 0.29*	-	-	r = 0.36*	-	-	r = 0.33*
Fiber size (μm ²)												
Type 1	ρ = 0.48**	-	-	ρ = 0.43**	-	-	ρ = -0.04	-	-	ρ = -0.46**	-	-
Type 2	-	r = 0.45**	-	-	r = 0.47**	-	-	r = -0.03	-	-	r = -0.51***	-
Mixed	-	-	r = 0.50***	-	-	r = 0.45**	-	-	r = 0.03	-	-	r = -0.44**
Myonuclei (n-fiber ⁻¹)												
Type 1	ρ = 0.40**	-	-	ρ = 0.50***	-	-	ρ = 0.15	-	-	ρ = -0.16	-	-
Type 2	-	r = 0.36*	-	-	r = 0.44**	-	-	r = 0.06	-	-	r = -0.26	-
Mixed	-	-	r = 0.35*	-	-	r = 0.40**	-	-	r = 0.07	-	-	r = -0.23
Myonuclear domain (μm ²)												
Type 1	r = 0.12	-	-	r = -0.08	-	-	ρ = -0.16	-	-	r = -0.33*	-	-
Type 2	-	r = 0.29	-	-	r = 0.22	-	-	r = -0.11	-	-	r = -0.48**	-
Mixed	-	-	r = 0.27	-	-	r = 0.14	-	-	r = -0.06	-	-	r = -0.37*

Statistically significant Pearson (r) or Spearman Rank (ρ) correlation: *P < 0.05, **P < 0.01, ***P < 0.001. CC, capillary contacts; C/Fi, capillary-to-fiber ratio; CFPE, capillary-to-fiber perimeter exchange; CD, capillary density; Type 1, type 1 muscle fibers; Type 2, type 2 muscle fibers; Mixed, mixed muscle fiber types; CSA, cross-sectional area.

3.5. Myonuclear content and domain size vs capillarization

Significant moderate positive correlations were observed between type 1 muscle fiber myonuclear content and type 1 muscle fiber CC or C/Fi ($P < 0.01$ and $P < 0.001$, respectively; Table 3). A weak and moderate positive correlation was observed between type 2 muscle fiber myonuclear content and type 2 muscle fiber CC ($P < 0.05$; Table 3) or C/Fi ($P < 0.01$; Table 3), respectively. No significant correlation was observed between type 1 or 2 muscle fiber myonuclear content and CFPE index (Table 3). No significant correlations were observed between type 1 muscle fiber myonuclear domain size and type 1 muscle fiber CC, C/Fi or CFPE index (Table 3). However, a weak negative correlation was observed between type 1 muscle fiber myonuclear domain size and type 1 muscle fiber CD ($P < 0.05$; Table 3). When expressed as mixed fiber type, a weak and moderate positive correlation was observed between mixed muscle fiber myonuclear content and CC ($P < 0.05$; Table 3) or C/Fi ($P < 0.01$; Table 3), respectively. Mixed muscle fiber myonuclear content did not correlate with mixed CFPE index or CD (Table 3). Type 2 muscle fiber myonuclear domain size tended ($P = 0.051$) to positively correlate with type 2 muscle fiber CC, while no significant correlations were observed with type 2 muscle fiber C/Fi or CFPE index (Table 3). A moderate negative correlation was observed between type 2 muscle fiber myonuclear domain size and type 2 muscle fiber CD ($P < 0.01$; Table 3). No significant correlations were observed between mixed myonuclear domain size and mixed muscle fiber C/Fi or CFPE index (Table 3), while a tendency was observed with mixed CC ($P = 0.091$; Table 3). A weak negative correlation was observed between mixed muscle fiber myonuclear domain size and CD ($P < 0.05$; Table 3).

3.6. Post-absorptive muscle protein synthesis rate vs capillarization

Post-absorptive muscle protein synthesis rates calculated based on intracellular precursor pool ranged between and 0.023 and 0.099%·h⁻¹ in healthy older men. No significant correlation was observed between post-absorptive muscle protein synthesis rates, based on plasma or IC precursor, with any indices of muscle fiber capillarization (Table 2 and Fig. 3AB).

4. Discussion

The present study shows that muscle fiber capillarization is associated with skeletal muscle mass on a whole body and muscle fiber level. In addition, this study is the first to show that specific muscle fiber capillarization indices are associated with myonuclear content and domain size but not with post-absorptive muscle protein synthesis rates in a large group of healthy, older men.

Skeletal muscle tissue perfusion is limited by the surface area of the

muscle fiber microvascular bed (Pittman, 1995). Hence, the importance of the capillary network for muscle maintenance, function, and health is becoming increasingly clear (Joanisse et al., 2017; Nederveen et al., 2017; Parise et al., 2019). Here, we show clear associations between various indices of muscle fiber capillarization and appendicular lean tissue mass in older individuals. These results are in line with the study of Prior and colleagues (Prior et al., 2016), showing a strong association between capillary to fiber ratio (C/Fi) and ALM_{BMI}, from which the latter is known to be an indicator of sarcopenia (Cawthon et al., 2014). We extend on these findings with equally strong associations between ALM_{BMI} and CD ($r = 0.45$, $P < 0.01$) or CFPE index ($r = 0.62$, $P < 0.001$), which have been suggested to be better representatives of muscle fiber perfusion capacity based on the anatomical structure of the capillary network (Hepple, 1997). Whereas CD has been suggested to relate mostly to metabolic substrate delivery (Lithell et al., 1981) and waste product removal (Tesch and Wright, 1983), CFPE index is suggested to reflect the oxygen flux capacity towards the skeletal muscle fiber (Hepple et al., 1997). These strong associations between measures of muscle mass and capillarization support the growing body of evidence that the substrate, in combination with oxygen, delivery may be a key factor in muscle mass maintenance (Joanisse et al., 2017; Nederveen et al., 2017; Parise et al., 2019). Interestingly, whereas in the study of Prior et al. (2016) the relationship between muscle fiber capillarization and ALM_{BMI} was observed by including both sarcopenic and non-sarcopenic older men and women, the present study shows similar results in a more homogenous group of healthy, mostly non-sarcopenic older men. Together these results provide further evidence that the muscle fiber capillary network may already be of major importance in muscle mass maintenance before sarcopenia becomes apparent in older adults.

On the muscle fiber level, aging is characterized by type II muscle fiber type specific atrophy (Nilwik et al., 2013), which is accompanied by a concomitant decline in myonuclear number (Snijders et al., 2020). In line with previous studies (Barnouin et al., 2017; Gavin et al., 2015; Croley et al., 2005), we show that muscle fiber size, both mixed as well as fiber type specific, is positively associated with CC and C/Fi suggesting that individuals with larger muscle fibers also have greater number of capillaries. However, we also observed a negative association between muscle fiber size and CD. Although this may seem contradictory, it has previously been suggested that the capillary network adapts to the fiber perimeter as opposed to the fiber area (Hepple, 1997; Poole and Mathieu-Costello, 1996). This is further substantiated by studies showing a rapid drop in oxygen pressure within the first few microns away from the red blood cell into the muscle fiber (Honig et al., 1992), which is attributed to the presence of metabolically active organelles in close proximity to the sarcolemma as opposed to being homogeneously distributed throughout the sarcoplasm (Pathi et al., 2012). Further

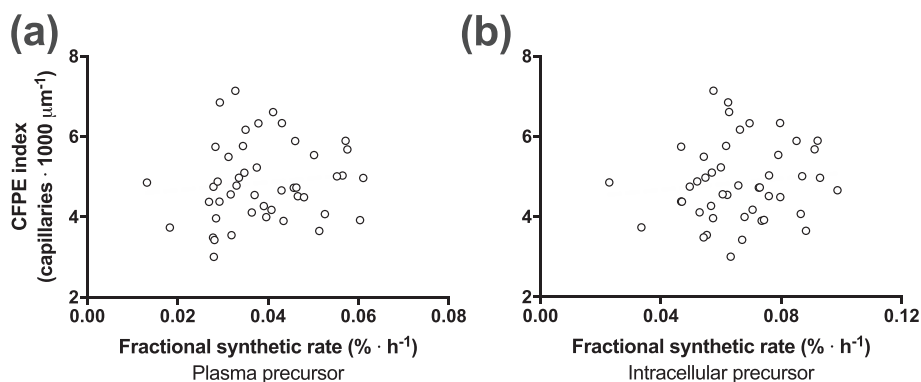


Fig. 3. Associations between muscle fiber capillary-to-fiber perimeter exchange (CFPE) index and post-absorptive muscle protein synthesis rates. Pearson correlations (r) between CFPE index and **A)** post-absorptive fractional synthetic rate calculated on the basis of the plasma precursor pool, **B)** post-absorptive fractional synthetic rate calculated on the basis of the intracellular precursor pool.

supporting this concept, we observed no significant correlation between muscle fiber size and CFPE index, indicating that capillary supply is similar between individuals with relatively small or large muscle fibers when corrected for fiber perimeter. Yet, the lack of any associations between CFPE index and type I, type II, or mixed muscle fiber size appears to contradict the positive correlations between CFPE index and ALM or ALM_{BMI} . However, ALM_{BMI} corrects for BMI and therefore shows, for example, lower values for individuals with a high BMI due to greater levels of fat mass (instead of lean mass). Thus, ALM_{BMI} takes leanness into account, while this is not the case for muscle fiber size. On the other hand, ALM would be expected to be more closely related to muscle fiber size. This discrepancy may be related to the correction for fiber perimeter when calculating CFPE index, which is directly related to the muscle fiber size (i.e. cross-sectional area) but not to ALM. In agreement, the correlations between CC or C/Fi and muscle fiber size or ALM are in line with each other.

The delivery of oxygen and metabolic substrates supports the transcription and translation machinery prerequisite for muscle fiber maintenance, as well as repair and growth (Allen et al., 1999). These processes are, in part, regulated by the large number of nuclei located at the periphery of the muscle fibers (Allen et al., 1999). It has been hypothesized that every myonuclei controls a certain volume of the muscle fiber cytoplasm, expressed as a ratio between muscle fiber size and myonuclear content and referred to as the myonuclear domain (Hall and Ralston, 1989). In general, a linear relationship is present between muscle fiber size and myonuclear content in humans (Allen et al., 1999). Previous studies have indicated that the loss in muscle fiber size occurs at a faster rate compared with age-related loss in myonuclear number, resulting in the typical observation of smaller myonuclear domain size in (frail) older adults (Snijders et al., 2020; Kramer et al., 2017). This may suggest that older individuals with a relatively small myonuclear domain size have suffered more from age-related muscle fiber atrophy and are at risk of developing sarcopenia. The observation of a negative correlation between CD and myonuclear domain size supports the current hypothesis that substrate delivery may be a key limiting factor in muscle tissue maintenance with aging. However, this remains speculation and additional research is warranted to further investigate the potential functional consequence of the observed negative relationship between muscle fiber capillarization and myonuclear content or domain size in humans.

Relevant gains or losses in skeletal muscle mass are attributed to a persistent change in muscle protein synthesis rates, breakdown rates or a combination of both. It is generally accepted that alterations in muscle protein synthesis play a more significant role than muscle protein breakdown in mediating long-term changes in muscle mass in healthy humans (Trommelen et al., 2019). As skeletal muscle is in a post-absorptive state for a considerable part of the day, subtle differences in post-absorptive muscle protein synthesis may become clinically relevant when calculating their impact over one or more decades before sarcopenia becomes apparent. Microvascular blood flow has been hypothesized to be an important contributing factor in the regulation of protein synthesis (Timmerman et al., 2010a; Timmerman et al., 2010b). This suggestion is, however, mostly based on acute changes in arterial and/or microvascular blood flow (Timmerman et al., 2010a; Timmerman et al., 2010b). Previously, Moro et al. (2019) reported no differences in post-absorptive muscle protein synthesis rates between two relatively small groups of older adults with either low or high muscle fiber capillarization based on CFPE index. The present study is the first to show no direct associations between various indices of muscle fiber capillarization and post-absorptive muscle protein synthesis rates in large homogenous group of healthy older men. The anatomical structure of the microvascular network (i.e. its potential perfusion capacity), therefore, does not seem to be a factor compromising post-absorptive muscle protein synthesis rates in senescent muscle. However, the structure and function of the microvascular network may become restrictive to muscle protein synthesis rates under conditions where

microvascular flow is greatly increased, such as following food ingestion (Phillips et al., 2015) or during and immediately after exercise (Hildebrandt et al., 2017). Additional research is warranted to assess the relationship between the anatomical structure and function of the muscle fiber capillary network and the capacity to increase post-prandial and post-exercise muscle protein synthesis rates in both young and older individuals.

As the present study is cross-sectional in nature, we cannot draw conclusions on cause and effect. However, it does provide insight in the temporal relationships between changes in capillarization and muscle mass or fiber characteristics in older adults. Animal studies have previously shown that endothelial apoptosis, capillary loss and/or impaired capillary function precede the decline in muscle mass (Vescovo et al., 1998; Wang et al., 2014). Furthermore, capillary proliferation seems to be a key factor for increasing muscle mass, as increasing capillarization is associated with increases in muscle mass in animals (Leiter et al., 2012). In support, muscle fiber repair and regeneration has been suggested to be enhanced with increased capillarization in both animal and human muscle tissue (Joanisse et al., 2016; Nederveen et al., 2018). Furthermore, it has been reported that CD does not significantly differ between sarcopenic and non-sarcopenic older adults, whereas CC and C/Fi were found to be significantly lower in sarcopenic individuals (Prior et al., 2016). This may suggest that muscle fiber atrophy either precedes or occurs simultaneously with capillary rarefaction in older adults. Together with the observations in the present study, it implies that older adults will likely benefit from augmenting the muscle fiber anatomical capillary network to support muscle tissue maintenance and/or facilitate muscle hypertrophy. An effective way to increase capillarization is aerobic-type exercise training, which shows more consistent angiogenic effects in the older population as compared to resistance-type exercise training (Snijders et al., 2017b; Gavin et al., 2007). Therefore, there may be a greater need to consider aerobic-type exercise training as a part of (resistance) exercise, nutrition and/or pharmacological interventions designed to increase muscle mass in older adults. This is also very relevant for disease populations that are known to have compromised skeletal muscle microvasculature, such as type 2 diabetic (Groen et al., 2014) and chronic obstructive pulmonary disease patients (Gouzi et al., 2013).

In conclusion, muscle fiber capillarization is associated with measures of skeletal muscle mass and muscle fiber characteristics in a large group of healthy older adults. This provides further evidence on the importance of the structure and function of the microvascular network in the regulation of skeletal muscle mass maintenance and suggests that targeting the microvascular network for intervention could be of particular benefit to support healthy aging.

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Author statement

Betz MW: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization, Project administration. **Aussieker T:** Investigation, Resources, Writing – Review & Editing. **Kruger CQ:** Investigation, Resources, Writing – Review & Editing. **Gorissen SHM:** Conceptualization, Methodology, Investigation, Writing – Review & Editing, Project administration. **van Loon LJC:** Conceptualization, Methodology, Writing – Review & Editing, Supervision. **Snijders T:** Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration.

Conflict of interest and funding disclosure

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analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion. No other conflicts of interest, financial or otherwise, are reported by any of the authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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