Effects of ageing, a high-fat diet and physical exercise on skeletal muscle morphology

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EFFECTS OF AGEING, A HIGH-FAT DIET AND PHYSICAL EXERCISE ON SKELETAL MUSCLE MORPHOLOGY

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‘Patience can't be acquired overnight.
It is just like building up a muscle.
Every day you need to work on it’.

Eknath Easwaran

(1910-1999)

ABSTRACT

Age-related loss of skeletal muscle mass and strength can lead to reduced independence, quality of life and life expectancy, which may be exacerbated by an increased high-fat intake and a low physical exercise. This thesis investigated the effects of ageing, high-fat diet (HFD) and regular physical training on muscle morphology. Specifically, in study I, we compared intramyocellular lipid (IMCL) levels, capillarisation, fibre type and size, and oxidative capacity of fibres in locomotor (soleus and EDL) and respiratory (diaphragm) muscles in 20- (young-adult) and 79-week-old (early ageing) mice. Early ageing was characterised by an absence of muscle wasting in soleus, the EDL atrophied while the diaphragm hypertrophied without changes in the capillary numbers supplying a fibre, or their oxidative capacity.

In study II, we studied the effects of a HFD on the morphology of the soleus, EDL and diaphragm in 20- and 79-week-old mice. Old mice were more susceptible to morphological alterations with a HFD compared to young mice. All fibre types showed similar adaptations in response to a HFD but they were muscle-specific with the EDL being least responsive.

In study III, we assessed fibre type grouping in the vastus lateralis of athletes and non-athletes (19 - 85 years old) and evaluated to what extent any observed grouping, indicative of cycles of denervation and reinnervation following motor neuron loss, is more than expected from the fibre type composition of the muscle. Since regular physical exercise may stimulate fibre reinnervation, we hypothesised that master athletes have larger fibre groups than age-matched non-athletes. An ‘enclosed fibre’ was any muscle fibre of a given type surrounded by fibres of the same type only. A ‘fibre group’ was defined as a group of fibres with at least one enclosed fibre. The prevalence of observed fibre type grouping was similar to that expected from the fibre type composition. No age-related effect on group size and group number in athletes or non-athletes was found.

In conclusion, the current thesis described the morphological changes of CD-1 mouse skeletal muscles during ageing as muscle specific. Additionally, using the same mouse model, HFD-induced muscle morphological alterations depending on diet duration and age, varied between muscles. Moreover, the results of the current thesis do not show evidence for improved reinnervation of muscle fibres with regular physical training. Nevertheless, histological examination may not provide the full extent of ageing related motor unit remodelling.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers:

I. **Messa, G. A. M.,** Piasecki, M., Hill, C., McPhee, J. S., Tallis, J. and Degens, H. (2019) 'Morphological alterations of mouse skeletal muscles during early ageing are muscle specific.' Exp Gerontol, Aug 7, 2019/08/11, p. 110684.

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CONFERENCE PRESENTATIONS

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LIST OF ABBREVIATIONS

AP-1: activator Protein-1

BB: biceps brevis

BMI: body mass index

CFD: capillary fibre density

CRP: c-reactive protein

CT: computed tomography

DEXA: dual-energy X-ray absorptiometry

DHPR: dihydropyridine receptors

EDL: extensor digitorum longus

ETC: electron transport chain

FCSA: fibre cross-sectional area

HFD: high-fat diet

HSF-1: heat shock factor-1

IGF-1: insulin-like growth factor-1

IMCL: intramyocellular lipid

IRS: insulin receptor substrate

LCFR: local capillary to fibre ratio

MAPK: mitogen-activated protein kinase

MHC: myosin heavy chain

MND: myonuclear domain

mtDNA: mitochondria deoxyribonucleic acid

mTORC2: mammalian target of rapamycin complex 2

MU: motor unit

MUNE: motor unit number estimation

NCAM: neuronal cell adhesion molecule

NF-κB: nuclear Factor-kappa B

Nrf2: nuclear factor erythroid 2-related factor 2

PDK1: 3-phosphoinositide-dependent kinase 1

PtdIns: Phosphatidylinositol

PtdIns (3,4,5)P3: Phosphatidylinositol 3,4,5-trisphosphate

RNS: reactive nitrogen species

ROS: reactive oxygen species

SCs: satellite cells

SDH-INT: succinate dehydrogenase integrated

SDH-OD: succinate dehydrogenase optical density

SKMDCs: skeletal muscle-derived cells

SR: sarcoplasmic reticulum

STA-MUNE: spike triggered averaging motor unit number estimation

TA: tibialis anterior

T2DM: type II diabetes mellitus

TnC: troponin C

TNF-α: Tumour necrosis factor alpha

TnI: troponin I

TnT: troponin T

VEGF: vascular endothelial growth factor

VL: vastus lateralis

CHAPTER 1

Introduction and Literature Review

1.1 General introduction

Age-related atrophy is accompanied by a series of morphological alterations that are associated with impairment of skeletal muscle function, including slowing of movement and muscle weakness. Consequently, older people suffer from increased fall risk, institutionalization, co-morbidity and premature death, which reduce the quality of life of the older persons and increases healthcare expenditures (McPhee et al., 2016; Musich et al., 2018). Mounting evidence suggests that not only muscle strength, but also muscle endurance decreases with advanced age (Ballak et al., 2015). Two important contributors of muscle endurance or muscle fatigue (the ability to maintain force output during contractions) are mitochondrial content (termed 'oxidative capacity') and the capillary network (Degens and Veerkamp, 1994). Age-related muscle mass loss and a decreased mitochondrial content, mitochondrial function and fatty acid oxidation capacity, contribute to the development of insulin resistance and type 2 diabetes (Rovira-Llopis et al., 2017; Szendroedi et al., 2011). In addition, it has been suggested that part of the development of insulin resistance could be caused by an accumulation of lipids in skeletal muscle fibres stored as intramyocellular lipid (IMCL) (Hancock et al., 2008; Montgomery et al., 2017; Bonen et al., 2015). Lipids accumulate in skeletal muscle fibres when energy intake exceeds the storage capacity of adipose tissue, and when fatty acid oxidation capacity cannot cope with elevated fatty acid uptake (Manco et al., 2004; Savage et al., 2007).

Master athletes maintain high levels of physical activity (Degens et al., 2013; Hannam et al., 2017) and are therefore considered a good model to disentangle the effects of reduced physical activity and ageing *per se* on skeletal muscle (Rittweger et al., 2009; Degens et al., 2013). The age-related reduction of muscle mass, which is thought to be partly attributable to a progressive loss of motor neurons, has been associated with reduced muscle fibre number and size, and repeating cycles of denervation and reinnervation resulting in fibre type grouping and expansion of surviving motor units (MUs) (Kanda and Hashizume, 1989; Lexell and Downham, 1991; Piasecki et al., 2016a; Rowan et al., 2012). However, the evidence is equivocal as to whether physical activity protects against ageing-related motor neuron loss and enhances reinnervation. The overall aim of this thesis was to define and gain a better understanding of the impact of ageing, a high-fat diet (HFD) and regular physical activity on the ageing-associated morphological changes in skeletal muscle.

1.2 Structure and function of skeletal muscle

1.2.1 Introduction

Skeletal muscle is the largest organ of the human body, comprising around 40% of its total body weight and 50-75% of all body proteins. It contains 75% water, 20% proteins and 5% other molecules such as inorganic salts, minerals, fat and carbohydrates (Frontera and Ochala, 2015). Skeletal muscle mass is usually regulated by the balance between protein synthesis and degradation, and both processes are influenced by factors such as ageing, dietary status, hormonal balance, physical activity/exercise and injury or disease. From a mechanical perspective, skeletal muscle is responsible for converting chemical energy into mechanical energy to generate force and power, maintain posture and produce movements. From a metabolic viewpoint, skeletal muscle has various roles including a contribution to glucose homeostasis, the production of heat for the maintenance of body temperature and the uptake of most oxygen and fuel used during physical activity and exercise. Different techniques have been used to quantify muscle mass, measure body composition and study muscle function non-invasively. These techniques comprise the measurement of urinary creatine concentration, ultrasonography, whole body counting-neutron activation, computed tomography (CT), magnetic resonance spectroscopy, bioelectrical impedance and dual-energy X-ray absorptiometry (DEXA) (Heymsfield et al., 2014).

1.2.2 Basic structure of skeletal muscle

A skeletal muscle consists of a well-defined arrangement of muscle fibres (also called myofibres or muscle cells) and associated connective tissue. In healthy individuals, the size of a muscle is determined by the number and size of individual muscle fibres. Skeletal muscle fibres are multinucleated cells, and each nucleus within a muscle fibre regulates the type of protein synthesised in that specific region of the cell. These specific regions are called nuclear domains and have a highly controlled, but not constant, size (Hikida, 2011). This characteristic of skeletal muscle fibres has led to a longstanding belief that a given nucleus controls a defined volume of cytoplasm, so that when a muscle grows (hypertrophy) or shrinks (atrophy) the number of nuclei change in proportion to size accordingly. The myonuclear domain (MND) hypothesis brought to light the theoretical idea that a single nucleus "supports" a determined amount of cytoplasm in order to grant the functionality of the entire fibre (Hall and Ralston, 1989). Satellite cells, the adult stem cells of skeletal muscle, are located between the sarcolemma and the basal lamina. While it is

widely accepted that hypertrophy of a muscle fibre is typically associated with a gain of new nuclei by the fusion of satellite cells (SCs) (Moss, 1968; Cabric and James, 1983; Egner et al., 2016; Van der Meer et al., 2011), the loss of nuclei by apoptosis/necrosis in atrophying muscle fibres has been controversial (Schwartz, 2018). Nevertheless, the SCs remain quiescent until activation by myogenic factors, after that they proliferate, differentiate and fuse with pre-existing fibres to create new nuclei (Bareja et al., 2014).

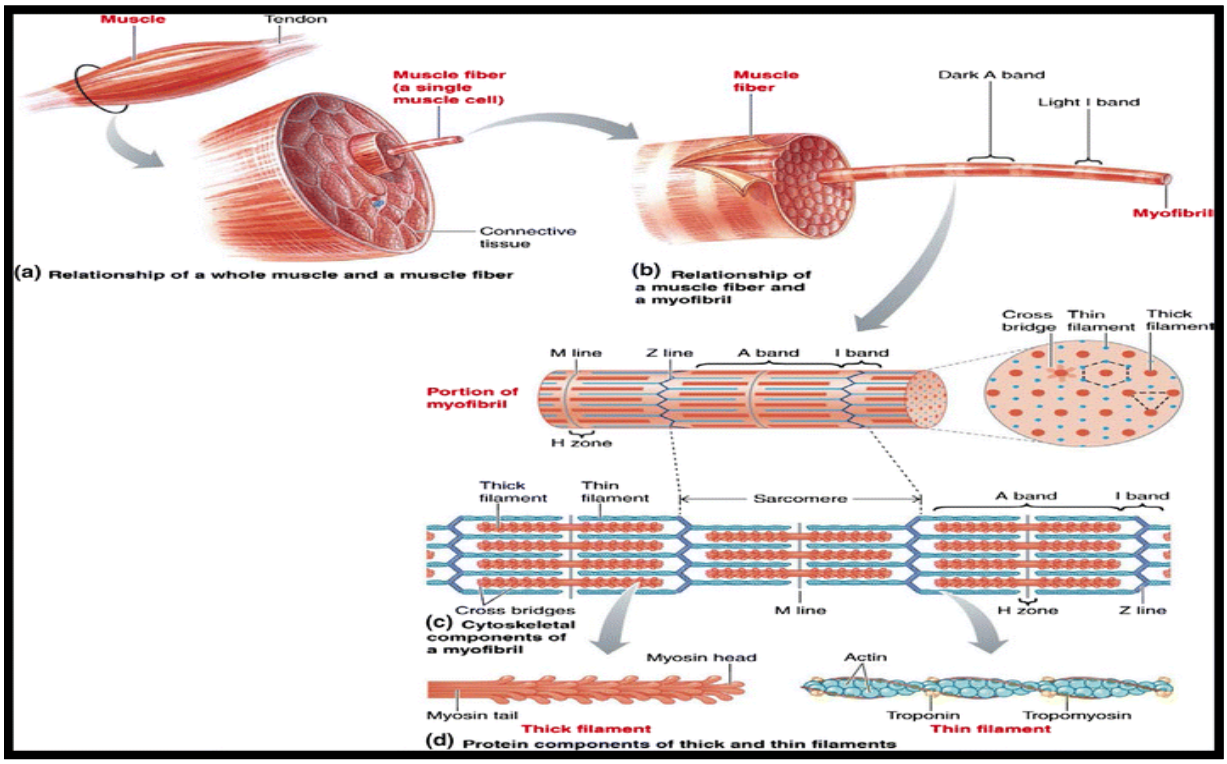


Figure 1.1 Structure of skeletal muscle (From: Frontera and Ochala (2015), Fig. 3)

1.2.2 Proteins and excitation-contraction (E-C) coupling in skeletal muscle

Skeletal muscle fibres are made up predominantly (~ 80%) of protein (contractile, regulatory, cytoskeletal) and sarcoplasm (~ 8%), not comprising their water content (Hoppeler et al., 1973). Each muscle fibre, which arises from fusion of many myoblasts, contains several hundred to several thousand myofibrils and billions of myofilaments (Fig. 1.1) (McCuller and Callahan, 2019). Myofilaments are assembled together in a well-defined pattern to form sarcomeres, the basic contractile units of skeletal muscle. Actin and myosin are the two most abundant myofilaments (proteins). Myosin is the prototype molecular motor and the myosin superfamily encodes 18 distinct classes of myosin motors, which are found ubiquitously in eukaryotes (Hartman and Spudich, 2012). The calcium-dependent troponin complex (including TnT, TnI and TnC) and tropomyosin are regulatory proteins associated with the

actin filament and play important roles in the activation process that results in myofilament sliding and force generation. Titin is an elastic protein, which is attached to the Z disk of the sarcomere and to myosin contributing to the stabilisation and alignment of the thick filament.

Excitation-contraction (E-C) coupling consists of a specific sequence of physiological events involved between the time of the generation of action potential on the muscle sarcolemma (plasma membrane) and the actual contraction. The muscle action potential spreads radially into the muscle fibre via invaginations of the sarcolemma that form the transverse tubular system (T-tubule system). As the action potential encounters dihydropyridine receptors (DHPR) in the T tubules, it causes the opening of the ryanodine calcium channels of the lateral sac of the sarcoplasmic reticulum (Rios and Brum, 1987), allowing calcium ions to diffuse out of the lateral sacs and into the region of the myofibrils. The Ca^{2+} released into the sarcoplasm then binds to the regulatory protein troponin C on the actin filament causing a conformational change in tropomyosin that exposes a myosin-binding site on the actin filament. Muscle contraction is generated by actin and myosin interactions that form the cross-bridges, whereby the heads of the myosin chains undergo a conformational change by ATP hydrolysis causing the thin actin filament to slide past the thick myosin filaments and shortens the muscle in a process referred to as the sliding filament mechanism.

1.2.3 Types of skeletal muscle fibre

Skeletal muscle is a heterogeneous organ containing different types of fibres with different characteristic movement rates, responses to neural inputs, and metabolic activities (Schiaffino and Reggiani, 2011; Bassel-Duby and Olson, 2006). To date, muscle fibres have been characterised based on 1) the colour of the muscle fibres (red vs. white) that relates with myoglobin, 2) the speed of contraction during a single twitch (fast vs. slow), 3) the degree of fatigability through sustained activation (fatigable vs. fatigue-resistant), 4) the predominance of particular metabolic or enzymatic pathways (oxidative vs. glycolytic), among others (Lamboley et al., 2014; Galpin et al., 2012). Skeletal muscle fibres have been regularly classified based on differential myosin heavy chain (MHC*)* gene expression as type I (slow-twitch) and type II fast-twitch. Within fast-twitch fibres, there are three major subtypes 2a, 2x, and 2b, although adult human skeletal muscles do not appear to have MHC expressing type 2b fibres (Schiaffino and Reggiani, 2011). With the use of protein electrophoresis or immune-reactivity to antibodies, it has been shown that a single muscle fibre may express more than one type of MHC (e.g. 2a and 2x or 2x and 2b together). These hybrid fibres have

been demonstrated to increase with exercise and ageing (Andersen, 2003; Klitgaard et al., 1990a).

1.2.4 Age-related loss of muscle mass and strength

One of the most striking effects of ageing is a loss of muscle mass, termed sarcopenia for the first time by Rosenberg (Rosenberg, 1997; Rosenberg, 1989). During periods of physical inactivity, the atrophy of muscle is substantially accelerated, as reflected by an approximately 1 kg of muscle mass loss in 10 days reported in immobilisation and bed rest studies (Kortebein et al., 2007; English and Paddon-Jones, 2010; Verdijk et al., 2014; Wall et al., 2013). This considerable loss of skeletal muscle mass leads to a substantial decrease in strength (Wall and van Loon, 2013; Wall et al., 2013), showing that extended periods of muscle disuse atrophy accelerate the decline in muscle performance, increasing the risk of physical disability at later life (Guralnik et al., 1994; Berger and Doherty, 2010; Fielding et al., 2011).

1.2.5 Age-related loss of muscle fibres and decrease in fibre size

The progressive age-associated loss of muscle mass, which has been attributed to a loss of muscles fibres and atrophy of the surviving fibres (Lexell et al., 1988; Larsson and Ansved, 1995; Faulkner et al., 2007; McPhee et al., 2018), enormously contributes to the loss of muscle power (force x velocity) (Lexell, 1995; Budui et al., 2015). A post-mortem study has indicated that in the *vastus lateralis* (VL) muscle there is a 30% loss of muscle mass by the age of 70 years, of which about one third is due to loss of muscle fibres, while the other 20% is attributed to fibre atrophy (Lexell and Downham, 1991). These observations were in line with other studies indicating substantially fewer fibres in the muscles from elderly people than young adults (Sjostrom et al., 1992; Lexell et al., 1988; Lexell et al., 1983b; Lexell et al., 1986). However, this is equivocal, as some investigators reported no age-related loss of muscle fibre in the VL (Nilwik et al., 2013) or biceps *brachii* (Klein et al., 2003). Of note, the number of fibres from the latter studies were estimated indirectly contrary to Lexell and collaborators (Lexell et al., 1988). The reasons of the discrepancy between indirect and direct determinations of muscle fibre number is uncertain but could include difference in activity level.

No studies have reported the effects of ageing on muscle fibre number in CD-1 female mice. Nevertheless, in C57BL/6 mice, a 10-15 % decrease in fibre number have been observed

in *extensor digitorum longus* (EDL) and *soleus* muscles of 24-27-month-old compared to 2-6-month-old mice (Sheard and Anderson, 2012; Zerba et al., 1990). Ballak et al. (2014b), by contrast, found no indication of fibre loss in plantaris muscle of 25- compared to 9-month-old mice of the same strain. The diaphragm C57BL/6 mice showed an age-related increase in the proportion of type IIa fibres with a concomitant decrease for type IIx and/or IIb fibres, and it has been suggested that this age-related reduction in the percentage of type IIx and/or IIb fibres may be attributable to the loss of these diaphragm fibres (Greising et al., 2013).

In humans, type II fibre cross-sectional area (FCSA) decrease with age while type I fibres appear to maintain their cross-sectional area in VL (Aniansson et al., 1980; Grimby et al., 1982; Larsson, 1978; Lexell et al., 1988; Jennekens et al., 1971; Aniansson et al., 1986; Oertel, 1986; Tomonaga, 1977; Barnouin et al., 2017; Verdijk et al., 2007; Martel et al., 2006; Snijders et al., 2009; McPhee et al., 2018). Similarly, there was an age-related decrease in FCSA in the quadriceps femoris (Overend et al., 1992; Klitgaard et al., 1990b), brachial biceps (Klitgaard et al., 1990b; Rice et al., 1989), triceps (Rice et al., 1989) and plantar flexor muscles (Rice et al., 1989). This loss in fibre size has been associated with an age-related decrease in growth factors (Barton-Davis et al., 1999), as well as a lack of mechanical stimuli for the fast twitch fibre. This is relevant because satellite cells are particularly reduced in type II skeletal muscle fibres in elderly (Verdijk et al., 2007). In animals, only one study assessed the effect of ageing on FCSA in female CD-1 mice in the gastrocnemius muscle and a reduction in FCSA in 22- vs. 6- month-old female CD-1 mice was found (Wagatsuma, 2006). In the plantaris muscle of C57BL6 mice, however, no age-related difference in FCSA was observed in 25-month-old compared to 9-month-old mice (Ballak et al., 2014b), as also seen by others in the extensor digitorum longus and soleus muscles of the same strain (Sheard and Anderson, 2012), suggesting that different muscles may show different age-related changes in morphology.

1.2.6 Mechanisms underlying sarcopenia in ageing

Extensive interest has been focused on the mechanisms underlying muscle fibre atrophy and muscle function in older people (Lexell, 1995; Lexell et al., 1995; Porter et al., 1995; Ryall et al., 2008; Bonaldo and Sandri, 2013; Gao et al., 2018). At physiological levels, reactive oxygen species (ROS) and /or reactive nitrogen species (RNS) play an important part in redox signalling and cell survival through activation or inhibition of various enzymes including mitogen-activated protein kinase (MAPK), phosphatases, and gene-dependent

cascades (Baumann et al., 2016; Altenhofer et al., 2015). An excess production of ROS and /or RNS observed in aged muscles have been indicated to play a part in the mechanisms underlying age-related skeletal muscle changes and sarcopenia (Vasilaki et al., 2006; Jackson and McArdle, 2016; Altenhofer et al., 2015). A skeletal muscle of old organisms also contains substantial amounts of oxidative damage to lipids, DNA and proteins compared to younger organisms (Vasilaki et al., 2006), and the repair systems, such as the proteasomal degradation of damaged proteins are compromised in aged muscles (Baumann et al., 2016). Based on experiments of global gene expression profiles in skeletal muscles of rat, Ibebunjo et al. (2013) demonstrated that sarcopenia is associated with loss of functional proteins associated with mitochondrial energy metabolism. Mitochondrial DNA (mtDNA) mutations accumulate with ageing (Park and Larsson, 2011; Joseph et al., 2016), and it has been reported that these mutations are triggered by ROS originated from the electron transport chain (Lightfoot et al., 2014). In aged skeletal muscle, ROS generated during contractile activity stimulate the activation of a number of redox-regulated transcription factors including Nuclear Factor-kappa B (NF-κB), Activator Protein-1 (AP-1), Heat Shock Factor-1 (HSF-1) and nuclear factor erythroid 2 -related factor 2 (Nrf2) (Ji et al., 2004; Vasilaki et al., 2006; Ristow et al., 2009). This is followed by an increased expression of regulatory enzymes and cytoprotective proteins (McArdle et al., 2001).

Circulating anti-inflammatory and pro-inflammatory cytokines, secreted by many cell types including monocytes, T-cells, fibroblasts and endothelial cells, activate or block signaling pathways, affecting protein synthesis and proteolysis. Multiple studies have shown that low-grade chronic systemic inflammatory cytokines increase throughout ageing causing redox imbalance, which may act as the underlying mechanism of age-related muscle waste and dysfunction (Roubenoff, 2003; White et al., 2012; Degens, 2010; Wust and Degens, 2007; Bartlett et al., 2012; Bruunsgaard, 2006; Chung et al., 2009). These observations have been evidenced by the continuous age-related accumulation of circulatory inflammatory cytokines such as interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and c-reactive protein (CRP) (Franceschi et al., 2000) concomitant with decreased levels of anti-inflammatory factors such as interleukin-10 (IL-10) (Lio et al., 2002). Investigating the inflammatory phenotype of differentiated primary skeletal muscle derived cells (SkMDCs) in young and older people, White et al. (2012) reported that in response to lipopolysaccharides (LPS), TNF-α and IL-1α were more upregulated in older cells, suggesting an age-related increase in the expression of pro-inflammatory cytokines.

1.2.7 Master athletes

1.2.7.1 Muscle mass and strength

Inactivity and disuse of the muscles contributes enormously to muscle morphology changes in many old people. The role of regular physical exercise in limiting the extent of sarcopenia has been demonstrated in studies where individuals who are less physical active have a greater chance of developing sarcopenia (Lee et al., 2018). Although physically active elderly individuals have only moderate losses in skeletal muscle mass (Valenzuela et al., 2018), the extent of the muscle atrophy as a consequence of ageing, a decrease in physical exercise or both is not elucidated.

Studies reported that people aged 60 years old or older can increase or maintain muscle mass with strength training (Fiatarone et al., 1990; Maltais et al., 2016; Tsuzuku et al., 2018; Klitgaard et al., 1990b), suggesting that the absence of muscle loading may have contributed to muscle atrophy in the aged muscles. Additionally, cross-sectional studies reported greater muscle mass and function in strength-trained master athletes compared with sedentary individuals of similar age (Sipila et al., 1991; Sipila and Suominen, 1991; Sipila and Suominen, 1993; McKendry et al., 2018; Klitgaard et al., 1990b). For instance, Klitgaard et al. (1990b) found that 70-year-old resistance-trained men (~ 20 years of training) had muscle mass and strength comparable to a group of 28-year-old untrained participants. Taken together, the above observations suggest that older strength-trained athletes may preserve muscle mass with age.

In contrast to cross-sectional studies of strength-trained master athletes that showed greater muscle mass compared to sedentary age-matched controls (Sipila and Suominen, 1993; Klitgaard et al., 1990b), it seems that lifelong endurance training does not maintain overall muscle mass or function in older age (Sipila and Suominen, 1991; Alway et al., 1996; Harridge et al., 1997; McKendry et al., 2018; Klitgaard et al., 1990b). In these studies, similar muscle mass and strength in old endurance-trained master athletes compared to older untrained people were reported, and a lower muscle mass compared with younger either untrained or young endurance-trained athletes. Overall, it appears that endurance training in older age does not prevent the age-related atrophy seen in sedentary ageing people, but the quantity and quality of the muscle may be improved through maintenance of contractile

tissue (Sipila and Suominen, 1991; Sipila and Suominen, 1993; McKendry et al., 2018; McKendry et al., 2019).

1.2.7.2 Muscle fibre type and size

Comparing type I, IIa and IIx fibres, Korhonen et al. (2006) found no difference in fibre type composition of the muscle VL between high-level young (18-33 years) and older (53-77 years) sprinters. These observations agree with a study by Reaburn (1994) where no significant difference in fibre type composition was seen between chronically (> 35 years) trained sprint- or endurance-trained master runners and body mass-matched younger runners.

The proportion of type I fibres in ageing endurance athletes appears to be maintained or increased (Coggan et al., 1990; Trappe et al., 1995; Pollock et al., 2018; Klitgaard et al., 1990b), typical of that observed in old non-athletes (Andersen et al., 1999). However, disagreement exists on whether a year of intense training can change fast twitch fibres into slow twitch fibres and vice versa. Neither 8 weeks of jump squats using either 30 or 80% of individuals' 1RM (McGuigan et al., 2002) nor 4 days a week of 3 sets of 3 second maximal sprints (Harridge et al., 1996) caused a change between fast and slow muscle fibre types in the quadriceps (Harridge et al., 1996; McGuigan et al., 2002) or the soleus (Harridge et al., 1996). These findings were consistent with the observations that 6, 9 (Carroll et al., 1998), or 19 (Adams et al., 1993) weeks of resistance training failed to induce a muscle fibre type shift. Taken together, these findings oppose the argument that intense training results in fibre type shifting from slow to fast twitch and vice versa. Yet, a 20-year-follow-up study showed that 6 of the 11 middle-aged male distance runners had a considerably larger percentage of slow-fibres than in baseline (Trappe et al., 1995), suggesting that certain training programs may result in exercise-induced muscle fibre type conversions.

Regarding the size of fibres, it has been reported that both chronic strength training (Korhonen et al., 2006) and endurance training (Harridge et al., 1997; Proctor et al., 1995; Klitgaard et al., 1990b) cannot inhibit the age-related muscle atrophy associated with a reduction in type II FCSA observed in sedentary ageing individuals. Korhonen et al. (2006) demonstrated in muscle VL that, while elite sprint runners exhibited significant reductions in the CSA of type II fibres, the declines were not different for type IIa (6.7% per decade) or type IIx (11.3% per decade), and fibre size was substantially larger in elderly sprinters than in untrained counterparts. In ageing endurance athletes, a typical observation is a reduced cross-

sectional area of type II fibres resulting from a shift towards a higher expression of myosin heavy chain (MHC) isoforms of slow fibres (Aagaard et al., 2007; Harridge et al., 1997; Proctor et al., 1995; Trappe et al., 1996). In 22-year longitudinal study, Trappe et al. (1996) demonstrated that elite master endurance runners (68.4 ± 2.7 years), despite having maintained high levels of endurance running training across 20 years, had similar muscle FCSA to age-matched sedentary men. Moreover, Klitgaard et al. (1990b) showed no maintenance of type II FCSA in older endurance runners or swimmers compared with the untrained counterparts. Taken together, it appears that the skeletal muscle atrophy in master athletes is due to both muscle fibre atrophy and loss of fibres. It also evident that the decrease in fibre size occurs mostly in the type II fibres, which may contribute largely to the age-associated reduction in anaerobic performance seen in master athletes.

1.3 Effects of ageing on the motor unit

1.3.1 Introduction

In humans, ageing is usually accompanied by substantial decline in neuromuscular function. Typical of this decrease is an age-associated reduction in skeletal muscle mass that results in decreased voluntary and electrically-induced contractile strength (Larsson, 1982; Lexell, 1993; Rogers and Evans, 1993; Vandervoort, 1992). Several studies suggest that the age-related decline in muscle mass is due to losses of alpha motor neurons in the spinal cord and denervation of their muscle fibres (Doherty et al., 1993; Doherty and Brown, 1993; Lexell, 1993; Mccomas et al., 1971). Thus, in these neurodegenerative conditions it could be expected that the MU pool in a given muscle exhibits substantial degeneration and restructuration.

1.3.2 Age-related motor unit numbers and physiological properties

The motor unit, as defined by Sherrington (Sherrington, 1925), is an alpha-motor neuron with its cell body in the ventral horn of the spinal cord, its single motor axon and all the muscle fibres innervated by the axon. Stimulation of MUs through signals from higher brain centres, or inputs from the periphery, directs the voluntary and reflex recruitment of motor neurons that elicit all postural and movement related contractions (Freund, 1983). The diversity of fibre types in skeletal muscle is thought to be dictated by the neurons innervating the MU (Buller et al., 1960), and that the muscle fibres within a healthy MU have the same phenotypic characteristics (i.e. slow-twitch or fast-twitch, type I or type II) and are activated

together. But the work of Salmons and Sreter (Salmons and Sreter, 1976) showed that it is the activity pattern that is more important than innervation.

Most studies related to the neuromuscular system have compared younger adults (~ 20-35 years) with those aged 60-65 years to over 90 years. Collectively, the reduction in muscle function (strength), which starts at around 30 years of age, accelerates after 60 years of age (Hepple and Rice, 2016). Interestingly, it appears that the age-related decline in muscle strength occurs before significant muscle loss and even after muscle loss takes place the decrease in strength is greater than can be due to muscle atrophy alone (Goodpaster et al., 2006).

Studies of whole muscle contractile function and analysis of muscle fibres from biopsy samples or whole muscle sections collected post-mortem have provided indirect evidence for age-associated decrease in the number and change in the physiological properties of human MUs. For example, Tomlinson and Irving (1977) investigated motor neurons on the lower limb segment by a direct count of alpha motor neurons taken from spinal sections post mortem of 47 deceased previously healthy individuals aged 13-95 years, and found no evidence of reduced numbers of motor neurons in the first seven decades. However, beyond the 7th decade there was significant decrease in motor neuron number, and specimens from those aged around 95 had approximately 32% fewer motor neuron supplying the lower limb muscles in comparison with those aged 10-60 years (Tomlinson and Irving, 1977).

Using incremental stimulation and the associated increase in the EMG signal, it was found in the extensor digitorum brevis (a small muscle in the foot) of people aged 3-96 years that MUs number were gradually lost with age (Campbell et al., 1973), as confirmed by others in the thenar muscle (Brown, 1972). Estimates of MU numbers with more recent EMG techniques have also revealed a lower MUs in older people in the tibialis anterior (McNeil et al., 2005; Power et al., 2010; Piasecki et al., 2016c; Piasecki et al., 2016a; Hourigan et al., 2015) and the muscle VL (Piasecki et al., 2019; Piasecki et al., 2016b), but not in the soleus (Dalton et al., 2008) and the biceps has shown mixed results (Galea, 1996; Power et al., 2012). Comparing three different age groups (23-89 years), McNeil (2005) reported a ~ 40% reduction in the motor unit number estimation (MUNE) in the *tibialis anterior* (TA) muscle of old (~ 65 years) compared with the young men (~ 25 years). Remarkably, in the very old (≥80 years), there was a significant loss of strength and a further decrease in the MUNEs

compared with the young and old individuals, supporting the observation by Tomlinson and Irving (1977) that the decline in MU may accelerate in old age. Collectively, it appears that age-related MU estimates in humans support the concept of more precipitous decline in muscle mass and function beyond the seventh decade possibly secondary to motor neuron loss.

1.3.3 Denervation and reinnervation in ageing skeletal muscle

1.3.3.1 Denervation and reinnervation process

As a result of the gradual loss of motor neurons (Tomlinson and Irving, 1977), denervated orphan fibres may express proteins and produce chemotactic signals that stimulate the sprouting of new dendrites from residual motor neurons. This process elicits reinnervation by the expansion of pre-existing MUs and is expected to restore the function of previously denervated muscle fibres. Over time the reinnervation of denervated fibres by the sprouting from close-by axons from other motor neurons would lead to a clustering of fibres within a specific motor unit (Larsson and Ansved, 1995) and the typical fibre type grouping, reported in muscles from older humans (Lexell and Downham, 1991; Kanda and Hashizume, 1989) and in oldest rodents (Caccia et al., 1979). However, some denervated fibres may not successfully be reinnervated and will progressively atrophy, becoming more angular throughout the process and will eventually disappear completely (Baloh et al., 2007). The observation of muscle fibre loss in some models of neuromuscular junction instability (Butikofer et al., 2011) suggests that motor neuron loss is not essential for fibre loss to occur. Yet, the increase accumulation of muscle fibres with peculiar small size and angulated appearance in old age in both human (Scelsi et al., 1980; Lexell and Taylor, 1991) and rodents (Purves-Smith et al., 2012; Rowan et al., 2012) is suggestive of a failed reinnervation at these more advanced ages.

1.3.3.2 Mechanisms of muscle denervation-reinnervation cycles in ageing

The denervation–reinnervation cycles in aged skeletal muscle elicit at the single myocyte level some modifications of the neuromuscular junction (NMJ) components such as decreased overlap of the pre- and postsynaptic structures, narrowing of the terminal axons, altered distribution of laminin and widespread sprouting of terminal axons (Balice-Gordon, 1997; Jang and Van Remmen, 2011; Chai et al., 2011; Samuel et al., 2012). These changes in NMJ structure have been observed in rats before fibre atrophy (Deschenes et al., 2010). In

mice, although some earlier changes were noticed at the level of the NMJ, it has been shown that the size and number of alpha motor neurons in the spinal cord are invariable until very late in life (Chai et al., 2011). In the very late life, the capacity of sprouting and reinnervation of denervated fibres by the motor neurons was diminished leading to smaller and more fatigable MUs with substantial muscle fibre atrophy (Luff, 1998; Glass and Roubenoff, 2010). It has also been highlighted Schwann cells (cells that myelinate axons) are involved in synaptic repair following denervation through their capacity to direct axonal regrowth, re-myelinate, and stimulate functional regain by secreting trophic and growth factors (Rangaraju et al., 2009; Kim et al., 2013; Hantai et al., 1995; d'Houtaud et al., 2009). Increased fragmentation of these cells may cause ineffective re-innervation and neuromuscular dysfunction in old muscles (Kawabuchi et al., 2011; Gordon et al., 2009). Moreover, in elderly people a reduction in the secretion of endocrine and paracrine IGF-1, which stimulates axonal sprouting, has been found (Grounds, 2002) while in rat, after the age of 300 days, neuronal cell adhesion molecule (NCAM) levels gradually increased in hindlimb muscles, suggesting an age-associated upregulation of certain NCAM isoforms with denervation of the fibres (Andersson et al., 1993).

1.3.3.3 Master athletes

Master athletes have regularly undertaken and continue to maintain high levels of structured exercise training. While in aged mice, lifelong exercise helps to preserve the stability of neuromuscular junctions (Deschenes, 2011; Nishimune et al., 2014), there is a little evidence of such effects in humans. Histological and electron microscopic examination of human VL biopsies revealed that in old master athletes, fibre type grouping is thought to be more pronounced because of a potential better capacity of reinnervation than in non-athletes (Mosole et al., 2014; Zampieri et al., 2015; Piasecki et al., 2019; Sonjak et al., 2019).

One appealing possibility is the maintenance of MU numbers and by extension, muscle size and function in master athletes. In contrast with other limb muscles (Johnson et al., 1973), a study reported that the soleus (a chronically active upright posture muscle in humans), composed predominantly of slow twitch fibres (> 85%) (Johnson et al., 1973) had only a minimal decrease in MU numbers in men aged ~ 75 years compared with those aged ~ 27 years (Dalton et al., 2008). In addition, Power (2010) using STA-MUNE showed that the numbers of MUs in the TA of the older aged (~ 65 years) endurance runners was similar compared to the young adults (~ 25 years) while in a follow-up study from the same master

runners, the numbers of MUs in the biceps brachii muscles (with presumably have lower degree of chronic activation) were not spared from loss (Power et al., 2012), suggesting indeed that physical activity may attenuate the age-related loss of motor units and motor neurons.

Piasecki and colleagues, however, demonstrated in the TA that the loss of MUs was similar in master endurance athletes and age-matched untrained controls (Piasecki et al., 2016c). In a recent study examining MU numbers in the VL and biceps brevis (BB) muscles across age groups of endurance and power athletes, the authors highlighted no difference in MUNE values between all groups of older men, regardless of their exercise discipline (control, endurance or power) (Piasecki et al., 2019). Remarkably, the latter study indicated similar MUNEs in the favoured arm of the master tennis players compared to the non-favoured arm. Combined, these results suggest, at least for the VL and BB, that long-term training does not avert age-associated decline in MU numbers, but may facilitate successful reinnervation of denervated fibres promoting an increase in MU size to preserve muscle function.

1.4 Skeletal muscle microcirculatory system and ageing

1.4.1 Introduction

The microcirculation consists of a branching network of vessels classified as arterioles, capillaries and venules. In skeletal muscle of mammals, a terminal arteriole feeds into a group of capillaries that run largely parallel to the longitudinal axis of muscle fibres, each muscle fibre being supplied by several different groups of capillaries from independent terminal arterioles (Emerson and Segal, 1997). The vascular casts in rat hind limb have revealed that the muscle capillaries are long and exhibit a degree of tortuosity (Newman et al., 1996), that increases the contact area with muscle fibres (Fig. 1.2). The function of capillaries is to exchange gasses (e.g., oxygen and carbon dioxide) and other molecules between the blood and the surrounding tissue. The capillary walls are very thin to facilitate diffusion. After leaving the capillaries, the blood is collected in the venules, which coalesce into progressively large veins, and the blood flows back into the heart.

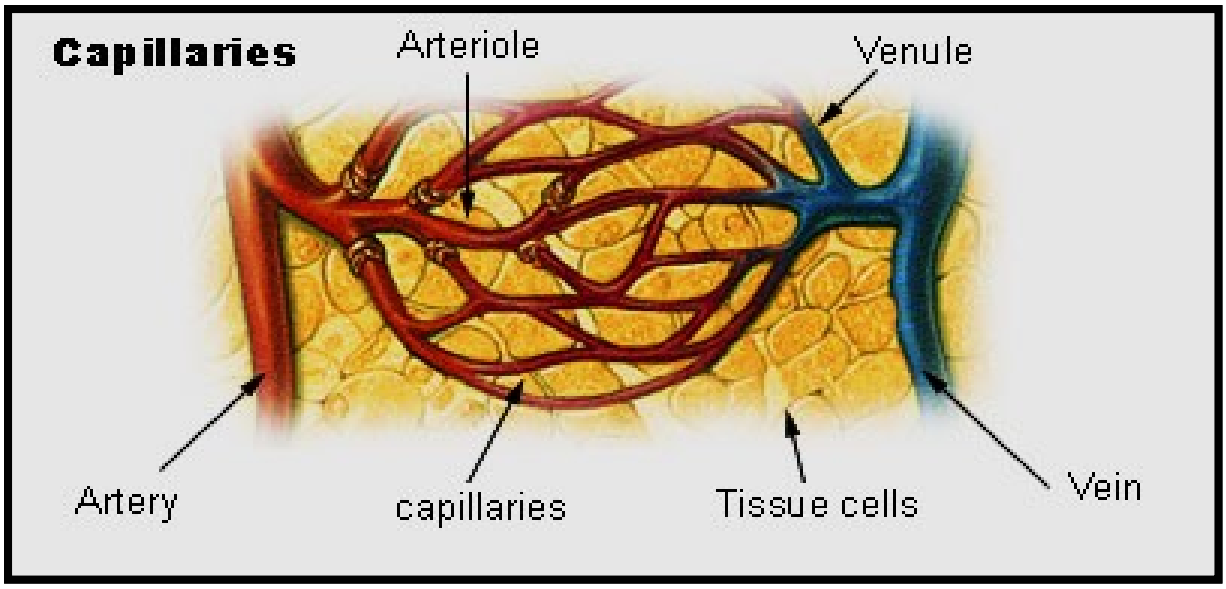


Figure 1.2 Arteries feed into muscle cells, supplying arterioles, each of which regulates a capillary network. Blood is then cleared from the capillaries via venules and veins (Image retrieved from homNes.com).

1.4.2 Methods of capillary domains

Capillaries are the main supplier of the oxygen for the mitochondria in the surrounding tissue cells. More capillaries have been observed in muscles with high oxidative capacity than those with low oxidative capacity, and even within a given muscle, areas with a higher oxidative capacity have a denser capillary bed (Degens et al., 2002; Degens et al., 1993b; Degens et al., 2003; Degens et al., 1994b; Degens et al., 1992; Gray et al., 1983). The method of capillary domains (Fig. 1.3) determines capillary domains that are defined as the area of a muscle cross-section surrounding an individual capillary delineated by equidistant boundaries from adjacent capillaries. The capillary domains approach has the capacity to provide indices of capillary supply to individual fibres, even when lacking direct contact with a capillary, and to estimate the maximal oxygen demand supported by a capillary (Bosutti et al., 2015). For example, the local capillary-to-fibre ratio (LCFR; the sum of the cumulative fraction of domains overlapping a fibre) and the capillary fibre density (CFD) are obtained from the cumulative overlap of capillary domains with individual muscle fibres (Egginton and Ross, 1989). Since a domain often overlaps more than one fibre (Fig. 1.3), it illustrates that one capillary supports more than one fibre. The LCFR thus gives the capillary supply to an individual fibre and is different from the overall capillary to fibre ratio (C:F) which only reveals the total number of vessels and cells included in the samples in relation to the total number of fibres in that same sample (Egginton and Ross, 1989). LCFR divided by fibre area gives the CFD or capillary density of a given fibre (Egginton and Ross, 1989).

1.4.3 Effect of ageing in skeletal muscle capillarisation

The decline in physical activity largely contributes to the development of sarcopenia, and may be associated with an ageing-associated reduction in capillarisation of skeletal muscle fibres that restrict substrate delivery. While muscle perfusion is controlled by macrovascular blood flow, which may decline with ageing, capillary numbers may be a limiting step in the perfusion of skeletal muscle and substrate delivery. Some publications also suggest that capillaries interact with satellite cells in the regulation of skeletal muscle morphology. For example, a study indicated not only a lower number of satellite cells in type II fibres in old people than in younger adults, but also that the distance between satellite cells and capillaries was greater in the older adults, which they suggested may compromise satellite cell activation through increased diffusion distances from the capillary to the satellite cell, hampering exposure to circulating growth factors (Nederveen et al., 2016). In contrast to the results of Nederveen and co-workers, McKendry et al. (2019) did not find a reduction in satellite cells or indices of capillarization between young untrained controls (YC) and untrained older controls (OC), which they suggest may be associated with the greater aerobic capacity of their OC cohort (~10.0 $mL \cdot kg^{-1} \cdot minute^{-1}$ greater compared to that reported in Nederveen and co-workers). In addition, Barnouin et al. (2017) reported lower capillary to fibre ratio in overall muscle fibres in older adults compared with younger adults, and this was associated with reduced FCSA. Similar observations were reported in other studies (Croley et al., 2005; Gavin et al., 2015; McKendry et al., 2019). These results indicate that capillary to fibre ratio and muscle fibre size are tightly linked across the spectrum of age, mostly in type II muscle fibres, which are more susceptible to age-related declines, as also confirmed by others (Ahmed et al., 1997; Wust et al., 2009; Barnouin et al., 2017; Bosutti et al., 2015).

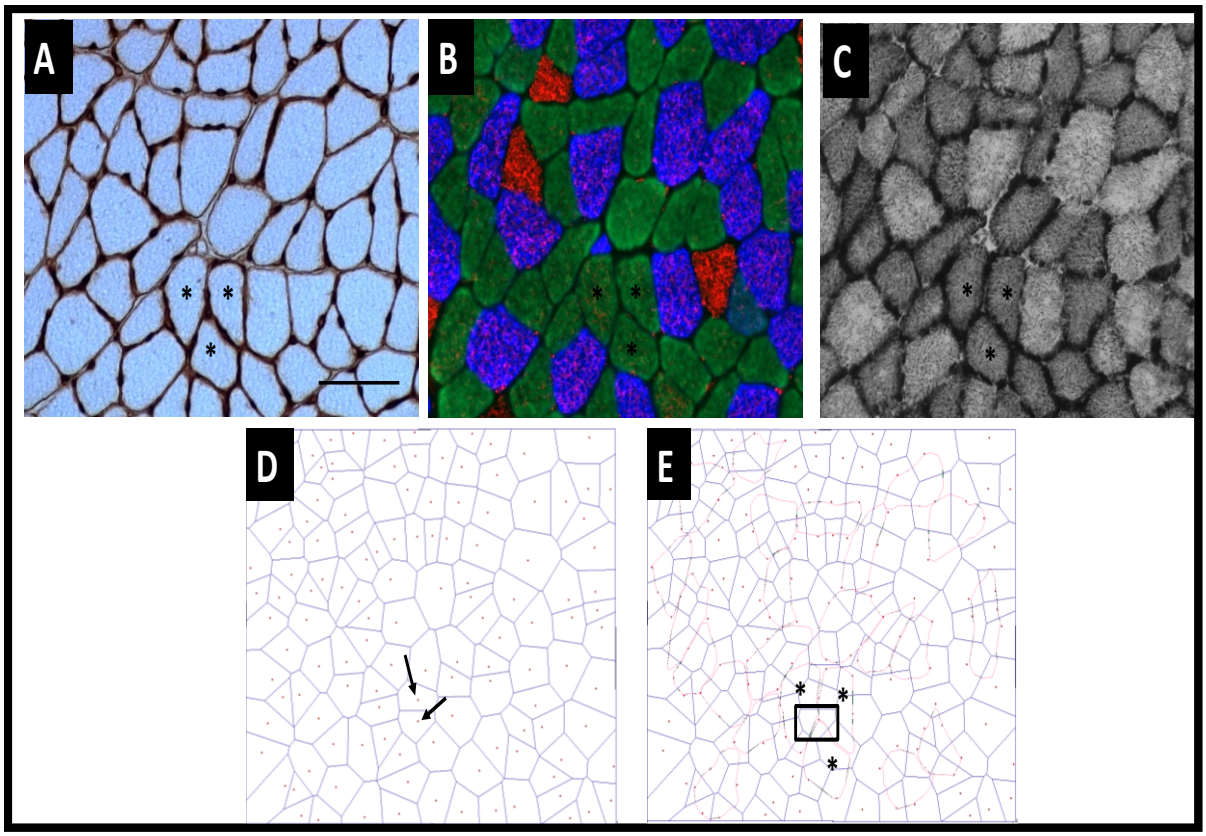


Figure 1.3 Fibre outlines and capillary domain areas. Serial frozen sections of mouse soleus stained with (A) lectin (Griffonia Bandeira simplicifolia) to depict capillaries (black dots around the fibres), (B) myosin heavy chain immunochemistry to distinguish fibre type (Blue: type I; Green: type IIa and Red: type IIx) and (C) succinate dehydrogenase (SDH) as an index of mitochondrial activity. (D): Capillary domains are illustrated, the contours outline the borders of the capillary domains and the red dots (showed by arrows) indicate the capillaries. (E): Overlap of domains with type IIa fibres; the region inside the rectangle illustrates overlap of domains and type IIa fibres. Asterisks identify the same fibre in each panel. Scale bars, 50μm.

Patterns of difference in the number of capillaries may inform the temporal associations between changes in capillarisation and muscle size. For example, if muscle fibre size declines prior to changes in capillarisation, capillary density would appear inflated in sarcopenic individuals (i.e., a similar number of capillaries with smaller muscle fibres). Assuming that capillary density will be similar in sarcopenic and non-sarcopenic adults (Prior et al., 2016), it appears that any decrease in fibre size is either preceded by a capillary rarefaction or decreases muscle fibre size occur in tandem with capillary rarefaction. Frontera et al. (2000) found a considerable decrease in the capillary to fibre ratio in old people (aged between 65 and 77 years old) on a time scale over a period of 12 years. A reduction in thigh muscle CSA was detected, but there were no changes in fibre CSA. According to the authors, the overall decrease in muscle CSA was due to a reduction in the number of muscle fibres, which suggests that capillary rarefaction may precede the decrease in fibre size with aging. These observations are supported by animal studies that show that endothelial cell apoptosis,

vascular rarefaction, and deficiencies in capillary function precede declines in muscle mass (Wang et al., 2014; Vescovo et al., 1998). These results offer evidence of a potential association between endothelial cell apoptosis and changes in muscle morphology. One additional study showed that muscle-specific vascular endothelial growth factor (VEGF) deficiency in a mouse model led to a 48% lower capillary-to-fiber ratio and reduced mass of the gastrocnemius muscle, but no change in gastrocnemius muscle fiber CSA was indicated (Olfert et al., 2009).

1.5 Skeletal muscle metabolism and ageing

1.5.1 Overview of skeletal muscle metabolism

Skeletal muscle contracts to produce force or movement, which is required for mobility. Metabolism in skeletal muscle provides the energy necessary for these contractions, and metabolic processes are increased in periods of need. Skeletal muscle can use glucose and fat as sources of energy, and particularly relies on fat β-oxidation during reduced energy intake (fasting) but is also the main tissue responsible for insulin-mediated glucose uptake (DeFronzo et al., 1981). Blood free fatty acids naturally sustains skeletal muscle with most of the fuel under low and moderate levels of exercise and the rate of glycogen depletion also augments with contraction (van Loon and Goodpaster, 2006). Both carbohydrate and fat are catabolised in mitochondria to produce ATP through aerobic respiration. Within the mitochondria, glucose, fats and proteins are hydrolysed to acetyl groups through a sequence of enzymatic reactions. The acetyl groups are then connected with Coenzyme A producing acetyl-coenzyme A (acetyl-CoA), which enters the tricarboxylic acid cycle (TCA) where it is oxidised and the products fed into the electron transport chain producing ATP via oxidative phosphorylation (Fig. 1.4).

The mitochondrial content of different muscle fibre types determines the metabolic profile of each muscle fibre type. Type I muscle fibres comprise a greater number of mitochondria with a high oxidative capacity, useful for endurance exercise activities. They are served via an extensive vascular network in order to supply the oxygen necessary for oxidative phosphorylation, and thus efficient generation of ATP, which provides the energy for all kinds of muscle work (Atalay and Hanninen, 2009). By contrast, type II muscle fibres have fewer mitochondria and are used for very short maximal intensity activities. They use anaerobic glycolysis where glucose is broken down to lactic acid to quickly generate ATP

(Fig. 1.4). Of note, the rate of ATP production is faster in anaerobic conditions, but the amount of generated ATP per glucose molecule is much less (2 ATP) than in aerobic (36 ATP) conditions.

Studies have shown that type I muscle fibres have a greater capillary to fibre ratio, a higher capillary density and more tortuous capillaries compared to type II muscle fibres (Murakami et al., 2010). Skeletal muscles with more capillaries are more oxidative whereas muscles with less capillaries provide less oxygen to the muscle fibre and are more reliant on anaerobic metabolism for ATP generation (Atalay and Hanninen, 2009). Glancy et al. (2014) showed that the embedding of the capillaries in the sarcolemma increased oxygen delivery to the muscle fibres as the mitochondrial pool was situated adjacent to the embedded capillaries.

1.5.2 Age-related changes in skeletal muscle metabolism function

The oxidative capacity of skeletal muscle reflects the ability of working muscle to produce ATP through aerobic metabolism, and has been shown to decrease in skeletal muscle during ageing (Coggan et al., 1992b; Essen-Gustavsson and Borges, 1986; Sugiyama et al., 1993), although not without exception (Rasmussen et al., 2003). The reduction in oxidative capacity of muscle fibres can be caused by a loss of mitochondrial content/or function. While it remains uncertain whether mitochondrial content decreases with ageing, with some studies reporting no change (Orlander and Aniansson, 1980; Mathieu-Costello et al., 2005) and others a decrease (Conley et al., 2000), an alteration of mitochondrial function is well established.

Mitochondria are a source of reactive oxygen species (ROS) in cells, and mitochondrial DNA (mtDNA) is particularly susceptible to ROS-induced damage due to the absence of histones, the proximity to the respiratory chain with electron leaks, and the relatively simple system of DNA repair. The age-related mitochondrial dysfunction is mainly due to the synthesis of abnormal electron transport chain (ETC) complexes secondary to damaged mtDNA (Wanagat et al., 2001; Trifunovic et al., 2004) and/or an accumulation of oxidatively damaged proteins (Rooyackers et al., 1996; Bota et al., 2002). Concerning the significance of age-associated DNA damage, it appears likely that a threshold of damage or mutation to the mitochondrial genome is necessary (60-90%) before this impairment affects mitochondrial function (Hayashi et al., 1991; Moraes and Schon, 1996; Rossignol et al., 2003). In mice, a study has

shown that alterations to the mitochondrial genome with ageing, which are associated with a lifetime of ROS production and damage, is strongly related to abnormalities in ETC with increasing age (Eimon et al., 1996). While from the available data the impact of mtDNA deletions on mitochondrial oxidative capacity with aging appears convincing, some studies (Szibor and Holtz, 2003; Stuart et al., 2005) did not support the idea of the involvement of mtDNA mutations and deletions altogether as major determinants to the decline in skeletal muscle oxidative capacity with aging.

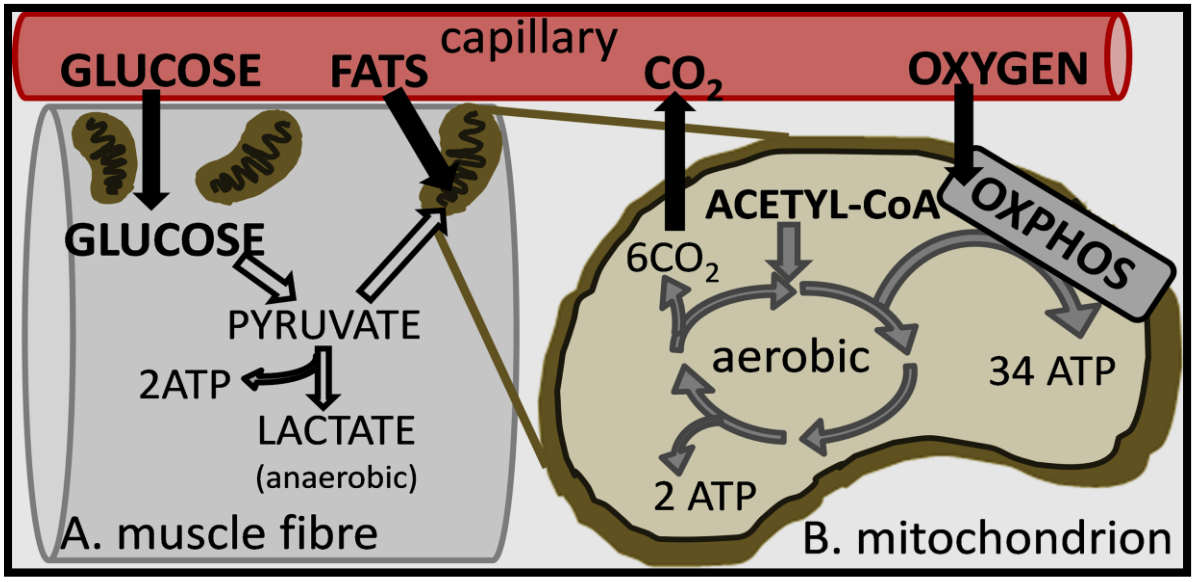


Figure 1.4 Microcirculation in skeletal muscle metabolism. Glucose and fats are the main source of energy for muscle fibres (A). Glucose is broken down to pyruvate that can be metabolized without oxygen to lactate, generating 2ATP (pathway used predominantly in fast muscle fibres). Pyruvate can also be transported into the mitochondria of the muscle fibre (B), where both pyruvate and fats can be converted to acetyl-CoA. This enters the TCA cycle and activates oxidative phosphorylation (OXPHOS), requiring oxygen from the blood (pathway common in slow muscle fibres). It produces much more ATP than anaerobic production of lactate, but also produces carbon dioxide, which must be removed by the blood. Thus, the microcirculation is essential for delivering glucose, fats and oxygen, and removing CO_2 from the muscle (Retrieved from Kolka (2016)).

1.5.3 Effect of exercise on skeletal muscle capillarisation and metabolism

Exercise, which necessitates increased ATP production, results in an increase in blood flow. The capillary density and the number of capillaries per muscle fibre increased by 20% and 25%, respectively in older individuals (60–70 years old) who participated in 10 months endurance training program that consisted of walking or jogging for 45 minutes per day, 3 times per week at 80% of their age-adjusted maximal heart rate (exercise stimulus was progressively increased over the period of study as the participants adapted to training) (Coggan et al., 1992a), suggesting that new capillaries were formed in the muscle. Similarly, an increase of 125% in the oxidative capacity was noted in 65-year-old men who performed

cycle ergometry at 70% of their age-adjusted maximal heart rate for 45 minutes per day, 3 days per week for 12 weeks (Meredith et al., 1989). In addition, the proportional increase in capillary number and fibre size observed in the quadriceps following 12-wk resistance training in young people reinforces the tightly relationship between the size and the capillary supply to a fibre (Green et al., 1999). Moreover, the observation that muscle fibre hypertrophy and angiogenesis have similar time course during overload (Egginton et al., 2011), further highlights that fibre size and fibre capillary supply are tightly linked and regulated by common factors.

1.6 High fat diet and obesity

1.6.1 Introduction

Overweight and obesity are due to excess calorie intake, and/or reduced energy expenditure. Dietary fat intake can increase both visceral and subcutaneous fat depots and alter muscle metabolism (Kim et al., 2003). In humans, high-fat diets (≥ 30% of energy from fat) can lead to obesity (Jequier, 2002; French and Robinson, 2003; Schrauwen and Westerterp, 2000; Smilowitz et al., 2010). Underreporting in epidemiological studies of diet and obesity in humans (Hebert et al., 2003; Poppitt et al., 1998; Voss et al., 1998) has led to the intensive use of animal models for experiments on diet-induced obesity (Young and Kirkland, 2007; Hurst et al., 2019; Speakman, 2019), with high-fat diets consisting of 30-78% of total energy intake from fat (Buettner et al., 2007). In rats (Ghibaudi et al., 2002; Lozano et al., 2016; Marques et al., 2016) and mice (Licholai et al., 2018; Tallis et al., 2017a; Hill et al., 2019) the proportion of high-fat in the diet correlates positively with an increase in body weight or fat gain.

Accumulation of fat mass in obesity may result from an increase in the number of adipocytes (hyperplasia) or their size (hypertrophy) or a combination of both (Bjorntor and Sjostrom, 1971; Jequier, 2002). Body mass index (BMI) has been traditionally used to categorize individuals as overweight or obese. In most animal studies, the degree of obesity has been assessed by matching body weight (or fat) of the animals fed a high fat or energy-dense diet with control animals that fed chow or low-fat diets (Rothwell and Stock, 1984; Ghibaudi et al., 2002; Harrold et al., 2000; Woods et al., 2003; Levin and Dunn-Meynell, 2002). The Lee index, as 1000* cubic root of body weight (g) divided by the nasoanal length (cm) (values > 310 indicating obesity), is also used to assess adiposity level of animals as it is highly

correlated with the proportion of the total body fat (Nations, 2012; H. Li et al., 1998), and corresponds to BMI in humans. In humans, the evaluation of body composition with air displacement plethysmography or dual energy X-ray absorptiometry (DEXA) provides a better indication of the degree of obesity compared to anthropometric measurements (Lindsay et al., 2001; Tzotzas et al., 2008).

1.6.2 Obesity and skeletal muscle inflammation

Skeletal muscle fibres express and secrete numerous cytokines including IL-6, IL-8, and IL-15 and other molecules such as irisin, myonectin, and myokines (Pedersen and Akerstrom, 2007; Eckardt et al., 2014). A number of adipokines have effects on muscle metabolism. For example, leptin can increase fatty acid oxidation in muscle fibres and thus protect against fat deposition. However, high levels of leptin with HFD have been associated with leptin resistance (Poppitt et al., 2006; Frederich et al., 1995) and the fact that hyperleptinemic animals and humans have a blunted response to exogenous leptin (Knight et al., 2010) demonstrates the association between leptin resistance and obesity. The effects of obesity on the expression of myokines have been controversial. Indeed, in rats, the expression of IL-6 and IL-15 in skeletal muscle was reduced with obesity (Shin et al., 2015). This observation is consistent with a study that observed a lower expression of IL-6 in cultured myocytes from skeletal muscle of obese subjects with impaired glucose tolerance or T2D compared to those from healthy controls (Green et al., 2011), but others found the opposite (Reyna et al., 2008; Corpeleijn et al., 2005; Ciaraldi et al., 2016). Another pro-inflammatory cytokine, TNF-α, was higher in skeletal muscle of rats fed a fructose-rich diet (Togashi et al., 2000), and may cause insulin resistance and mitochondrial dysfunction in myocytes (Austin et al., 2008). Hence, the obesity-related increases in TNF-α secretion by myocytes may stimulate myocyte insulin resistance via autocrine effects. Taken together, obesity is associated with elevated inflammation in myocytes, which may generate increased levels of pro-inflammatory molecules and contribute to muscle inflammation.

1.6.3 Insulin resistance

1.6.3.1 The insulin-signalling pathway

Glucose homeostasis is maintained by controlling the hepatic glucose production through glycogenolysis and gluconeogenesis (Fig. 1.5). Following feeding, the beta cells of the pancreas release insulin, which inhibits hepatic glucose production while stimulating glucose

uptake into skeletal muscle and adipose tissue. In the liver, glucose is released through the glucose transporter isoform Glut2 while the insulin-sensitive Glut4 stimulates glucose uptake in skeletal muscle and fat. Upon binding to its cell surface receptors, insulin triggers signalling cascades that promote the maintenance of blood glucose level in the circulation and activate a key protein kinase designated as Akt (Boucher et al., 2014). This Akt protein is largely involved in the insulin regulation of the pathways that regulate systemic glucose homeostasis, including glucose transport in adipocytes and skeletal muscle (Whiteman et al., 2002).

Circulating insulin binding to its extracellular α subunit receptor protein activates its intrinsic tyrosine kinase activity that phosphorylates Insulin Receptor Substrate (IRS) proteins on tyrosine residues which then act as anchoring sites for the p85 regulatory subunits of p85/p110 PI-3 kinase at the cell membrane (Boucher et al., 2014). This leads to the formation of phospholipid phosphatidyl 3,4,5 phosphate (PtdIns3,4,5P3) from PtdIns 4,5 P2 in the membrane. Phosphorylation of 3' position enables recruitment and interaction between proteins that contain pleckstrin homology (PH) domains including 3'-phosphoinositide dependent protein kinase-1 (PDK1) and Akt/PKB by direct binding to $PI(3,4,5)P_3$, resulting in the phosphorylation (and activation) of Akt on threonine 308. Akt is phosphorylated by a second protein kinase, mTORC2, for full activation (Fig. 1.5).

1.6.3.2 Skeletal intramyocellular lipids (IMCL) metabolism and insulin resistance

A high-fat diet (HFD) may lead to hyperlipidemia, promoting the storage of excess fat in non-adipose tissues such as skeletal muscle (ectopic fat storage) (van Hees et al., 2010). All types of lipids within muscle fibres are referred to as intramyocellular lipids (IMCLs), and are composed mainly of triacylglycerol, but also comprise diacylglycerol, ceramide, sphingolipid, and phospholipid. Several groups have reported an increase IMCL content following a HFD in both humans (Bachmann et al., 2001; Schrauwen-Hinderling et al., 2005; Sakurai et al., 2011) and rodents (Komiya et al., 2017; Morton et al., 2016; Baek et al., 2018).

A muscle type switch to more glycolytic fibres has been indicated in obese mice (Kemp et al., 2009). The IMCL content of individual muscle fibres was twice as abundant in obese than in lean subjects (Malenfant et al., 2001), and IMCL has been associated with altered metabolism in vivo (Virkamaki et al., 2001). While this increased lipid content may lead to mitochondrial dysfunction (Toledo and Goodpaster, 2013), IMCL accumulation itself may not alter

metabolism (Goodpaster et al., 2001). For example, skeletal muscle of endurance athletes has more oxidative muscle fibres, associated with a higher capillary density, but also higher IMCL levels, the latter phenomenon is known as the athlete's paradox (Goodpaster et al., 2001; van Loon and Goodpaster, 2006; Phielix et al., 2012). In this context, the accumulation of IMCL content is explained by the increase availability of free fatty acids indispensable to meet the high oxidative requirement during intense work. By contrast, obese people also have high IMCL, but less oxidative muscle fibres and a reduced capillary density. Thus, it appears more likely that IMCL only compromises metabolism when the lipid supply is in excess of demand. While obesity has been associated with a decrease capillary density (Czernichow et al., 2010), both reduced skeletal muscle capillary density and a shift to glycolytic fibre types are possible contributors of in vivo insulin resistance in obese humans (Lillioja et al., 1987).

Accumulating evidence supports the close coupling of IMCL and the development of insulin resistance in both humans (Bachmann et al., 2001; Boden et al., 2001) and animals (Chalkley et al., 1998; Griffin et al., 1999; Yu et al., 2002). Of these, Bachmann et al. (2001) demonstrated that in male subjects (~ 28 years old) a 3-day HFD increased IMCL levels in the TA and reduced insulin sensitivity. Several mechanisms have been proposed about the relationship between IMCL accumulation and insulin resistance in skeletal muscle. One hypothesis is that skeletal muscle mitochondria contribute to the pathogenesis of type 2 diabetes if a primary impairment in mitochondrial function causes intramyocellular accumulation of toxic lipid metabolites that would then suppress insulin signalling resulting in insulin resistance (Koves et al., 2008; Hirabara et al., 2010). It has been reported that the skeletal muscle of insulin-resistant, obese or Type 2 diabetes individuals have a reduced mitochondrial oxidative capacity compared with lean, healthy controls (Kelley et al., 2002; Petersen et al., 2004; Petersen et al., 2003). However, it is still unclear whether the reduced oxidative capacity present in insulin-resistant states is a result of reduced mitochondrial mass, deficiency in mitochondrial function or both. Cross-sectional studies have demonstrated in lean but insulin-resistant offspring, of type 2 diabetics (T2DM) patients increased IMCL, reduced mitochondrial oxidative capacity and decreased skeletal muscle mitochondrial density and content (Petersen et al., 2004; Petersen et al., 2003), supporting the concept of mitochondrial dysfunction as the primary pathology underlying insulin resistance and the progression to diabetes. Nevertheless, data of studies on skeletal muscle mitochondrial respiration reveal controversial evidence. One study performed in isolated myofibrils indicated that skeletal muscle mitochondria function in T2DM is normal, and that the reduced

skeletal muscle oxidative capacity of these patients is attributed to a decrease in mitochondrial content (Boushel et al., 2007). Kelley et al. (2002) have shown that in T2DM muscle NADH-Q2 oxidoreductase activity (complex 1) is reduced, resulting in NADH/NAD^+ redox imbalance, reflecting decreased functional activity of the respiratory chain, and subsequent diminished mitochondrial respiration.

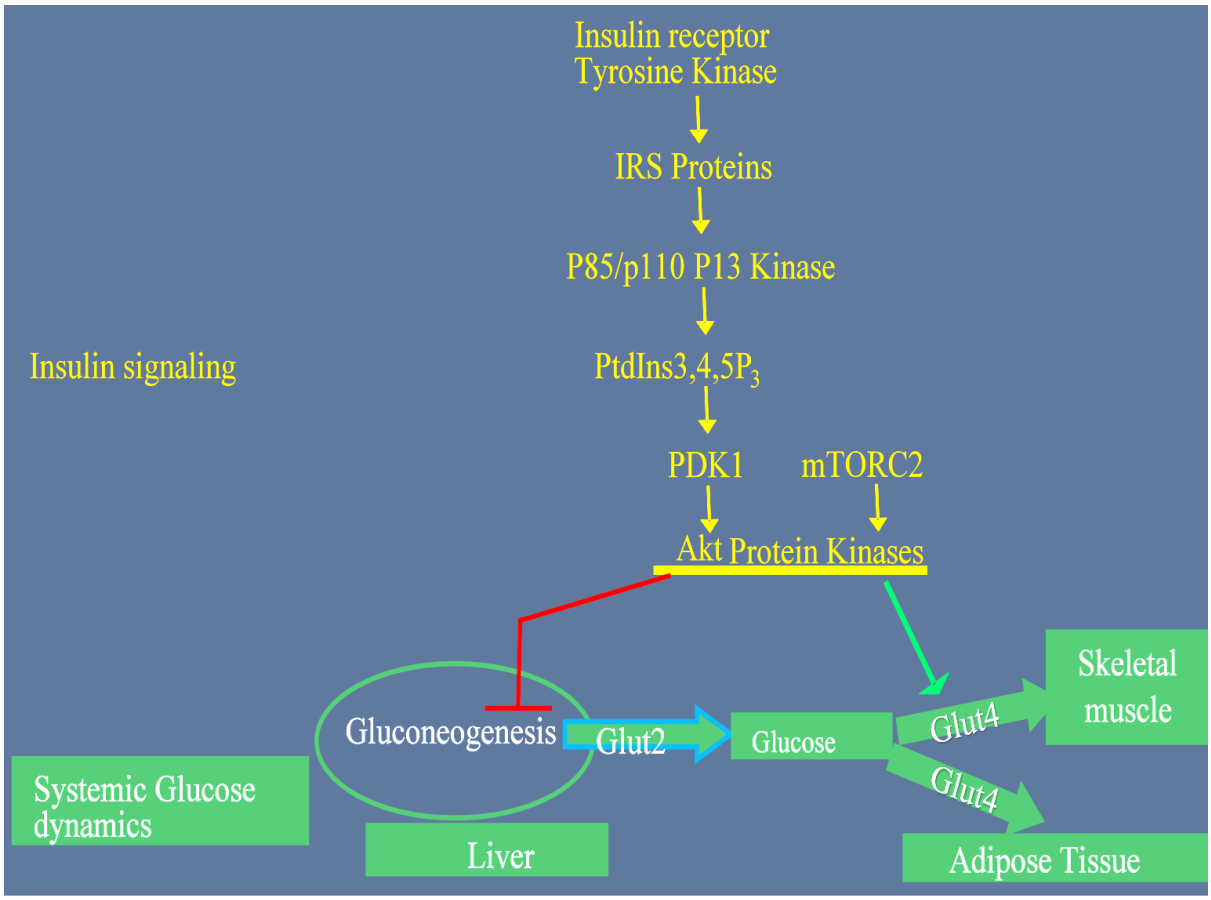


Figure 1.5 Insulin signaling pathway and systemic glucose dynamics. Glucose homeostasis is controlled by coordinating the production of glucose in the liver through the pathways of glycogenolysis and gluconeogenesis in times of fasting with the disposal during feeding of glucose into skeletal muscle through glycogen synthesis and glucose metabolism, and to a much lesser extent, with adipose tissue. Insulin inhibits hepatic glucose output while enhancing glucose uptake into muscle and adipose tissue. Glucose is released through the glucose transporter GLUT2 in the liver, whereas the insulin-sensitive GLUT4 mediates glucose uptake in muscle and fat. The insulin signalling cascade required for this maintenance of blood glucose concentrations activates a protein kinase Akt, which is required for insulin regulation of systemic glucose homeostasis, including glucose transport in adipocytes and muscle, inhibition of hepatic gluconeogenesis. The binding of insulin to its receptor protein activates its intrinsic tyrosine-kinase activity, which phosphorylates insulin-receptor substrate (IRS) proteins on tyrosine residues that then serve as anchoring sites for the p85 regulatory subunits of p85/p110 PI-3K kinase at the cell membrane. This results in the formation of the phospholipid phosphatidyl 3,4,5-phosphate (PtdIns(3,4,5)P_3) from PtdIns(4,5)P_2 in the membrane, which enables recruitment and interaction between the protein kinases PDK1 and Akt. This in turn leads to the phosphorylation (and activation) of the latter on threonine 308. Additional activation of Akt occurs upon its phosphorylation by a second protein kinase, mTORC2.

1.7 Rationale for research models used in this thesis

Experimental Animals

Study 1 and study 2 used female CD-1 mice for the experiments. The CD-1 mouse is an inexpensive and robust strain and has been well characterized for ageing research. The CD-1 stock was particularly chosen due to its outbred nature aligning more closely to the heterogeneous variability of human populations in comparison to the more commonly used C57Bl6/J strain (Pagala et al., 1998; Moran et al., 2005; Graber et al., 2015; Brooks and Faulkner, 1994; Lynch et al., 2001). Despite the large differences in their body size and lifespan, humans and mice show similar patterns in organ and systemic physiology. Their cells contain similar molecular structures that regulate the functioning of cells. Mice are valuable model organisms due to their small size, fast reproduction and short lifespans (Gorbunova et al., 2008). Moreover, the skeletal muscle function and molecular mechanism of ageing in mice resemble to those of humans (Demetrius, 2006). For instance, mice have a life expectancy of only a few years, and approaches for intervening in ageing can be tested by examining their effects on ageing parameters during the relatively short period of PhD project (Mitchell et al., 2015). Also, mouse model of diet-induced obesity and insulin resistance continue to be used frequently in experimental studies (Speakman, 2019; Wang and Liao, 2012). Mice can develop obesity by consumption of palatable and high calorie food and develop obesity as in humans. Moreover, the diet-induced obesity model closely mimics the cumulative accessibility of the high-fat/high-density nutrients in modern-day society over the past two decades, which are main contributors to the obesity trend in humans (Wang and Liao, 2012). These experiments have contributed to expand our current understanding that early ageing and high-fat diet exert differing effects in respiratory and limb muscles.

1.8 Hypotheses for each experimental study that include a theoretical explanation of the expected outcome of the experiment

Previous reports have indicated that in humans, ageing-related muscle weakness result from both losses of muscle mass and decrease in force or power per muscle cross-sectional area (McPhee et al., 2018). In mice, an ageing-related reduction in specific tension reported for both locomotor and diaphragm muscles (Ballack et al 2014; Chan and Head, 2010; Hill et al., 2018) occur in many cases in the absence of muscle atrophy. Additionally, in the soleus and white region of the rat gastrocnemius muscle, an ageing-related reduction in oxidative capacity occurs that is proportional larger than the loss of capillaries resulting in a

capillarisation in relative excess to the oxidative capacity of muscle fibres (Hepple and Vogel, 2004). These muscles also showed atrophy, but in the red region of the gastrocnemius, where hypertrophy occurred, there was no excessive capillarisation (Hepple and Vogel, 2004). These observations demonstrate that effects of ageing on skeletal muscle morphology may differ between muscles, and it remains to be established whether excessive capillary supply and loss of oxidative capacity are early signs of ageing.

Intramyocellular lipid content may increase (Schwenzer et al., 2009; Rahemi et al., 2015) and evidence suggest that this would further contribute to the lower specific tension in advanced age. However, data on ageing-related changes in IMCL content are lacking and may be muscle-specific, as suggested by a substantial accumulation of IMCL after a HFD + denervation in mouse soleus and EDL muscle fibres (Komiya et al., 2017). The experimental study I investigated the relationship between the fibre capillary supply with fibre type, size oxidative capacity and IMCL content in the soleus, EDL and diaphragm of 20- and 79-weeks old CD-1 mice. It was hypothesised that the effects of early-ageing on skeletal muscle morphology are muscle-specific. It was also expected that in early-ageing skeletal muscle morphological changes occur in the absence of significant atrophy and are more pronounced in locomotor muscles than in the diaphragm.

The prevalence of obesity in the Western World is alarming (Batsis and Villareal, 2018). However, the effects of obesity on skeletal muscle morphology may not be systemic as reflected by the different response in locomotor and respiratory muscles (Tallis et al. 2018). In addition to a reduction in force generating capacity, fatigue resistance has also been found to be reduced in obese people (Syed and Davis, 2000; Maffiuletti et al., 2007). Given the positive correlation between muscle fatigue resistance and oxidative capacity in motor units and single muscle fibres (Degens and Veerkamps, 1994), part of the lower fatigue resistance in skeletal muscle of obese individuals may be due to a reduced oxidative capacity (Koves et al., 2005).

However, some studies in humans and rodents indicated an increase in oxidative capacity with HFD rather than a decrease. Additionally, adaptations to a HFD may be muscle-specific and dependent on age. It has been reported that in young-adult mice fatigue resistance is reduced in the EDL, but not in the diaphragm and soleus (Hurst et al., 2019) while in old mice 9 weeks HFD did not induce any changes in fatigue resistance in the EDL, soleus or diaphragm. Reports on the effects of a HFD on the morphology of skeletal muscle rarely

consider aged animals. However, data on the effect of an extended HFD on in vivo skeletal muscle morphology and lipid content in aged rats indicated an inverse association between hind limb muscle volume and muscular lipid content (Bollheimer, 2012). These observations suggest that high-fat feeding and subsequent elevated muscular lipids may contribute to age-associated muscle atrophy.

During the experimental study II, the effects of a HFD and the duration of HFD on the fibre type and size, capillarisation, oxidative capacity and IMCL content were compared in 20-and 79-week-old CD-1 mice. It was expected that a HFD induces in all muscles an elevated IMCL, and that locomotor muscles will show a decrease in oxidative capacity and capillarization while the diaphragm shows an increase in oxidative capacity and capillarization. It is also proposed that the HFD-induced changes will increase with duration of feeding and be more pronounced at old age.

With the increase number of older people in the western populations, particularly those over 85, it becomes ever more important to expand knowledge on ageing muscle in order to develop strategies to alleviate the ageing-related decline in muscle mass and function in order to delay of functional limitations (McPhee et al., 2016). While it is accepted that physical inactivity leads to muscle atrophy (Ingram, 2000; Degens and Alway, 2006), it is not known to what extent muscle wasting and weakness in old age are due to reduced physical activity levels. Since master athletes maintain high physical activity levels (Degens et al 2013; Hannam et al 2017), they are considered a good model to assess the effects of ageing *per se* on reduced levels of skeletal muscle physical activity (Rittweger et al 2009; Degens et 2013; Harridge and Lazarus, 2017).

Several studies have interpreted increased motor unit size (Piasecki et al., 2019; 2016a) and large fibre type groups (Mosole et al 2014; Carraro et al 2017) as evidence of improved reinnervation in master athletes. Others, however, have reported small motor unit size, indicative of less collateral reinnervation in master athletes (Power et al 2016). Therefore, it is equivocal to whether regular physical activities protect against ageing-related motor neuron loss and facilitate reinnervation. Based on previous data on the size of fibre groups (Mosole et al 2014; Carraro et al 2017) and the loss of motor units (Piasecki et al. 2019) in master athletes, it is expected that both fibre group size and number of groups increase with increasing age beyond that predicted from muscle fibre type composition. As it has been suggested that master athletes may have better reinnervation capacity than non-athletes

(Mosole et al., 2014; Piasecki et al. 2019), it is also expected that group size is larger in master athletes than non-athletes.

The following three studies are novel research data to address each of these hypothesises and objectives in turn. This work forms part of the project funded by Manchester Metropolitan University and is exclusively the work of Guy Anselme Mpaka Messa.

1.9 Summary of the thesis

This thesis deals with the question how muscle ageing is affected by diet and physical activity. Ideally, sarcopenia (the age-related loss of muscle wasting) is even prevented, and therefore in chapter 2, I studied muscular changes in a mouse model of early ageing, representing a situation preceding sarcopenia. In chapter 3, I studied in mice of a similar age to what extent a high fat diet worsened the age-related muscle wasting even during early ageing. Besides diet, another important factor thought to contribute significantly to ageing is the age-related reduction in physical activity (Degens et Alway, 2006). To investigate this, I used muscle biopsies from master athletes and non-athletes. An additional lifestyle factor that may have significant impact on the age-related rate of muscle wasting is smoking (Rom et al., 2012) and initially I also had the intention to study the effects of smoking in ageing mice, and this work that I co-authored has indeed been published (Ajime et al., 2020). Thus, these studies are concerned with the question how different lifestyle factors – diet, physical activity (and originally smoking also) – affect sarcopenia.

CHAPTER 2

Morphological alterations of mouse skeletal muscles during early ageing are muscle specific

Results presented in this chapter are based on a published article: **Messa, G. A. M.,** Piasecki, M., Hill, C., McPhee, J. S., Tallis, J. and Degens, H. (2019) 'Morphological alterations of mouse skeletal muscles during early ageing are muscle specific.' Exp Gerontol, Aug 7, 2019/08/11, p. 110684.

Abstract

Background

One of the hallmarks of ageing is muscle wasting that may be preceded by morphological changes, such as capillary rarefaction. Muscle-specific changes in morphology in early ageing may differ between locomotor and respiratory muscles.

Methods

We compared capillarization, fiber type composition, fiber cross-sectional area (FCSA) and oxidative capacity of individual fibers of the soleus ($n = 6/5$ for 20- and 79 weeks, respectively), extensor digitorum longus (EDL: $n = 3/3$) and diaphragm ($n = 7/5$) muscles in 20- (mature) and 79-week-old (early ageing) CD-1 female mice.

Results

There was no significant loss of soleus and EDL mass. The FCSA was larger and the capillary density lower at 79 than 20 weeks in the diaphragm, while in the EDL the opposite was found (both $p \leq 0.002$) with no significant ageing-related differences in the soleus. The heterogeneity in capillary spacing, which may negatively impact on muscle oxygenation, was highest in muscles from 20-week-old mice, irrespective of muscle ($p \leq 0.011$). Succinate dehydrogenase activity, indicative of oxidative capacity, and capillary to fiber ratio did not significantly change with age in any muscle. At all ages, the capillary supply to a fiber was positively related to FCSA in each muscle.

Conclusion

Despite previously reported early age-related reductions in specific tension in both locomotor and respiratory muscles, morphological changes show a muscle-specific pattern in early ageing CD-1 mice. Specifically, early ageing was associated with 1) diaphragm hypertrophy 2) and fiber atrophy in the EDL that was not accompanied by angiogenesis, capillary rarefaction or reductions in oxidative capacity.

2.1 Introduction

With increasing age, mammals progressively lose muscle mass (referred to as sarcopenia) and strength. This weakness and loss of muscle mass has been attributed to a loss of fibers, preferential type II atrophy and a reduction in specific tension (Lexell et al., 1988; Andersen, 2003; Degens and Korhonen, 2012; Fragala et al., 2015; Barnouin et al., 2017; McPhee et al., 2018; Larsson et al., 2019). In addition to muscle weakness, older muscle may also suffer from an earlier onset of muscle fatigue, particularly during repeated and shortening contractions (Allman and Rice, 2002; Callahan and Kent-Braun, 2011). The consequences of progressive weakness are many and varied and together contribute significantly to frailty, reduced mobility and quality of life, often leading to the loss of independence and social isolation (McPhee et al., 2016). To develop effective countermeasures, it is important to improve understanding of the effects of ageing on skeletal muscle. In addition, recognizing early signs of sarcopenia will help inform preventative measures.

In previous studies, it has been shown that ageing-related muscle weakness in humans is a consequence of both loss of muscle mass and a reduction in force or power per muscle cross-sectional area (McPhee et al., 2018). In mice, an ageing-related reduction in specific tension has been reported for both locomotor and diaphragm muscles (Ballak et al., 2014a; Chan and Head, 2010; Hill et al., 2018), in many cases such age related changes occur in the absence of muscle (fiber) atrophy. This indicates that a reduction in specific tension may well be an early hallmark of ageing.

Different muscles may show different ageing-related changes. For instance, whilst quadriceps strength is reduced by almost 40% between the 2nd and 7th decade of life (Klitgaard et al., 1990a; McPhee et al., 2018; Young et al., 1985), the strength of the diaphragm is reduced by about 25% over the same period (Tolep et al., 1995; Polkey et al., 1997). Furthermore, in contrast to the locomotor muscles (Degens and Veerkamp, 1994; Allman and Rice, 2002; Degens and Alway, 2006), there is some evidence that diaphragm fatigability is unaltered with age in humans (Tolep et al., 1995; Polkey et al., 1997) or even elevated, at least during early ageing, in mice (Tallis et al., 2014). In addition to different patterns of use related to different functional requirements, an increased load on the diaphragm due to the ageing-related increase in chest wall stiffness (Teramoto et al., 1995) may offer protection to the diaphragm from the effects of disuse and ageing.

Besides a loss of muscle strength, also other ageing-related changes occur, such an ageing-related reduction in oxidative capacity that is proportionally larger than the loss of capillaries, resulting in a capillarization in relative excess to the oxidative capacity of muscle fibers in the soleus and white region of the rat gastrocnemius muscle (Hepple and Vogell, 2004). These muscles also showed atrophy, while in the red region of the gastrocnemius, where fiber hypertrophy occurred, there was no excessive capillarization (Hepple and Vogell, 2004). These data indicate that the effects of ageing on muscle morphology may differ between muscles, and it remains to be seen if excessive capillary supply and loss of oxidative capacity are early hallmarks of ageing.

The significance of capillaries for oxygen supply to mitochondria is demonstrated by the fact that muscles or muscle regions with a high oxidative capacity have a denser capillary network than those with low oxidative capacity (Degens et al., 1992; Larsson et al., 2019). Yet, the number of capillaries supplying a fiber is more strongly associated with fiber size than with fiber type (Ahmed et al., 1997) or oxidative capacity (Bosutti et al., 2015). This intricate interrelationship between size and the capillary supply to a fiber is also indicated by the proportional (Green et al., 1999) and similar temporal (Holloway et al., 2018) increase in capillary number and fiber size in the quadriceps after 12 weeks resistance training in young men, and the similar time course of muscle fiber hypertrophy and capillary proliferation in rat models of compensatory hypertrophy (Plyley et al., 1998; Egginton et al., 2011). It has been suggested that the ageing-related muscle atrophy may be preceded by capillary loss (Larsson et al., 2019).

Although the distribution of capillaries has a significant impact on tissue oxygenation (Degens et al., 1994a; Degens et al., 2006), it is rarely considered in studies on the effects of ageing on muscle capillarization. The absence of any significant changes in the heterogeneity of capillary spacing during maturational (Degens et al., 2006) or hypertrophic muscle growth (Degens et al., 1992), suggest that the neoformation of capillaries is a controlled process. There is, however, some indication that the heterogeneity of the capillary spacing was higher in 5- than 25-month-old rat plantaris muscle, which was linked to the increased heterogeneity in fiber size (Degens et al., 2009). Given that there is a lower heterogeneity in capillary spacing in slow oxidative than fast muscles (Egginton et al., 1988), it remains to be seen whether there are any muscle-specific effects of early ageing on the heterogeneity of capillary spacing in slow-oxidative and fast-glycolytic limb muscles, and the diaphragm.

Another part of the ageing-related decrement in specific tension is attributable to intermuscular fat infiltration (Delmonico et al., 2009; Hogrel et al., 2015). Also, intramyocellular lipid (IMCL) content may increase (Schwenzer et al., 2009; Rahemi et al., 2015) and modelling data indicate that this would further contribute to the lower specific tension in old age (Rahemi et al., 2015). Besides the effect of a larger IMCL content at the expense of myofibrils, apoptosis induced by intracellular lipids via elevated oxidative stress (Kob et al., 2015a) may further contribute to the decreased specific tension. However, information about changes in IMCL content during ageing is lacking and may be muscle specific, as suggested by a larger accumulation of IMCL after a high fat diet + denervation in mouse soleus than extensor digitorum longus (EDL) muscle fibers (Komiya et al., 2017).

The aim of the present study was to investigate the effects of early ageing on skeletal muscle morphology and more specifically the relationship between the fiber capillary supply with fiber type, size, oxidative capacity and IMCL in a postural slow oxidative muscle (the soleus), a muscle that is intermittently active (the fast, more glycolytic EDL) and a muscle that is constantly active (the diaphragm, highly oxidative with a mixed fiber type composition). It was hypothesized that early-ageing-related changes in muscle morphology occur in the absence of significant atrophy and are more pronounced in locomotor muscles than in the diaphragm.

2.2 Materials and methods

2.2.1 Animals

The effects of early ageing on skeletal muscle may be masked by maturational changes when using not fully-matured animals as the control group. An example of this masking of ageing effects is the absence of a difference in force generating capacity between the plantaris muscles from 5- and 25-month-old rats, while there was a significant reduction between the age of 13 and 25 months (Degens et al., 1993a). Since the mass of the extensor digitorum longus muscle (EDL) was higher in 30- than 10-week-old CD-1 mice and specific tension lower (Tallis et al., 2014), we have chosen 20-week-old CD-1 mice in the present study as the fully matured young-adult group to minimize bias of maturation and 79-week-old mice representing early ageing, as they already show a reduction in specific tension, but without loss of muscle mass (Hill et al., 2018). The CD-1 stock was selected as it is outbred enough to display a genetic heterogeneity similar to that found in humans (Rice and Obrien, 1980;

Aldinger et al., 2009), thus replicating the genetic heterogeneity found in humans more closely than inbred strains as the C57BL/6J mouse.

Fifty-nine female CD-1 mice (Charles River Ltd., Harlan, UK), housed 8-10 per cage at Coventry University, were maintained on a 12/12 h light/dark cycle at 20-22°C. They were fed *ad libitum* a low-fat standard chow diet (CRM(P); SDS/Dietex International Ltd, Whitham, UK; calories provided by protein 17.49%, fat 7.42%, carbohydrate, 75.09%; gross energy 3.52 kcal/g; metabolizable energy 2.57 kcal/g). At the age 20 or 79 weeks, animals were weighed, sacrificed by cervical dislocation [in accordance with British Home Office Animals (Scientific Procedures) Act 1986, Schedule 1] and then snout-to-anus length was determined using digital calipers (Fisher Scientific^{TM} 3417, Fisher Scientific, Loughborough, UK) to calculate the body mass index (BMI) as body mass (kg) divided by snout-to-anus length (cm) squared (Hill et al., 2019; Sjogren et al., 2001). All experimental procedures were carried out in compliance with the local ethical review board of Coventry University.

The removal of the soleus (20 w: n = 6; 79 w: n = 5), extensor digitorum longus (EDL; 20w: n = 3; 79w: n = 3) and right part of the diaphragm (20 w: n = 7; 79 w: n = 5) muscles were performed as outlined previously (Tallis et al., 2017a). After excision, the muscles were blotted dry and weighed (except the diaphragm, as only strips were excised), embedded in Tissue-Tek optimal cutting temperature freezing medium (Leica Biosystems, Nußloch, Germany), frozen in liquid nitrogen-cooled isopentane (Sigma Aldrich, Steinheim, Germany) and stored at -80°C until use.

2.2.2 Histological analysis and microscopy

Serial 10-µm thick cross-sections of the soleus, EDL and diaphragm muscles were cut with a cryostat (CM3050S; Leica, Nußloch, Germany) at -21°C and collected on Superfrost Plus microscope slides. Serial sections were stained for intramyocellular lipid (IMCL), myosin heavy chain (MHC), capillaries, or succinate dehydrogenase (SDH).

Intramyocellular fat. Sudan Black B was used to stain IMCL. The Sudan Black B dye stains mainly neutral lipids (mainly triglycerides) with a blue-black tint. Briefly, air-dried sections were fixed in 10% formalin for 10 min. Sections were then washed three times for 1 min in distilled water before incubation in propylene glycol for 3 min. Sections were then incubated in the Sudan Black B solution (preheated at 60°C) for 7 min, differentiated in 85% propylene

glycol for 3 min and subsequently washed three times for 1 min in distilled water. Sections were cover-slipped using glycerol gelatin.

Fiber typing. Serial sections were immunohistochemically stained for type I, IIa, IIx or IIb MHC using mouse monoclonal primary antibodies BA-D5 (1 $\mu g \cdot mL^{-1}$), SC-71 (1 $\mu g \cdot mL^{-1}$), 6H1 (10 $\mu g \cdot mL^{-1}$) and BF-F3 (5 $\mu g \cdot mL^{-1}$), respectively (Development Studies Hybridoma Bank, Iowa, USA). One section was co-stained for type I, IIa and IIx MHC and a serial section for type IIb MHC.

Sections were fixed with ice-cold acetone for 15 min and then blocked for 45 min with 10% goat serum in phosphate-buffered saline (PBS) at room temperature. Following a wash with PBS, the sections were incubated with the primary antibody for 90 min in a humid chamber. The sections were subsequently washed in PBS and incubated in the dark for 60 min with Alexa 350 IgG anti-mouse (2 $\mu g \cdot mL^{-1}$, Invitrogen, UK) or Alexa 488 IgG anti-mouse (2 $\mu g \cdot mL^{-1}$, Invitrogen, UK) for type I and IIa fibers, respectively and Alexa 555 IgG anti-mouse (2 $\mu g \cdot mL^{-1}$ Invitrogen, UK) for type IIx and IIb fibers. Sections were washed, dried and mounted using Prolong Diamond antifade mounting medium (Life Technologies, UK). Sections without the primary antibodies served as negative controls. Images were taken with a Carl Zeiss AxioMRc Camera (Gottingen, Germany) on a Zeiss fluorescence microscope (10x objective).

Succinate dehydrogenase. The succinate dehydrogenase (SDH) activity was assessed according to the protocol described by Wüst et al. (2009). Sections were incubated for SDH in 37.5 mM sodium phosphate buffer (pH 7.6), 74 mM sodium succinate and 0.4 mM tetranitro blue tetrazolium in the dark at 37°C for 20 min. The reaction was stopped with 0.01 N HCl for 10 s. The slides were then washed with two changes of distilled water, mounted in glycerol gelatin and stored in the dark until measurement of the staining intensity within two days. All samples were processed simultaneously in the same incubation solution, ensuring that all samples were subjected to the same conditions.

Capillary staining. Capillaries were visualized using lectin as described previously (Ballak et al., 2016). Briefly, air-dried sections were fixed with ice-cold acetone for 15 min, and blocked with 0.1% bovine serum albumin (BSA) diluted in 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) for 60 min. Subsequently, the sections were treated with a peroxide solution for 30 min and incubated with biotinylated *Griffonia (Bandeira) simplicifolia* lectin (GSL I; Vector Laboratories, Peterborough, UK; 50 $\mu g \cdot mL^{-1}$ diluted in 1%

BSA/HEPES) for 60 min. A 5-min wash with HEPES was conducted between each step. Sections were then treated with avidin-biotinylated horseradish peroxidase (Vectastain ABCkit, Vector Laboratories) for 60 min, washed with HEPES, and incubated with Horse Radish Peroxidase Substrate Diaminobenzidine (Vectastain DAB kit, Vector Laboratories) for 5 min. After a wash in distilled water, the sections were mounted in glycerol gelatin (Sigma-Aldrich, UK). Characteristic staining of diaphragm in old mouse is shown in Fig. 2.1.

2.2.3 Morphometry

Stained sections were photographed with a digital camera (Carl Zeiss) on a light microscope (Carl Zeiss, Germany). Two images per muscle cross-section were taken and 200 ± 85 (soleus, 186 ± 81; EDL, 194 ± 61; diaphragm, 216 ± 99) complete fibers were analyzed per sample.

Intramyocellular lipid. The IMCL content of individual fibers was determined using a microscope (Carl Zeiss, Germany) with a 20× objective and bright field settings. Images were digitally captured using a black and white AxioCam ICMI camera (Carl Zeiss) and analyzed with ImageJ (National Institutes of Health, USA, https://imagej.nih.gov/ij/). The fiber of interest was outlined, and the grey levels were converted to optical density (OD) using a calibration curve constructed from a series of filters of known OD. For each section, a separate calibration curve was constructed, and all images were taken at the same exposure with the same microscope settings. The OD of the Sudan Black B stain was determined in individual fibers and the background OD for each fiber was subtracted from the OD measured; the higher the OD for the Sudan Black B stain, the higher the IMCL content of the fiber.

Fiber type composition and fiber size. The fiber outlines and capillary centers were collected with a digitizing program (Btablet, BaLoH Software, Ooij, the Netherlands) and the data analyzed with AnaTis (BaLoH Software, http://www.baloh.nl). The fiber-type composition was expressed as number percentage. The fiber cross-sectional area (FCSA) was calculated for each fiber. An increased variation in fiber size may be an early hallmark of ageing, where small fibers might be atrophied as a consequence of denervation following motor neuron loss, and larger fibers may reflect compensatory hypertrophy. To investigate this, we also compared the standard deviation of the FCSA between the three muscles and the 20- and 79-week-old mice.

Succinate dehydrogenase. Photomicrographs of sections stained for SDH were taken on a light microscope with a 660-nm interference filter and a black and white AxioCam ICMI camera (Göttingen, Germany). All images were taken at the same exposure with the same microscope settings. Images were analyzed using ImageJ (National Institutes of Health, USA). To measure the optical density (OD) of a given fiber, the outline of the fiber was drawn, and the background OD subtracted. For each session, a separate calibration curve was made with filters of known OD (A_{660}). The calibration curve was used to convert the absorbance values of the SDH staining into OD values. It has been shown in single muscle cells that the intensity of the staining is linearly related to the maximal oxygen consumption of the fiber (van der Laarse et al., 1989).

To assess the SDH activity (SDH-OD), the OD (A_{660}) was converted to the rate of staining and expressed as the increase in absorbance at 660 nm (A_{660}) per µm section thickness per second of incubation time ($\Delta A_{660} \cdot \mu m^{-1} \cdot s^{-1}$). The SDH-OD multiplied by the FCSA yielded the integrated SDH activity (SDH-INT in $\Delta A_{660} \cdot \mu m\ s^{-1}$):

$$\text{SDH-INT} = \text{SDH-OD X FCSA}$$

Capillarization. The capillarization in the muscles was determined with the method of capillary domains as described previously (Degens et al., 1992; Degens et al., 2006; Hoofd et al., 1985) with AnaTis. In short, a capillary domain is defined as the area of a muscle cross-section surrounding an individual capillary delineated by equidistant boundaries from adjacent capillaries. The capillary domain provides a good estimate of the capillary oxygen supply area, even in muscles with mixed fiber type composition (Al-Shammari et al., 2014). In addition to the overall parameters of muscle capillarization, including capillary density (CD; number of capillaries per mm^2) and the capillary to fiber ratio (C:F), this method allows to define the capillary supply to individual fibers even when they lack direct capillary contact. The local capillary to fiber ratio (LCFR), the sum of the fractions of the capillary domains overlapping a particular fiber, provides a continuous, rather than a discrete value of the capillary supply to a fiber and takes into consideration that a capillary supplies more than one fiber (Barnouin et al., 2017). The ratio of LCFR to the FCSA provides the capillary density for a given fiber, defined as the capillary fiber density (CFD).

The radius (R) of a domain, calculated from a circle with the same surface area, provides an indication of the maximal diffusion distance from the capillary to the edge of its domain. R shows a lognormal distribution, and thus the Log_RSD (logarithmic standard deviation of the

domain radius) is a measure of the heterogeneity of capillary spacing, where a larger value indicates a larger variability in the capillary domain sizes, and hence a less homogeneous distribution of the capillaries in the tissue. Model calculations have shown that increasing the heterogeneity of capillary spacing results in poorer muscle oxygenation (Degens et al., 2006; Hoofd et al., 1985).

2.2.4 Statistical analysis

All statistical analyses were performed using IBM SPSS version 25. The Shapiro-Wilk test showed that all data were normally distributed. Where appropriate, a three-way (factors: age, muscle and fiber type) or two-way (factors: age and muscle) ANOVA was applied. Three-way interactions were excluded. If a main effect of age, muscle, fiber type or interactions was found, LSD post-hoc tests were performed to locate the differences. To assess the extent the capillary supply to a fiber was determined by the oxidative capacity of the fiber (SDH-OD), FCSA, fiber type, muscle of origin and/or age, a stepwise regression was performed. Statistical significance was accepted as $p < 0.05$. A measure of statistical power (sp) for each p-value was included. Data are expressed as mean ± SD.

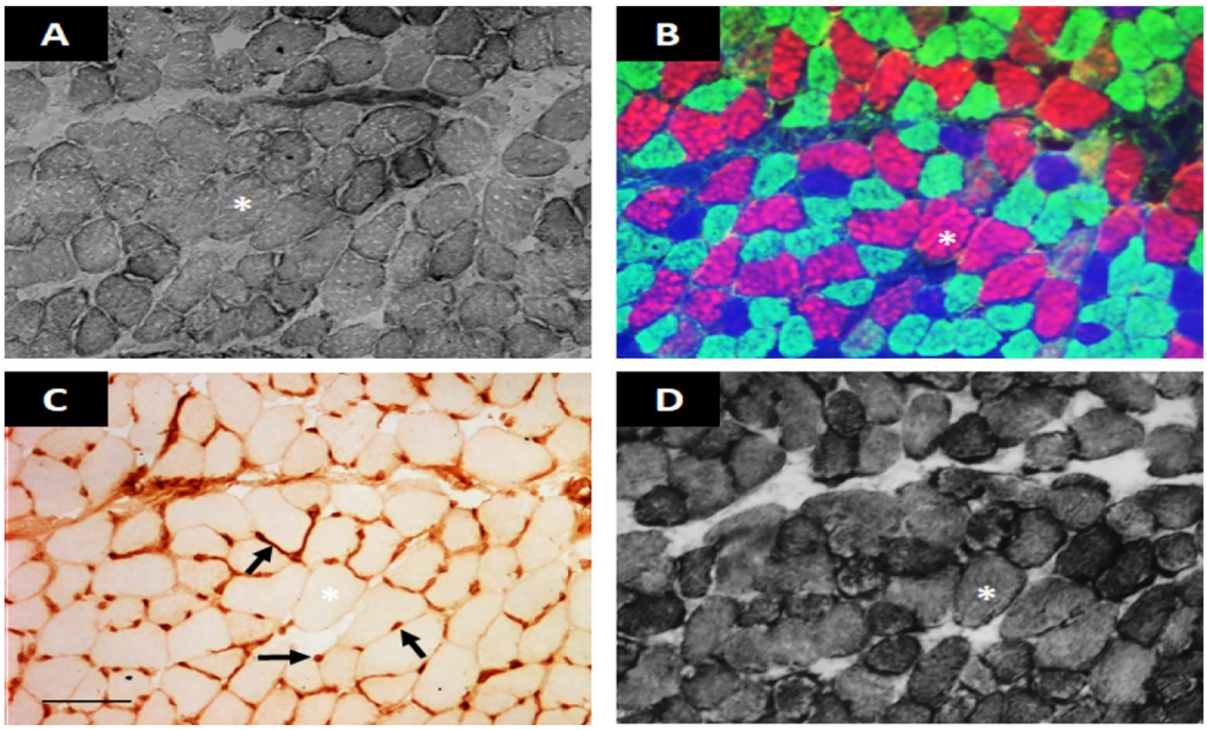


Figure 2.1 Serial sections of diaphragm from a 79-week-old mouse stained for (A) intramyocellular fat, (B) myosin heavy chain (MHC), (C) capillaries and (D) succinate deydrogenase (SDH) activity. Note that type IIa fibers (green) had a higher SDH activity than type I (blue) and type IIx (red) fibers. *: indicates same fiber in the four panels. Arrows indicate examples of capillaries. Scale bar = 100 µm.

2.3 Results

2.3.1 Mice characteristics

The body mass was greater in 79- than 20-week-old mice ($p < 0.001$; Table 1), but the BMI was lower in 20- than 79-week-old mice ($p = 0.002$; Table 2.1). The mass of the soleus and EDL did not differ significantly between mice of different ages (Table 2.1). The MM/BM of the soleus and EDL were lower in 79- compared to 20-week-old mice ($p = 0.002$; Table 2.1).

Table 2.1 Body and muscle mass in 20- and 79-week-old female CD-1 mice

	20 weeks (n = 29)	79 weeks (n = 30)
BM (g)	38.5 (4.9)	47.2 (8.6)[a]
BMI ($kg \cdot m^{-2}$)	3.04 (0.34)	3.37 (0.41)[a]
Soleus (mg)	10.1 (1.6) (n =10)	9.4 (1.4) (n =10)
Soleus MM/BM ($mg \cdot g^{-1}$)	0.26 (0.04) (n =10)	0.20 (0.04)[a] (n =10)
EDL (mg)	10.0 (2.1) (n =10)	10.6 (1.8) (n =10)
EDL MM/BM ($mg \cdot g^{-1}$)	0.28 (0.06) (n =10)	0.24 (0.05)[a] (n =10)

BM: Body mass; BMI: body mass index (body mass divided by snout-to-anus length squared); EDL: extensor digitorum longus muscle; MM/BM: muscle mass divided by BM. [a] different from 20 weeks at $p \leq 0.002$. Data are mean ± SD.

2.3.2. Muscle fiber type composition and fiber cross-sectional areas

Fiber type composition. Figure 2.2 shows the fiber type composition in the soleus (Fig. 2.2A), EDL (Fig. 2.2C) and diaphragm (Fig. 2.2E) in 20- and 79-week-old mice. The proportion of hybrid fibers was smaller than 5% in any of the muscles and hybrid fibers were therefore excluded from statistical analyses.

Type IIxb and type IIb fibers were only observed in the EDL. The proportion of type I fibers was higher in the soleus than in the diaphragm and EDL ($p < 0.001$; sp = 0.998), while for type IIx fibers the opposite was found ($p < 0.001$; sp = 0.995). The percentage of type IIa fibers was larger in the diaphragm than in the soleus and EDL ($p < 0.001$; sp = 0.999).

The proportion of type I, IIx and IIb fibers did not change significantly with age in any of the muscles. The muscle x age interaction ($p = 0.015$; sp = 0.765) for the type IIa fiber type proportion was reflected by a lower proportion of type IIa fibers in the soleus of 79- compared to 20- week-old mice ($p = 0.014$; sp = 0.770) and no significant age-related differences in the EDL and diaphragm.

Fiber cross-sectional area (FCSA). Figure 2.2B, D and F show the FCSA in soleus, EDL and diaphragm fibers, respectively. In the diaphragm and EDL type I and IIa fibers were smaller than type IIx and IIb fibers ($p \leq 0.002$; sp = 0.998), while in the soleus type I fibers were larger than type IIa and IIx fibers ($p \leq 0.032$; sp = 0.996).

The muscle x age interaction for FCSA ($p = 0.002$; sp = 0.901) was reflected by larger fibers in 79- than 20- week old mice in the diaphragm ($p < 0.001$; sp = 0.994), while in the EDL the opposite was found ($p = 0.001$; sp = 0.955). There was no significant effect of age on the FCSA in the soleus.

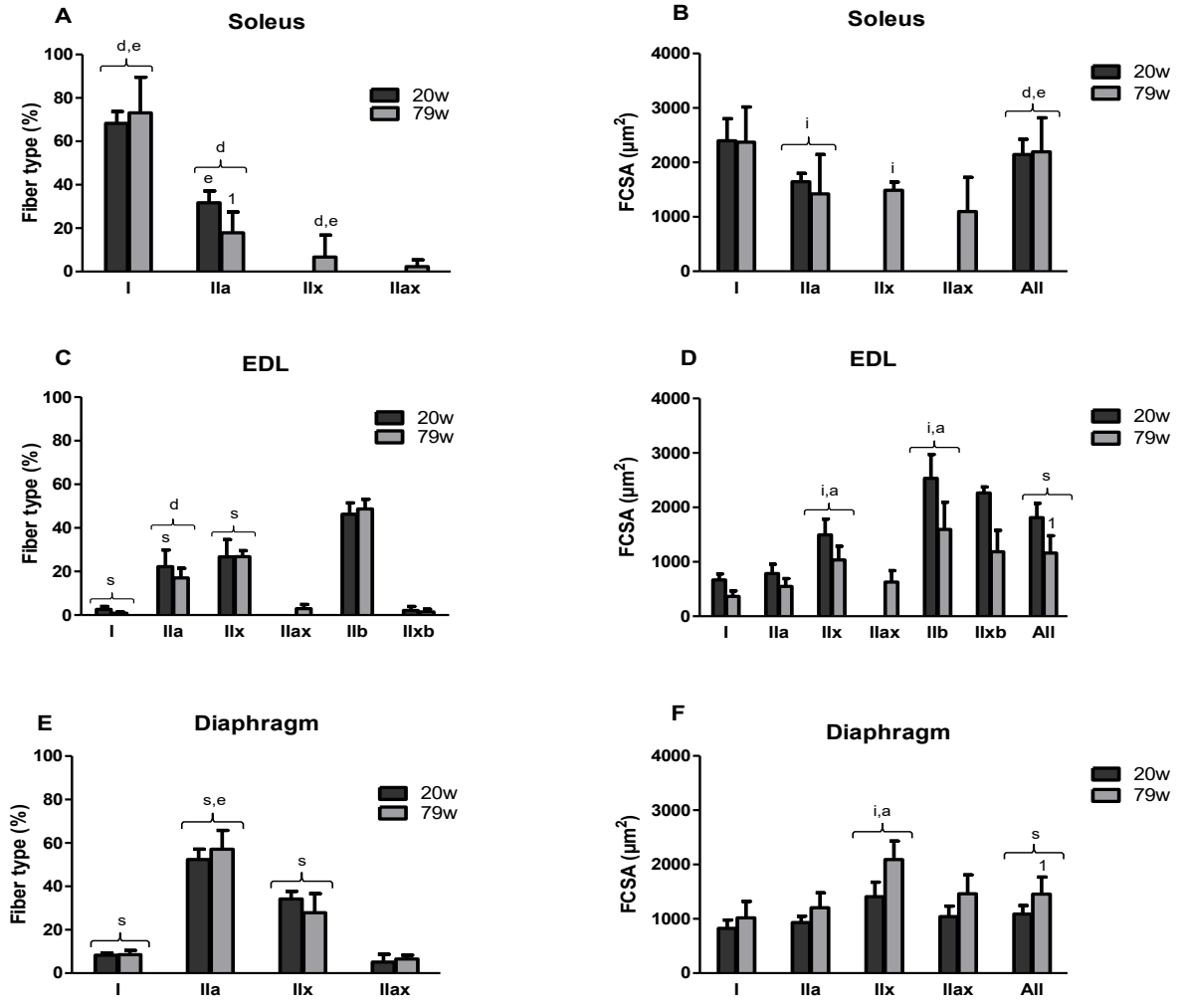


Figure 2.2 Fiber type composition (A, C and E) and fiber cross-sectional area (FCSA) (B, D and F) in the (A–B) soleus, (C–D) extensor digitorum longus (EDL) and (E–F) diaphragm muscles of 20- and 79-week-old mice. Values are means ± SD (n = 3–7). [s] different from soleus; [d] different from diaphragm; [e] different from EDL at $p < 0.001$; [i] different from type I fibers at $p \leq 0.032$; [a] different from type IIa at $p \leq 0.002$; [1] different from 20 weeks at $p \leq 0.014$.

Variation in fiber size (SD FCSA). There was an effect of muscle on the SD FCSA ($p = 0.003$; $sp = 0.916$), but also a muscle x age interaction ($p = 0.044$; $sp = 0.608$; Table 2.2) that was reflected by a larger SD FCSA in the diaphragm than in the soleus and EDL in 20-week-old mice ($p < 0.001$; $sp = 1.000$), but no significant difference in SD FCSA between the muscles in the 79-week-old mice. The SD FCSA was larger in the EDL of 20- than 79-week-old mice ($p = 0.044$; $sp = 0.589$), with no significant age-related difference in the SD FCSA in the soleus and diaphragm.

2.3.3 Intramyocellular lipid (IMCL) levels

The IMCL content was higher in the diaphragm than in the soleus and EDL ($p < 0.001$; $sp = 0.999$), but there were no fiber type or ageing-related differences in IMCL levels in any of the muscles.

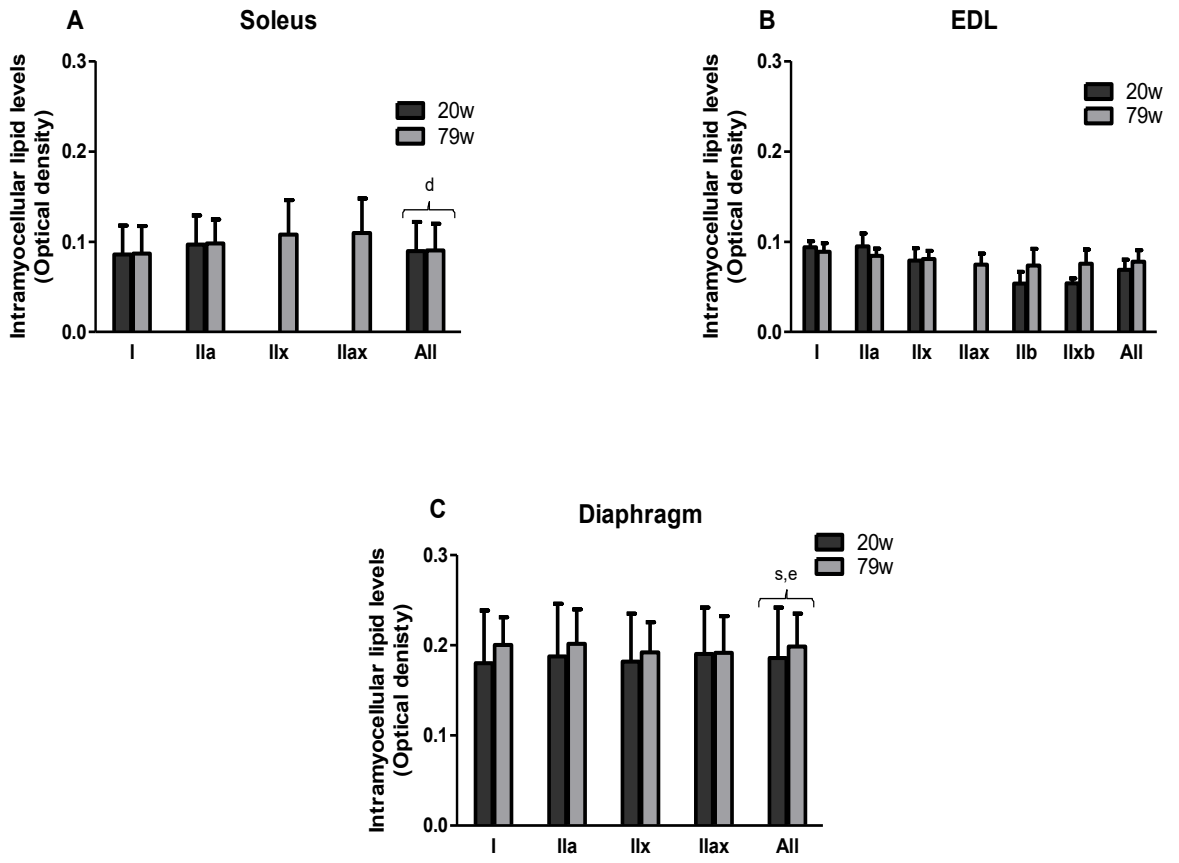


Figure 2.3 Intramyocellular lipid levels in the A) soleus, B) extensor digitorum longus (EDL) and C) diaphragm muscles of 20- and 79-week-old mice. Values are means ± SD (n = 3-7). [s] different from soleus; [d] different from diaphragm; [e] different from EDL at $p < 0.001$

2.3.4 Succinate dehydrogenase (SDH) activity (SDH-OD)

The SDH-OD decreased in the following order: IIa>I,IIx>IIb ($p \leq 0.027$; sp = 0.997). The SDH-OD of fibers in the diaphragm was higher than that in the soleus and EDL ($p < 0.001$; sp = 0.999). The SDH-OD in muscle fibers did not differ significantly between 20- and 79-week-old animals.

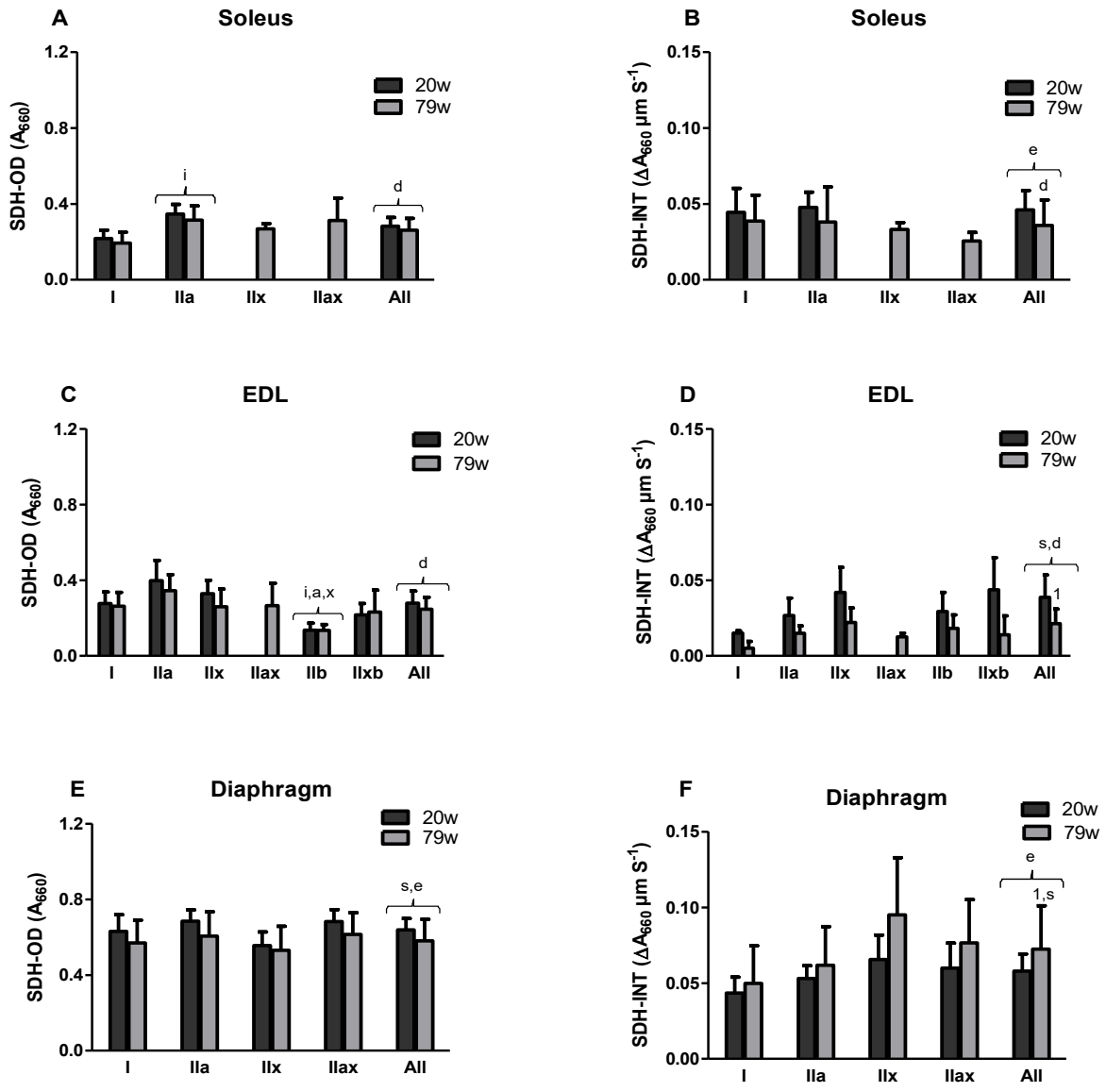


Figure 2.4 Succinate dehydrogenase (SDH) optical density (A, C and E) and integrated succinate dehydrogenase activity (SDH-INT) (B, D and F) in the (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of 20- and 79- week-old mice. Values are means ± SD (n = 3-7). [s] different from soleus; [d] different from diaphragm; [e] different from EDL at $p \leq 0.035$; [i] different from type I fibers at $p \leq 0.027$; [a] different from type IIa at $p < 0.001$; [x] different from type IIx fibers at $p < 0.001$; [1] different from 20 weeks at $p < 0.046$.

The SDH-INT of muscle fibers was higher in the diaphragm and soleus than in the EDL ($p \leq 0.035$; sp = 0.775). While the SDH-INT was lower in the diaphragm of 20- than 79-week-old mice ($p = 0.046$; sp = 0.523), in the EDL the opposite was found ($p = 0.010$; sp = 0.858) with no significant age-related difference in the SDH-INT in the soleus.

2.3.5 Muscle capillarization

Indices of global capillary supply. The C:F was soleus>diaphragm>EDL ($p \leq 0.002$; sp = 0.996) (Table 2.2). The C:F did not differ between age groups for any muscle. The CD was higher in the diaphragm than in the soleus and EDL ($p \leq 0.001$; sp = 0.999). The CD was lower in the diaphragm of 79- than 20-week-old mice ($p = 0.027$; sp = 0.648), while in the EDL the opposite was found ($p = 0.012$; sp = 0.901) with no significant age-related differences in the soleus.

The heterogeneity of capillary spacing, indicated by the Log_RSD was EDL>soleus>diaphragm ($p \leq 0.043$; sp = 0.622) (Fig. 2.5A). The Log_RSD was higher in muscles of 20- than 79-week-old mice, irrespective of muscle ($p \leq 0.011$; sp = 0.750). The Log_RSD in the muscles was positively corrected with the SD FCSA ($p = 0.030$; Fig. 2.5B).

Local capillary to fiber ratio (LCFR). Figure 2.6 A, C and E show the LCFR. The LCFR of type I fibers was soleus>diaphragm>EDL ($p < 0.001$; sp = 0.999) and that of type IIa fibers was higher in the soleus than in the diaphragm and EDL ($p < 0.001$; sp = 0.999). The LCFR of type IIx fibers was larger in the diaphragm and soleus than in the EDL ($p < 0.034$; sp = 0.903). The LCFR of all fibers pooled was larger in the soleus than in the diaphragm and EDL ($p \leq 0.002$; sp = 0.998). Overall, there were no significant age-related differences in LCFR in any of the muscles.

Capillary fiber density (CFD). Figure 2.6 B, D and F show the CFD. Overall, the CFD of type IIb was smaller than that of type IIx, IIa and type I fibers ($p \leq 0.002$; sp = 0.999). There was a significant muscle x age interaction ($p < 0.001$; 0.997). At 20 weeks of age, the CFD was larger in the diaphragm than in the soleus and EDL ($p < 0.001$; sp = 0.972). In the diaphragm, the CFD was lower in 79- than in 20-week-old mice ($p < 0.001$; sp = 0.998), while in the EDL the opposite was found ($p < 0.002$; sp = 0.605) with no significant difference in CFD in the soleus.

Table 2.2 Indices of capillary supply in mouse soleus, extensor digitorum longus (EDL) and diaphragm

	Soleus		EDL		Diaphragm		Effect (p-values)		Interaction (p values)
	20w (n = 6)	79w (n = 5)	20w (n =3)	79w (n = 3)	20w (n = 7)	79w (n = 5)	Age	Muscle	A x M
C:F ratio	2.89[d,e] (0.20)	2.89 [e] (0.59)	1.80[s,d] (0.29)	1.73[s] (0.23)	2.45[s,e] (0.32)	2.31 (0.37)	0.633	< 0.001	0.966
CD (mm^{-2})	951[d] (106)	1066 (220)	625[d] (65)	1193[1] (213)	1659[s,e] (322)	1245[1] (173)	0.302	< 0.001	0.001
SD FCSA pooled	814[d] (146)	977 (404)	965[d] (132)	572[1] (194)	443[s,e] (104)	612 (246)	0.821	0.003	0.044

A x M, age × muscle interaction; Inter., interaction; w = weeks; CD, numerical capillary density; C:F ratio, ratio between the number of capillaries and number of fibers; values between brackets indicate standard deviation; FCSA, fiber cross-sectional area. Data are presented as mean ± SD. [s] different from soleus at $p \leq 0.002$; [d] different from diaphragm at $p < 0.001$; [e] different from EDL at $p \leq 0.001$; [1] different from 20 weeks at $p < 0.05$.

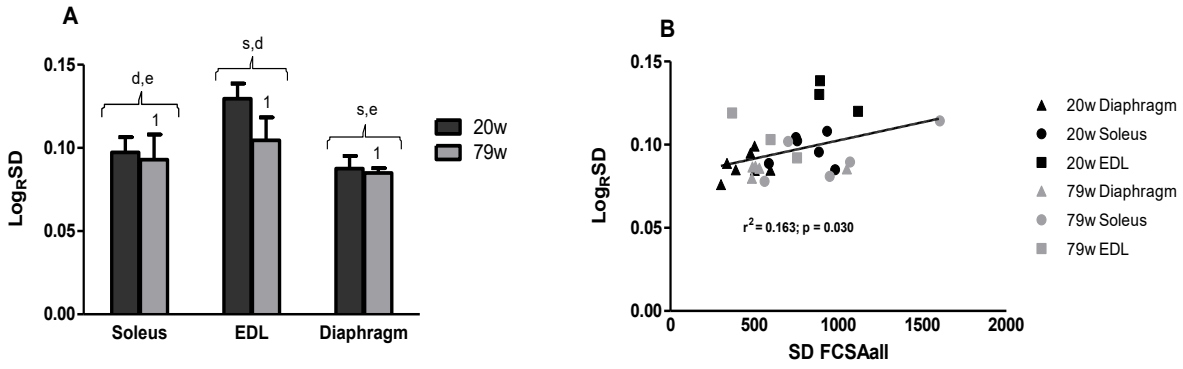


Figure 2.5 Heterogeneity of capillary spacing (Log_RSD) (A) and relationship between Log_RSD variation in fiber size (SD FCSA) (B) in the soleus, extensor digitorum longus (EDL) and diaphragm muscles of 20- and 79-week-old mice. Values are means ± SD (n = 3-7). [s] different from soleus at $p \leq 0.043$; [d] different from diaphragm at $p \leq 0.043$; [e] different from EDL at $p < 0.001$. [1] different from 20 weeks at $p \leq 0.011$.

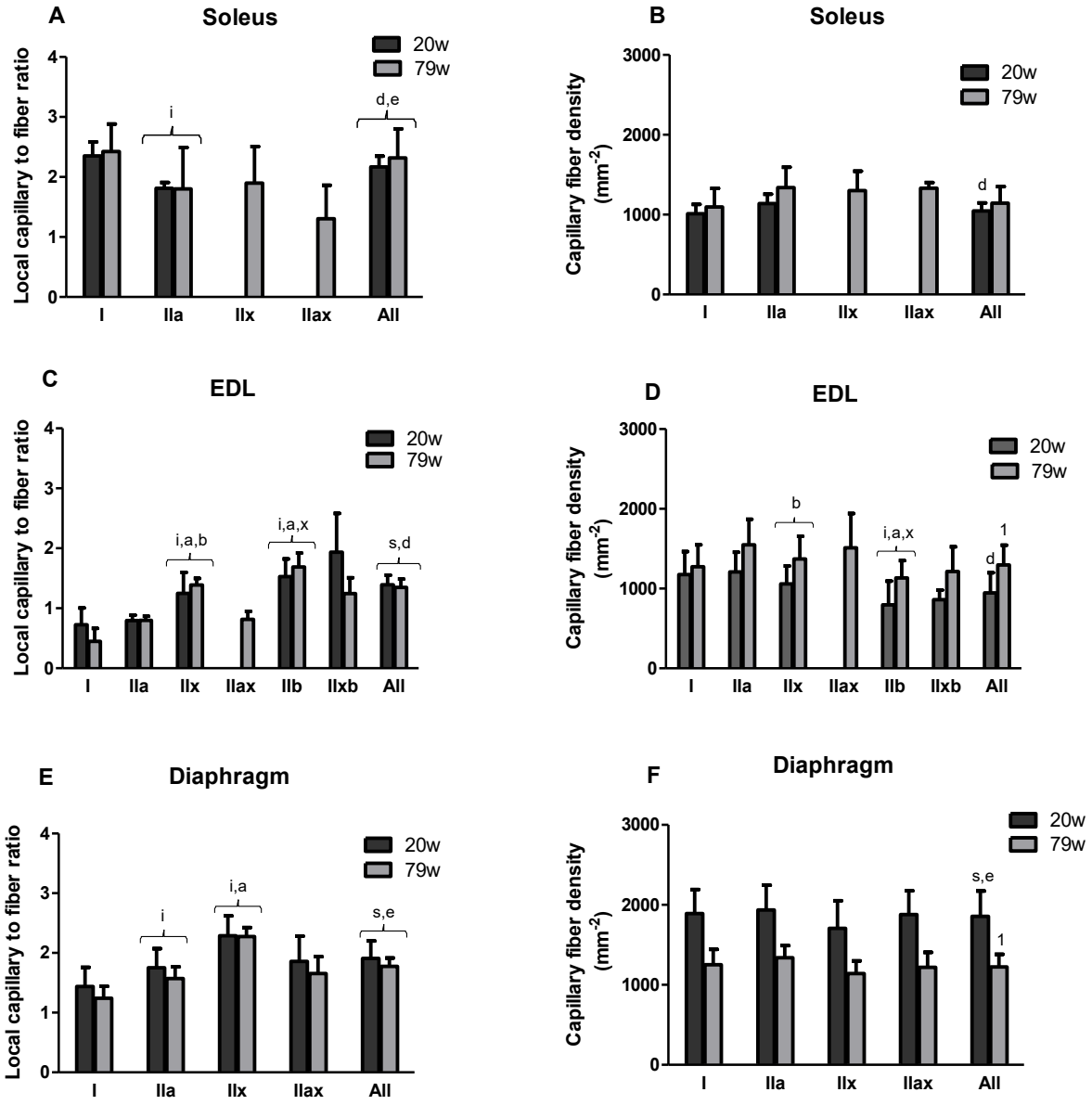


Figure 2.6 Local capillary to fiber ratio (LCFR) (A, C and E) and capillary fiber density (CFD) (B, D and F) in the (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of 20- and 79-week-old mice. Values are means ± SD (n = 3-7). [s] different from soleus; [d] different from diaphragm; [e] different from EDL at $p \leq 0.002$; [i] different from type I fibers; [a] different from type IIa; [x] different from type IIx fibers; [b] different from type IIb fibers at $p \leq 0.036$; [1] different from 20 weeks at $p \leq 0.002$.

2.3.6 Determinants of fiber capillary supply

To assess differences between muscles, fiber types and age in the matching of oxygen supply (LCFR) and demand (SDH-INT) of a fiber, the LCFR/SDH-INT was calculated as a measure of the supply:demand ratio. The LCFR/SDH-INT was higher in type IIb fibers in comparison to all other fiber types ($p<0.001$; sp = 0.935). There was a significant age x muscle interaction

(p = 0.001; sp = 0.785), which was reflected by a higher LCFR/SDH-INT in the EDL of 79- than in 20-week-old mice (p ≤ 0.006; sp = 0.845), while in the diaphragm the LCFR/SDH-INT the opposite was found (p = 0.019; sp = 0.617).

It appeared that the LCFR was primarily determined by FCSA (adjusted R^2 = 0.550; p < 0.001) and a small contribution of muscle of origin that increased the adjusted R^2 to 0.731 (p < 0.001) (Fig. 2.8). We also performed the analysis on all individual fibers (n = 4663), and this gave essentially the same outcome: FCSA was the main determinant (adjusted R^2 = 0.423; p < 0.001), and muscle of origin increased the adjusted R^2 to 0.533 (p < 0.001). There were no significant contributions of age or SDH-OD, suggesting that the qualitative and quantitative relationships between size and oxidative capacity of fiber with capillary supply do not change during early ageing.

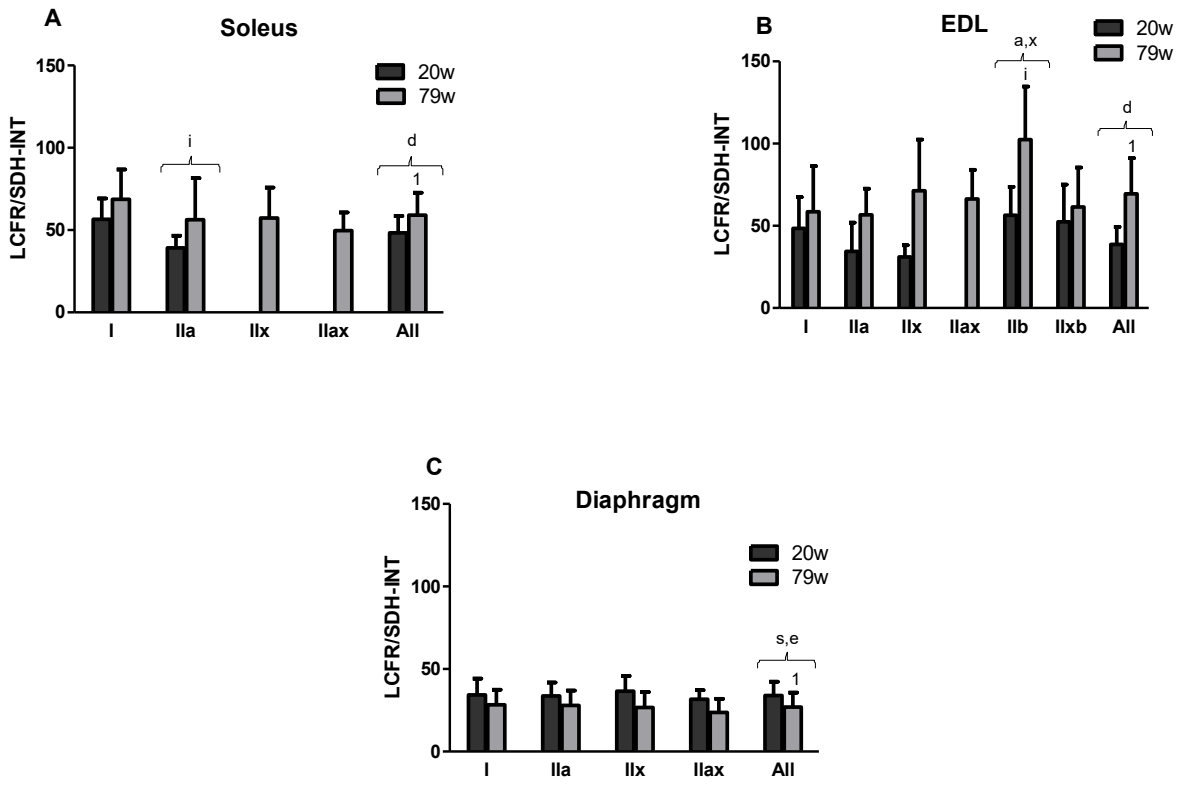


Figure 2.7 Local capillary to fiber ratio (LCFR):integrated succinate dehydrogenase activity (SDH-INT) in the (A) soleus, (B) extensor digitorum longus (EDL) and (C) diaphragm muscles of 20- and 79-week-old mice. Values are means ± SD (n = 3-7). [s] different from soleus; [d] different from diaphragm; [e] different from EDL at p ≤ 0.022; [i] different from type I fibers; [a] different from type IIa; [x] different from type IIx at p < 0.001; [1] different from 20 weeks at p < 0.02.

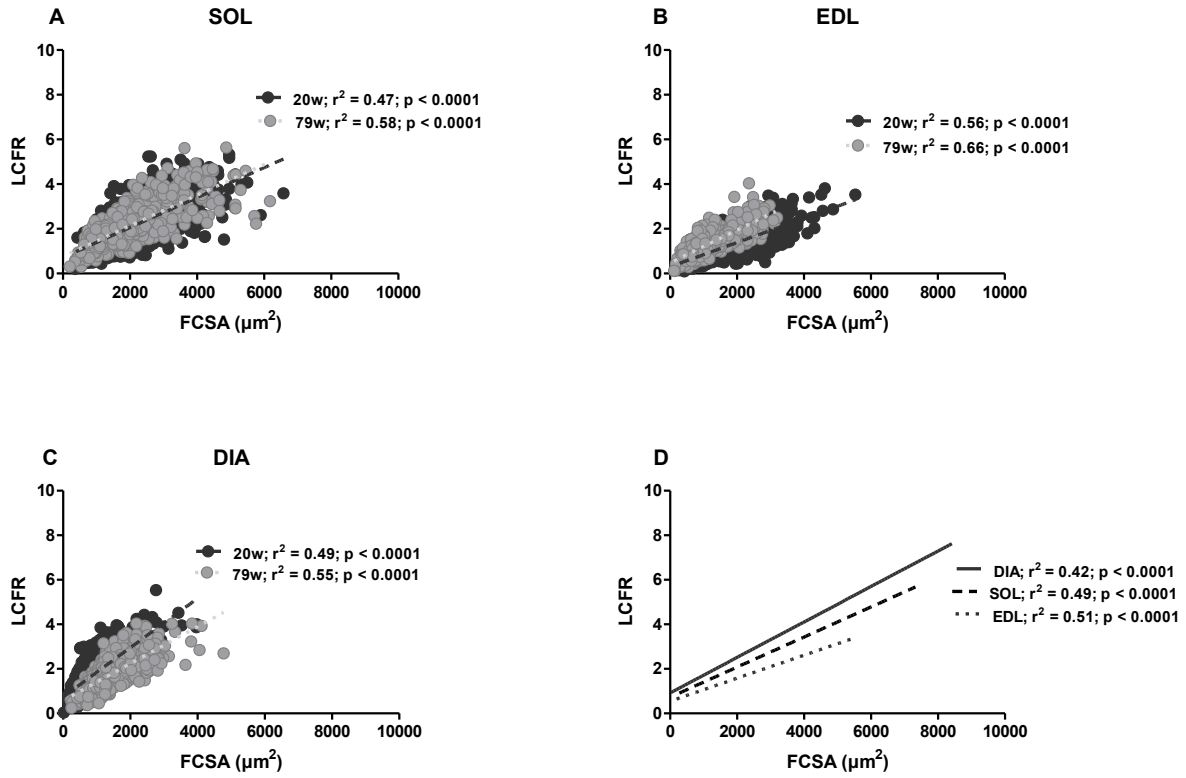


Figure 2.8 Relationship between the local capillary-to-fiber ratio (LCFR) and the fiber cross-sectional area (FCSA) in soleus (A), EDL (B) and diaphragm (C) muscles of 20- and 79-week-old mice. The FCSA and the LCFR was significantly correlated (soleus, n = 1663; EDL, n = 981; diaphragm, n = 2015). Each data point comes from an individual fiber.

2.4 Discussion

The main observations of the present study were that in mice, early ageing (between 20 and 79 weeks), characterized by an absence of a significant loss of hind limb muscle mass, was associated with hypertrophy in the diaphragm. This hypertrophy resulted in a reduction in the capillary supply to a fiber relative to oxidative capacity that was already lower in the diaphragm than the extensor digitorum longus (EDL) and soleus muscle. The capillary supply to a fiber relative to the oxidative capacity did increase in the EDL, indicative for a capillary supply in relative excess of oxidative capacity. These changes were almost entirely explicable by fiber hypertrophy in the diaphragm and fiber atrophy in the EDL, while no changes in morphology were seen in the soleus. Combined with previous observations in CD-1 mice these data indicate that in addition to a reduction in specific tension (Hill et al., 2018; Tallis et al., 2014) 1) diaphragm hypertrophy is a hallmark of early ageing, 2) fiber atrophy is not necessarily preceded by capillary rarefaction and reductions in oxidative capacity, and 3) different muscles undergo different patterns of ageing.

Morphological differences between muscles

In line with previous studies (Greising et al., 2013; Omairi et al., 2016), we found that, irrespective of age, the mouse soleus contains mostly type I and IIa fibers, whereas the EDL is predominantly composed of type IIb fibers and the diaphragm has primarily type IIa and type IIx fibers. Overall, the mouse soleus had the largest and the diaphragm the smallest fibers. Larger fibers in the soleus than the EDL have been observed previously in mice (Omairi et al., 2016). Irrespective of fiber type, the oxidative capacity of fibers was higher in diaphragm compared to those in the soleus and EDL, something also seen in rats (Smith et al., 1988). Part of the explanation of the higher oxidative capacity and IMCL content in fibers of the diaphragm may be that it is continuously active and comparable to a trained muscle that has higher IMCL stores and fatty acid oxidative capacity (van der Vusse and Reneman, 1996). Whatever the cause, these observations indicate that the properties of the fibers of a given type are dependent on the muscle of origin.

In agreement with a previous study on muscle capillarization (Murakami et al., 2010), we found that the capillary to fiber ratio (C:F) was higher in soleus compared with EDL and diaphragm. The CD was, however, highest in the diaphragm. The heterogeneity of capillary spacing, reflected by the logarithmic standard deviation of the capillary supply radius (Log_RSD) (Degens et al., 1993b; Barnouin et al., 2017; Hoofd et al., 1985) has a significant effect on tissue oxygenation (Degens et al., 2006; Degens et al., 1994a; Goldman et al., 2006) and was in 20-week-old mice smaller in the diaphragm than the soleus and EDL, and may be related to the higher oxidative capacity of the diaphragm requiring a more homogeneous oxygen tension in this than the other two muscles.

Early ageing

In contrast to the average life expectancy of 26.7 months (115.7 weeks) of C57BL/6 mice (Ballak et al., 2014a), the 50% survival of female CD-1 mice is 78-80 weeks (Navarro et al., 2002). However, it has been found that the specific tension and power output normalized to muscle mass of the EDL and diaphragm is lower in 30- and 50- than 10-week-old mice, but without significant differences in maximal tetanic force or muscle atrophy (Tallis et al., 2014; Hill et al., 2018). The absence of significant differences in the mass of the soleus and EDL from 20- and 79-week-old mice in our study suggests that the observed reductions in specific tension are early signs of muscle ageing.

With ageing, selective type II atrophy is common in both human (Lexell et al., 1988; Barnouin et al., 2017; McPhee et al., 2018) and mouse muscles (Brooks and Faulkner, 1988). At first glance, the atrophy of the EDL muscle fibers without significant atrophy of fibers in the diaphragm and soleus between 20 and 79 weeks of age seems to support this idea. However, within the EDL atrophy was not limited to type IIb fibers, as type I and IIa fibers also atrophied. Furthermore, in the diaphragm, in contrast to Greising et al. (2013), we observed an increase in the size of type IIx fibers. The discrepancy may be due to the comparison of different age groups of rodents, where we compared 20- with 79-week-old mice and Greising et al. (2013) compared 20- with 100-week-old mice, suggesting that diaphragm hypertrophy during early ageing is followed by diaphragm atrophy at an advanced age. The initial hypertrophy of the diaphragm during ageing may be an adaptation to the increased cost of breathing as a consequence of an ageing-related reduction in lung compliance (Sharma and Goodwin, 2006). Although another explanation for the discrepancy might be the use of C57BL/6 x 129 mice by Greising et al. (2013), where we used CD-1 mice with a shorter life span (Navarro et al., 2002), one would expect more pronounced atrophy in the diaphragm of our mice. Overall, it appears that the increase in fiber size during ageing in the respiratory muscles is opposite to the decreases in fast limb muscles, and the changes in muscle morphology during early ageing are more related to functional demands on the muscle than fiber type composition.

The oxidative capacity of the fibers in the soleus and the diaphragm did not change between 20 and 79 weeks. In the EDL muscle, the age-related decrease of the integrated SDH activity per fiber is explicable by both a reduction (though not significant) in the mass-specific oxidative capacity (SDH-OD) and FCSA. The increase in fiber size in the diaphragm during early ageing was not accompanied by an increase in mitochondria, as indicated by the similar integrated SDH, a measure of the total number of mitochondria in a cell.

It has been found previously in mice of similar ages that during early ageing the specific tension and power normalized to muscle mass of the EDL and soleus was reduced (Hill et al., 2018). In older obese mice there was a reduction in specific tension and power of the diaphragm (Hill et al., 2019), something also reported for the vastus lateralis muscle in older obese adults where this was associated with intramyocellular fat accumulation (Choi et al., 2016). We found, however, no age-related increase in IMCL content in any of the three muscles, corresponding with their largely unchanged oxidative capacity.

It has been suggested that capillary loss may precede the age-related fiber atrophy (Larsson et al., 2019). However, the absence of an age-related difference in C:F in any of the muscles, as also reported by others in humans (Snijders et al., 2017), suggests this is not the case, and in the EDL the CD was even increased during early ageing. This increased CD in the EDL was explicable by a decrease in the FCSA, while in the diaphragm the reduced CD was attributable to an increase in FCSA during early ageing, without any indications of angiogenesis or capillary rarefaction, respectively.

In contrast to previous observations in rats (Degens et al., 2009), we found that the heterogeneity of capillary spacing did, if anything, decrease during early ageing, suggesting a more homogeneous distribution of capillaries. It should be noted, however, that the increased heterogeneity occurred in rats that were at a relatively more advanced age than our oldest mice, and was associated with an increased variation in fiber sizes in the muscle (Degens et al., 2009). While here we also showed that the heterogeneity of capillary spacing increased with an increase in the variation in fiber size (Fig. 5B), there was no significant change in the variation in fiber size during early ageing in the muscles of our mice. Overall, this corresponds with the suggestion that 1) the constraint of capillary positioning at the periphery of a fiber is one determinant of the heterogeneity of capillary spacing (Degens et al., 2009), and 2) that the location of capillaries is not random but controlled (Degens et al., 2006), at least up to early ageing, to maintain sufficient muscle oxygenation.

These data indicate that the ageing-related changes in muscle morphology differ markedly between muscles; there is fiber hypertrophy (~30%) in the diaphragm and fiber atrophy (~30%) in the EDL. Such changes during early ageing may be masked if the young control group has not yet fully developed muscles, stressing the importance of selecting appropriate age groups when using rodent models to study (early pre-sarcopenic) muscle ageing (Ballak et al., 2014a).

Determinants of fiber capillary supply

In line with others (Degens et al., 1992; Bosutti et al., 2015; Barnouin et al., 2017), we found that the main determinant of the number of capillaries supplying a fiber (LCFR) was fiber size. This relationship differed somewhat between muscles, as reflected by the higher CFD in the diaphragm than the soleus and EDL, suggesting that the relationship between fiber size and capillary supply to a fiber is somewhat modulated by the metabolic surrounding of the

fiber. In contrast to common assumptions, we have previously reported that the capillary supply to a fiber is not determined by the oxidative capacity of the fiber itself (Bosutti et al., 2015; Barnouin et al., 2017). Similarly, in the present study, the oxidative capacity did not significantly contribute to the capillary supply of a fiber. To investigate the relationship between supply and demand further we estimated the maximal oxygen demand of a fiber as the integrated SDH activity (FCSA * SDH-OD) and calculated the capillary supply (LCFR) to demand ratio (LCFR/SDH-INT) for each fiber. In contrast to expectations, the diaphragm had the lowest and the EDL the highest supply to demand ratio. Similar to hypertrophied hearts (Des Tombe et al., 2002), the lower supply to demand ratio in the diaphragm may indicate that oxygen supply may be at risk of being compromised in maximally working diaphragm, and less so in soleus and EDL muscles, which becomes even more compromised during ageing, as reflected by the lower ratio in the diaphragm of 79- than 20-week-old mice. On the other hand, the higher supply to demand ratio in the EDL than in the soleus and diaphragm suggests that there is an excessive capillary supply in the EDL in relation to its oxidative capacity.

During early ageing, the capillary supply to demand ratio increased in the soleus, but not in the diaphragm, suggesting that, as seen in ageing rat muscle (Hepple and Vogell, 2004), the capillary supply becomes even more in excess to oxidative capacity, particularly in the soleus muscle. This again supports the notion that the oxidative capacity is not the main determinant of the capillary supply to a fiber, but other functions, such as removal of heat and waste products, and substrate delivery are more important. It is even possible that the capillary supply limits for these reasons fiber size, rather than fiber size determining the capillary supply (Larsson et al., 2019; Hendrickse and Degens, 2019).

Conclusion

The current study showed that in mouse muscles during early ageing, characterized by an absence of significant loss of muscle mass in the hind limb muscles, the EDL fibers had atrophied, and interestingly, the diaphragm hypertrophied without changes in the number of capillaries supplying a fiber or their oxidative capacity. It, therefore, appears that early ageing exerts differing effects in respiratory and limb muscles, where atrophy is not necessarily accompanied with, or preceded by, capillary rarefaction. Nevertheless, at any age and in all muscles the fiber size was the main determinant of capillary supply to a fiber, with no significant contribution of oxidative capacity.

Limitations of study I

The experimental study I has described muscle fibre composition, size and capillarity of mouse soleus, extensor digitorum longus and diaphragm muscles of 20- and 79-week-old CD-1 mice. Although there are numerous descriptions of the effects of ageing on structure and function of mouse muscles, most have used C57BL/6 mice. Thus, the timing and the magnitude of the age-associated morphological changes in CD-1 mice are of interest. We have chosen 20-weeks old mice as the control group to study the effects of early-ageing on skeletal muscle morphology. This age of mice was selected as the fully matured young-adult group to minimize bias of maturation given that the mass of EDL was higher in 30- than 10-week-old CD-1 mice and specific tension lower (Tallis et al. 2014). However, the study would be strengthened significantly by additional age groups to indicate the time course of the age-related muscle morphology declines in female CD-1 mice.

Summary of study I

With increasing ageing, mammals gradually lose muscle mass and strength, which contribute to frailty, reduced mobility and quality of life, often leading to the loss of independence and social isolation (McPhee et al., 2016). Since skeletal muscle morphology is an important factor for health and endurance performance, it is important to understand the physiological components of skeletal muscle morphology and how they are affected by increasing age. Additionally, knowing early signs of sarcopenia will help inform preventive measures.

Capillary density and mitochondrial content of skeletal muscle are important determinants of the aerobic capacity to generate ATP and the fatigue resistance of a muscle. The skeletal muscle oxidative capacity and fibre type composition are to a large extent genetically determined, but are also significantly affected by ageing, diet and level of physical activity.

In the first study the effects of early ageing on skeletal muscle morphology were investigated in the CD-1 mouse, an outbred strain that displays, in contrast to other strains that are often used in ageing research, a genetic heterogeneity comparable to that found in humans. Different muscles may undergo a different rate of ageing, due to specific usage in daily life. For instance, the diaphragm will be used continuously, while the extensor digitorum longus muscle (EDL) will most likely show a more pronounced decrease in activity during ageing and may thus show more age-related changes than the diaphragm. Therefore, the relationship between the fibre capillary supply with fibre type, size, oxidative capacity and IMCL was studied in the soleus (a postural slow oxidative muscle), EDL (intermittently active more

glycolytic muscle) and diaphragm (highly oxidative with a mixed fibre type composition muscle) of 20-week-old and 79-week-old CD-1 mice. The 20-week-old CD-1 mice were selected as the fully matured young-adult group to minimize bias of maturation and 79-week-old mice represented early ageing since they already show a reduction in specific tension without loss of muscle mass (Hill et al 2018).

It has been suggested that obesity may exacerbate the effects of ageing on skeletal muscle structure and function (Tallis et al., 2018), but it is not yet clear how the morphology of different muscles is affected by a HFD in young-adult and early-ageing muscles. To extend knowledge of these issues, the effects of a HFD on CD-1 mouse skeletal muscle morphology will be examined in the following chapter.

CHAPTER 3

The impact of a high-fat diet in mice is dependent on duration and age, and differs between muscles

Results presented in this chapter are based on an article published in the Journal of Experimental Biology: **Messa, G. A. M.,** Piasecki, M., Hurst J., Hill, C., Tallis, J. and Degens, H. (2020)

Abstract

Background

Prolonged high-fat diets (HFD) can result in an accumulation of intramyocellular lipids (IMCL) that may negatively affect muscle function. The duration of a HFD required to instigate these changes is poorly defined, and effects may be muscle-specific and aggravated in older age.

Methods

Muscle morphology was determined in the soleus, extensor digitorum longus (EDL) and diaphragm muscles from female CD-1 mice divided into 5 groups: young fed a 8 weeks HFD (YS-HFD, n = 16), young fed a16 weeks HFD (YL-HFD, n = 28) and young control (Y-CON, n = 28). The young animals were 20 weeks old at the end of the experiment. Sixty 70-week-old female CD-1 mice received either a normal diet (O-CON, n = 30) or a HFD for 9 weeks (OS-HFD, n = 30).

Results

Body mass, body mass index and IMCL content increased in old mice fed HFD for 9 weeks ($p \leq 0.003$). In the young mice, this increase was only seen in YL-HFD and not YS-HFD ($p \leq 0.006$). The fibre cross-sectional area (FCSA) in young YL-HFD was larger in the soleus and diaphragm ($p \leq 0.004$) compared to CON, while old mice had a larger FCSA in the soleus compared to CON after only 9 weeks on a HFD ($p < 0.001$). There were no significant differences between groups in FCSA in the EDL. Oxidative capacity of fibres increased in young only, irrespective of HFD duration ($p < 0.001$), and at all ages the ratio of number of capillaries : oxidative capacity of muscle fibres was lower following a HFD, irrespective of HFD duration ($p = 0.003$).

Conclusion

Morphological changes induced by HFD occur earlier in the muscles from old animals when compared to young. Although all fibre types show similar adaptations to HFD, they are muscle-specific with the EDL being least responsive.

3.1 Introduction

More than 1.9 billion adults (approximately 24.6% of the world's population) over the age of 18 are overweight or obese (WHO, 2018). Under obesogenic conditions, such as excessive and prolonged consumption of high-fat diets (HFD), energy intake exceeds expenditure, causing an accumulation of lipids in adipose tissues and ectopically in non-adipose tissues, including skeletal muscle where excess lipids are stored as intramyocellular lipids (IMCL; lipid *within* the muscle fiber) (Unger et al., 2010; Addison et al., 2014). In addition to systemic inflammation and insulin resistance associated with obesity (Cesari et al., 2005) that have a negative effect on myogenesis, muscular lipid accumulation itself can cause detrimental effects to metabolic and contractile functioning of skeletal muscle (Hancock et al., 2008; Montgomery et al., 2017; Bonen et al., 2015; Choi et al., 2016).

In line with the potential detrimental effects of obesity on skeletal muscle it has been reported that the age-related loss of muscle strength is larger in obese than non-obese women (Tomlinson et al., 2014). The prevalence of sarcopenic obesity, the presence of sarcopenia and obesity in an individual, is increasing in the Western world (Batsis and Villareal, 2018). Yet, the absolute strength and mass of postural and locomotor muscles may be larger in obese than non-obese individuals (Abdelmoula et al., 2012; Maffiuletti et al., 2007; Tomlinson et al., 2014), which may be due to the higher load on the postural muscles during standing and locomotion (Garcia-Vicencio et al., 2016). Nevertheless, muscle strength normalized to body mass is lower in obese than non-obese adolescents (Lee et al., 2012), young (Maffiuletti et al., 2007) and old adults (Zoico et al., 2004; Tomlinson et al., 2014).

The effects of obesity on skeletal muscle are not systemic as reflected by the different response in locomotory and respiratory muscles (Tallis et al., 2018). In a study on obese men and women, the predominant upper-body fat distribution was not associated with a significant impairment of respiratory muscle strength (Magnani and Cataneo, 2007). In the lower leg, however, muscle strength is at least partly attributable to a lower specific tension (maximal muscle force per cross-sectional area) in obese older adults (Choi et al., 2016) and obese adult rodents (Kemmochi et al., 2018; Tallis et al., 2018). Part of the lower specific tension may be caused by a larger volume fraction of IMCL, that may be as high as 5% of the muscle fiber volume in obese individuals (Malenfant et al., 2001).

In addition to a decrease in force-generating capacity, fatigue resistance has also been reported to be reduced in obese people (Syed and Davis, 2000; Maffiuletti et al., 2007). Given the positive relationship between muscle fatigue resistance and oxidative capacity in motor units and single muscle fibres (Degens and Veerkamp, 1994), part of the lower fatigue resistance in skeletal muscle of obese people may be due to a reduced oxidative capacity (Koves et al., 2005; Sparks et al., 2005; Crunkhorn et al., 2007; Shortreed et al., 2009a). However, several studies in humans and rodents showed an increase in oxidative capacity with HFD (Miller et al., 1984; Iossa et al., 2002; Hancock et al., 2008; Sadler et al., 2012; Li et al., 2016; Garcia-Roves et al., 2006), rather than a decrease. This discrepancy may be due to differences in diet duration as the effects of a HFD are time-dependent: a 4-week period of HFD resulted in little effect on the EDL oxidative capacity, but a significant increase after a 12-week period (Eshima et al., 2017). In addition, adaptations to a HFD may be muscle specific and dependent on age. For instance, in young-adult mice, it has been reported that fatigue resistance is reduced in the EDL, but not in the diaphragm and soleus muscle (Hurst et al., 2019), while in old mice 9 weeks HFD did not induce any changes in fatigue resistance in the EDL, soleus or diaphragm.

A HFD-induced shift from glucose to fatty acid metabolism may require an enhanced oxygen supply as fatty acid oxidation requires approximately 8% more oxygen than carbohydrate oxidation for each ATP generated (Silvennoinen et al., 2013). In mice, this shift in metabolism has been suggested to stimulate angiogenesis as reflected by the higher capillary density (CD) and capillary to fiber ratio (C:F) in gastrocnemius and quadriceps femoris muscles after 19 weeks HFD (Silvennoinen et al., 2013), which may be stimulated via the leptin-induced increase in VEGF-A expression (Nwadozi et al., 2019). Others, however, found no difference in the C:F or CD in the plantaris muscle of rats fed a HFD for 8 weeks (Roudier et al., 2009), and it may thus be that only after prolonged exposure to a HFD angiogenesis occurs. The effects of a HFD on the heterogeneity of capillary spacing that has an important impact on tissue oxygenation (Degens et al., 2006) is yet to be elucidated.

The majority of current available data on the effects of a HFD on skeletal muscle morphology do not consider aged animals (Eshima et al., 2017; Silvennoinen et al., 2013; Nwadozi et al., 2016; de Wilde et al., 2008; Bonnard et al., 2008). However, one study that examined the effect of an extended HFD on in vivo skeletal muscle morphology and lipid content in aged rats reported an inverse association between hind limb muscle volume and muscular lipid

content (Bollheimer et al., 2012). This observation suggests that high-fat feeding and subsequent elevated muscular lipids may contribute to age-related muscle atrophy.

The aim of the present study was to comprehensively analyze the effects of a HFD and the duration of HFD on the morphology of the soleus, EDL and diaphragm muscles in mature (20 weeks old) and early ageing (79 weeks old) mice. We hypothesized that a HFD 1) induces in all muscles an increased IMCL, and 2) that the locomotory muscles will show a *decrease* in oxidative capacity and capillarization while 3) the diaphragm shows an *increase* in oxidative capacity and capillarization, where 4) the HFD-induced changes will increase with duration of feeding and 5) be more pronounced at old age.

3.2 Methods

3.2.1 Animal and diets

We compared the effect of HFD in the diaphragm, soleus and EDL in female CD-1 mice (Charles River, Harlan Laboratories, UK) that were 20 or 79 weeks old at the end of the experiment. The 79-week-old female mice are a model of early ageing, where survival is 50% (Navarro et al., 2002) and the muscles show morphological changes suggestive of early ageing (Messa et al., 2019), further supported by a significant declines in soleus and EDL specific tension and specific power compared to 10-week-old animals (Hill et al., 2018). In addition, the CD-1 stock is heterogeneous and therefore have greater genetic variability than many other strains of lab mice (Aldinger et al., 2009; Rice and Obrien, 1980) and as such reflect the genetic variability seen in humans.

The female CD-1 mice were housed and aged at Coventry University in cages of 8–10 individuals in 12-12h light-dark cycle at 50% relative humidity. Fifty-six 4-week-old mice and sixty 68-week-old mice were randomly allocated to either HFD or normal diet.

The 4-week-old mice were provided with normal chow (Y-CON, n=28), or a HFD for 16 weeks (YL-HFD, n=28). An additional group of mice was provided with a HFD for 8 weeks from the age of 12 weeks (YS-HFD, n = 16).

Animals used for the ageing element of the study were purchased at 9 weeks of age and provided access to a standard lab chow only during ageing. At the age of 70 weeks they either continued with regular chow (O-CON, n=30) or were given a HFD (OS-HFD, n=30) for a duration of 9 weeks.

All experimental groups provided a HFD were simultaneously provided with the standard lab chow in the form of a self-selected forage diet (Hurst et al., 2019; Hill et al., 2019). Access to each diet and water was provided *ad libitum* for all groups.

The caloric composition of the standard chow was: protein 17.5%, fat 7.4%, carbohydrate, 75.1%; gross energy 3.52 kcal.g^{-1}; metabolizable energy 2.57 kcal.g^{-1} (CRM(P) SDS/Dietex International Ltd, Whitham, UK). The caloric composition of the HFD was: protein 18.0%, fat 63.7%, carbohydrate, 18.4%; gross energy 5.2 kcal.g^{-1}; metabolizable energy 3.8 kcal.g^{-1} (Advance protocol PicoLab, Fort Worth, USA).

At the age of 20 or 79 weeks, mice were sacrificed by cervical dislocation. All experimental procedures were carried out in compliance with the local ethical review of the Coventry University under UK Home Office project license held in accordance with the Animals (Scientific procedures) Act 1986. Animals were weighed, and snout-to-anus length determined to calculate the body mass index (BMI), as body mass (kg) divided by length (cm) squared.

The removal and processing for analysis of muscle morphology of the soleus, EDL and right part of the diaphragm muscles were performed as outlined previously (Tallis et al., 2017a; Messa et al., 2019). After excision, the muscles were blotted dry, weighed, embedded in Tissue-Tek freezing medium (Leica Biosystems, Nußloch, Germany), frozen in liquid-nitrogen-cooled isopentane (Sigma-Aldrich, Steinheim, Germany) and stored at -80°C. Only one muscle per animal was collected.

3.2.2 Histological analysis and microscopy

Serial 10-µm thick cross-sections of the soleus, EDL and diaphragm muscles were cut with a cryostat (CM3050S; Leica, Nußloch, Germany) at -21°C and collected on Superfrost Plus microscope slides. Serial sections were stained for intramuscular cellular lipid (IMCL), myosin heavy chain (MHC), capillaries, or succinate dehydrogenase (SDH) (Messa et al., 2019).

Intramyocellular fat. Sudan Black B was utilized to stain IMCL. The Sudan Black B dye stains mainly neutral lipids (mainly triglycerides) with a blue-black tint. Briefly, air-dried sections were fixed in 10% formalin for 10 min. Sections were then washed three times for 1 min in distilled water before incubation in propylene glycol for 3 min. Sections were then incubated in the Sudan Black B solution (preheated at 60°C) for 7 min, differentiated in 85%

propylene glycol for 3 min and subsequently washed three times 1 min in distilled water. Sections were cover-slipped using glycerol gelatin.

Fiber typing. Serial sections were immunohistochemically stained for type I, IIa, IIx or IIb MHC using mouse monoclonal primary antibodies BA-D5 (1 $\mu g \cdot mL^{-1}$), SC-71(1 $\mu g \cdot mL^{-1}$), 6H1 (10 $\mu g \cdot mL^{-1}$) and BF-F3 (5 $\mu g \cdot mL^{-1}$), respectively (Development Studies Hybridoma Bank, Iowa, USA). One section was co-stained for type I, IIa and IIx MHC and a serial section for type IIb MHC.

Sections were fixed with ice-cold acetone for 15 min and then blocked for 45 min with 10% goat serum in phosphate-buffered saline (PBS) at room temperature. Following a wash with PBS, the sections were incubated with the primary antibody for 90 min in a humid chamber. The sections were subsequently washed in PBS and incubated in the dark for 60 min with Alexa 350 IgG anti-mouse (2 $\mu g \cdot mL^{-1}$, Invitrogen, UK) and Alexa 488 IgG anti-mouse (2 $\mu g \cdot mL^{-1}$, Invitrogen, UK) for type I and IIa fibers, respectively and Alexa 555 IgG anti-mouse (2 $\mu g \cdot mL^{-1}$ Invitrogen, UK) for both type IIx and IIb fibers. Sections were washed, dried and mounted using Prolong Diamond anti-fade mounting medium (Life Technologies, UK). Sections without the primary antibodies served as negative controls. Images were taken with a Carl Zeiss Axio MRc Camera (Göttingen, Germany) on a Zeiss fluorescence microscope (10-x objective).

Capillary staining. Capillaries were visualized using lectin as described previously (Ballak et al., 2016). Briefly, air-dried sections were fixed with ice-cold acetone for 15 min, and blocked with 0.1% bovine serum albumin (BSA) diluted in 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) for 60 min. Subsequently, the sections were treated with a peroxide solution for 30 min and incubated with biotinylated *Griffonia (Bandeira) simplicifolia* lectin (GSL I; Vector Laboratories, Peterborough, UK; 50 $\mu g \cdot mL^{-1}$ diluted in 1% BSA/HEPES) for 60 min. A 5-min wash was conducted between each step. Sections were then treated with avidin-biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories, Peterborough, UK) for 60 min, washed with HEPES, and incubated with Horse Radish Peroxidase Substrate Diaminobenzidine (Vectastain DAB kit, Vector Laboratories, Peterborough, UK) for 5 min. After a wash in distilled water, the sections were mounted in glycerol gelatin (Sigma-Aldrich, Aldrich, UK).

Succinate dehydrogenase. The succinate dehydrogenase (SDH) activity was assessed according to the protocol described by Wüst et al. (2009). Sections were incubated for SDH

in 37.5 mM sodium phosphate buffer (pH 7.6), 74 mM sodium succinate and 0.4 mM tetranitro blue tetrazolium in the dark at 37°C for 20 min. The reaction was stopped with 0.01 Normal hydrochloric acid for 10 s. Then, the slides were washed with two changes of distilled water, mounted in glycerol-gelatin and stored in the dark until measurement of the staining intensity within two days. All samples were processed simultaneously in the same incubation solution, ensuring that all samples were subjected to the same conditions. Characteristic staining of EDL in an old mouse is shown in Fig. 3.1.

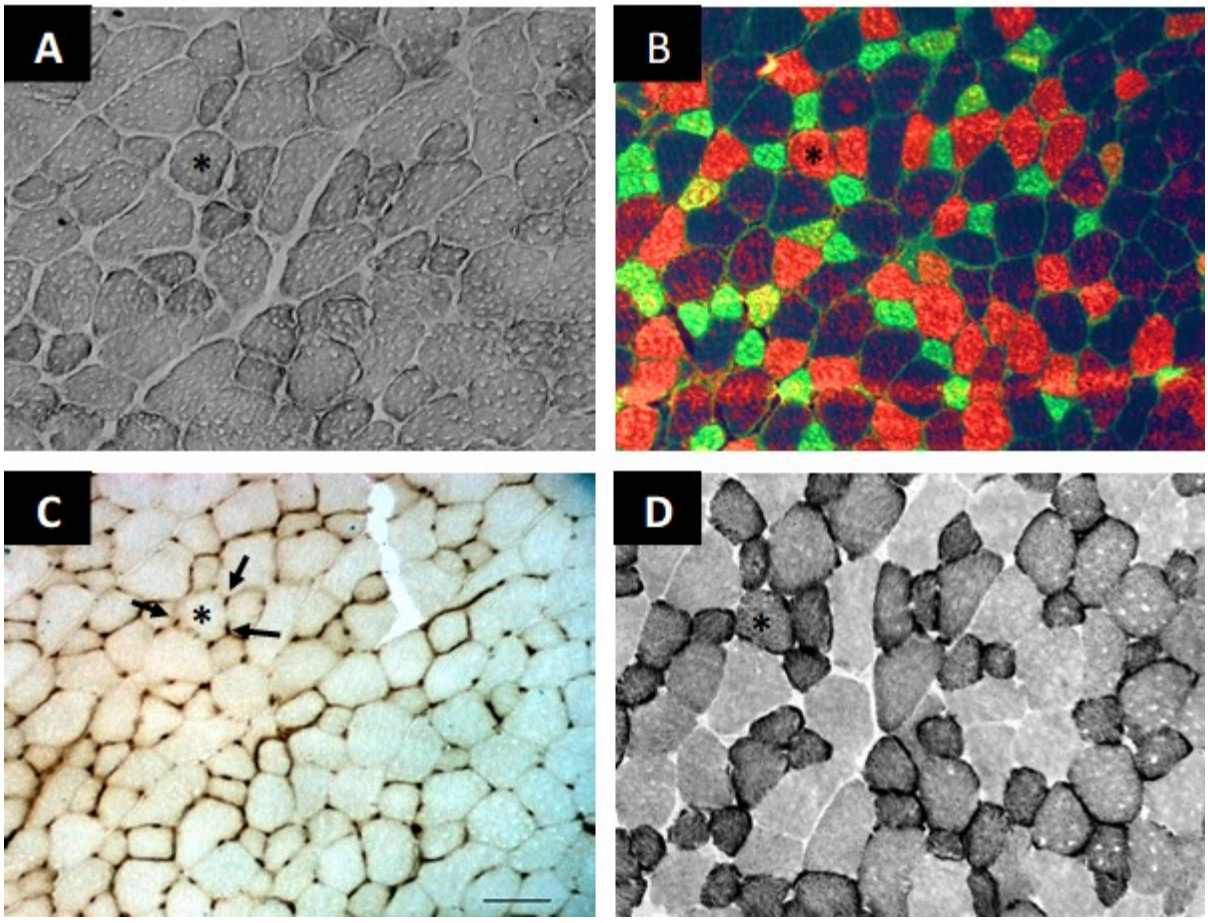


Figure 3.1 Serial sections of extensor digitorum longus of a 79-week-old mouse fed a high-fat diet for 9 weeks stained for (**A**) intramyocellular fat, (**B**) myosin heavy chain, (**C**) capillaries (arrows) and (**D**) succinate dehydrogenase (SDH) activity. *: indicates same fibre in the four panels. Green: type IIa, red: type IIx and unstained: type IIb fibers. Scale bar = 100 µm.

3.2.3 Morphometry analysis

Stained sections were photographed with a digital camera (Zeiss AxioCam MRc) on a light microscope (Carl Zeiss, Göttingen, Germany; 20x objective). At least two images per muscle cross-section were taken and 245 ± 74 complete fibers were analyzed per sample.

Intramyocellular lipid. The IMCL content of individual fibers was determined using a microscope with a 20× objective and bright-field settings. Images were digitally captured using a white and black AxioCam ICMI camera (Göttingen, Germany) and analyzed with ImageJ (National Institutes of Health, USA, https://imagej.nih.gov/ij/). The fiber of interest was outlined, and the grey levels were converted to optical density (OD) using a calibration curve constructed from a series of filters of known OD. For each section, a separate calibration curve was constructed, and all images were taken at the same exposure with the same microscope settings. The OD of the Sudan Black B stain was determined in individual fibers and the background OD for each fiber was subtracted from the OD measured. The higher the net OD for the Sudan Black B stain, the higher the IMCL in the fiber.

Fiber type composition and fiber size. The fiber outlines and capillary centres were collected with a digitizing program (Program Btablet, BaLoH Software, Ooij, The Netherlands, http://www.baloh.nl) and the data analyzed with AnaTis (BaLoH Software). The fiber cross-sectional area (FCSA) was calculated for each fiber. The fiber-type composition was expressed as number percentage.

Capillarization. The capillarization in the muscles was determined with the method of capillary domains (Degens et al., 1992; 2006; Hoofd et al., 1985) using AnaTis. Briefly, a capillary domain is defined as the area of a muscle cross-section surrounding an individual capillary delineated by equidistant boundaries from adjacent capillaries. The capillary domain provides a good estimate of the capillary oxygen supply area, even in muscles with mixed fiber type composition (Al-Shammari et al., 2014). In addition to the overall parameters of muscle capillarization, including capillary density (CD; number of capillaries per mm^2) and the capillary to fiber ratio (C:F), this method enables the identification of the capillary supply to individual fibers even when they lack direct capillary contact. The local capillary to fiber ratio (LCFR), the sum of the fractions of the capillary domains overlapping a particular fiber, provides a continuous, rather than a discrete value of the capillary supply to a fiber and takes into consideration that a capillary supplies more than one fiber (Barnouin et al., 2017). The ratio of LCFR to the FCSA provides the capillary density for a given fiber, defined as the capillary fiber density (CFD). The radius (R) of a domain, calculated from a circle with the same surface area, provides an indication of the maximal diffusion distance from the capillary to the edge of its domain. R shows a lognormal distribution, and thus the Log_RSD (logarithmic standard deviation of the domain radius) is a measure of the heterogeneity of

capillary spacing, which plays a vital role in muscle oxygenation (Degens et al., 2006; Hoofd et al., 1985).

Succinate dehydrogenase. Photomicrographs of stained sections of SDH were taken on a light microscope with a 660-nm interference filter and a white and black AxioCam ICM1 camera. All images were taken at the same exposure with the same microscope settings. Images were analyzed using ImageJ (National Institutes of Health, USA). To measure the OD of a given fiber, the outline of the fiber was drawn and the background OD subtracted. For each session, a separate calibration curve was made with filters of known OD (A_{660}). The calibration curve was used to convert the absorbance values of the SDH staining into OD values.

To assess the SDH activity (SDH-OD), the OD (A_{660}) was converted to the rate of staining and expressed as the increase in absorbance at 660 nm (A_{660}) per µm section thickness per second of incubation time ($\Delta A_{660} \cdot \mu m^{-1} \cdot s^{-1}$). The SDH-OD multiplied by the FCSA yielded the integrated SDH activity (SDH-INT in $\Delta A_{660} \cdot \mu m\ s^{-1}$):

$$\text{SDH-INT} = \text{SDH-OD} \times \text{FCSA}$$

It has been shown that the mass-specific maximal oxygen uptake (VO_{2max} in $ml \cdot kg^{-1} \cdot min^{-1}$) is proportional to SDH-OD and that the SDH-INT is linearly related to the maximum rate of oxygen uptake ($VO_{2max\ fibre}$ in $ml \cdot min^{-1}$) of the muscle fiber (van der Laarse et al., 1989)

3.2.4 Statistical analysis

All statistical analyses were performed using IBM SPSS version 25 (IBM SPSS Statistics for Windows, IBM Corp, Armonk, NY, USA). The Shapiro-Wilk test indicated that all data were normally distributed. The effect of diet and HFD duration were evaluated in the young-adult and old mice separately. A four-way ANOVA with the factors diet, age, muscle and fiber type - where appropriate - applied and three-and four-way interactions were excluded. If a main effect of diet or interactions with diet were found, LSD post-hoc tests were performed to locate the differences. To assess to what extent the capillary supply to a fiber was determined by the oxidative capacity of the fiber (SDH-OD), FCSA, fiber type, muscle of origin, age and/or diet we performed a stepwise regression. Hybrid (type IIax and IIxb) were excluded from the analysis as they occurred infrequently. Statistical significance was accepted at $p < 0.05$. A measure of statistical power (sp) for each each p-value was included. Data are expressed as mean ± SD.

3.3 Results

The data of the young-adult and old control muscles, and the effects of ageing have been published previously (Messa et al., 2019) and the ageing comparison is not repeated here. However, we did consider age * diet interactions, where a significant interaction indicates that the response to a HFD differs between young-adult and old mice.

In many cases, the collected muscles were not suitable for histological analysis due to damage during the preparation, freezing artefacts and/or large areas where muscle fibers were cut longitudinally; conditions that precluded adequate morphological analysis. Because of this, no data were collected for the EDL from YS-HFD mice. Nevertheless, we included them in Table 1, as they were still applicable when calculating the body and muscle masses.

3.3.1 Mice characteristics

In young-adult mice, an increase in body mass, BMI and soleus and EDL mass were only seen after 16 weeks of a HFD ($p \leq 0.001$; Table 3.1), with no significant differences from CON after 8 weeks HFD. In old mice, 9 weeks of a HFD induced an increase in body mass, BMI and soleus and EDL mass ($p \leq 0.001$; Table 3.1) while the muscle mass to body mass ratio was not significantly different between the HFD and CON. There were no significant diet * fiber type interactions for any of the parameters, indicating that fibers of all types responded similarly to diet. Whole animal fat pad mass was greater in the HFD groups compared with CON ($p \leq 0.001$; Table 3.1), but body length did not differ significantly between the HFD and CON mice.

Table 3.1 Characteristics of young-adult and old female CD-1 mice fed with a control (CON) or high fat diet (HFD)

	Body mass (g)	Body length (cm)	Fat pad mass (g)	BMI ($kg \cdot m^{-2}$)	Soleus MM (mg)	Soleus MM/BM ($mg \cdot g^{-1}$)	EDL MM (mg)	EDL MM/BM ($mg \cdot g^{-1}$)
Young-adult								
CON (n=29)	38.5 (0.9)	11.3 (0.1)	0.07 (0.08)	3.04 (0.06)	10.1 (0.5)	0.26 (0.01)	10.0 (0.7)	0.28 (0.02)
YS-HFD (n=16)	40.2 (2.2)	10.9 (0.1)	2.97 (0.60)^{c}	3.38 (0.22)	10.6 (0.6)	0.31 (0.02)		
YL-HFD (n=28)	52.7 (2.3)^{c}	11.6 (0.1)	5.24 (0.52)^{c}	3.93 (0.15)^{c,h}	13.0 (0.8)^{c,h}	0.25 (0.02)	15.4 (1.3)^{c}	0.28 (0.03)
Old								
CON (n=30)	47.2 (1.6)	11.8 (0.1)	3.58 (0.53)	3.37 (0.08)	9.4 (0.4)	0.20 (0.01)	10.6 (0.5)	0.24 (0.02)
OS-HFD (n=30)	58.6 (1.9)^{c}	12.4 (0.1)	7.85 (0.70)^{c}	3.80 (0.09)^{c}	10.9 (0.5)^{c}	0.19 (0.01)	12.5 (0.4)^{c}	0.22 (0.01)

YL-HFD: 16 weeks HFD; YS-HFD: 8 weeks HFD; OSL-HFD: 9 weeks HFD; BM: Body mass; BMI: body mass index (body mass divided by snout-to-anus length squared); EDL: extensor digitorum longus muscle; MM/BM: muscle mass divided by BM. Data are mean ± SEM. ^{c} different from standard diet at $p \leq 0.001$; ^{h}: different from HFD at $p \leq 0.011$.

3.3.2 Intramyocellular lipid (IMCL) level

In young-adult mice, the IMCL levels were elevated after 16 weeks HFD above CON, but not after 8 weeks HFD, in the soleus, EDL and diaphragm ($p \leq 0.006$; sp = 0.901). In the old mice 9 weeks, HFD resulted in an elevated IMCL in all three muscles ($p \leq 0.003$; sp = 0.805; Fig. 3.2).

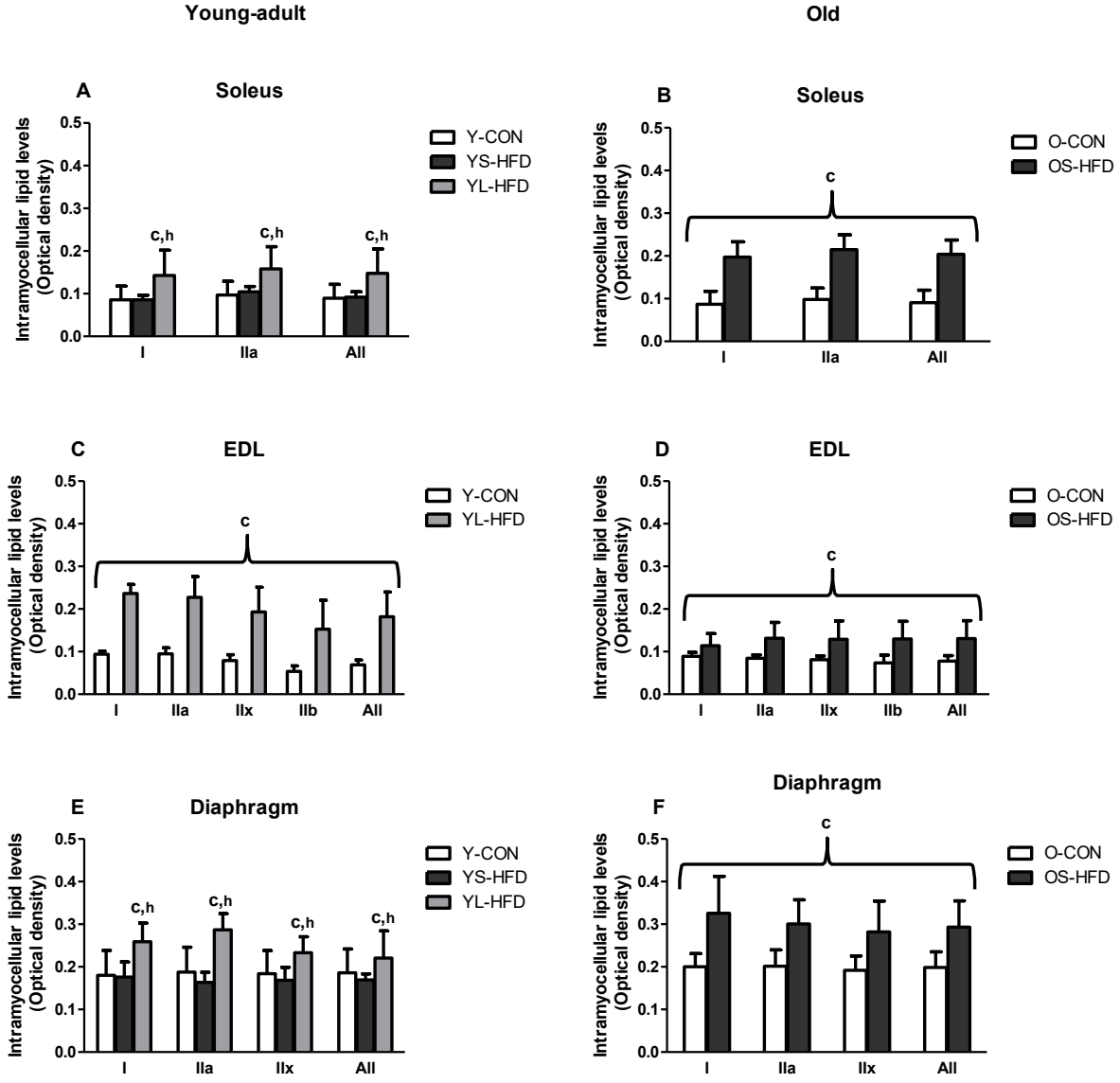


Figure 3.2 Intramyocellular lipids in the (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks of high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20-week-old mice; right: 79-week-old mice. [c] different from control; [h] different from YS-HFD at $p \leq 0.006$. Values are means ± SD (n = 3-7).

3.3.3 Muscle fiber type composition and fiber cross-sectional areas

Fiber type composition. Figure 3.3 shows that the fiber type composition in the soleus (Fig. 3.3A & B), EDL (Fig. 3.3C & D) and diaphragm (Fig. 3.3E & F) muscles was not significantly affected by either 8-9 or 16 weeks of HFD in neither young-adult nor old mice.

Fiber cross-sectional area (FCSA). Young-adult animals on 16 weeks HFD had larger FCSAs in the soleus and diaphragm ($p \leq 0.004$; sp = 0.766), however, no significant difference in FCSA was seen between CON mice and mice 8 weeks on a HFD (Fig 3.4A & E). Old mice had a larger FCSA in the soleus after 9 weeks on a HFD ($p < 0.001$; sp = 0.969; Fig. 3.4B). In the EDL muscle, no significant differences in FCSA were observed between HFD and control groups in either young-adult or old mice (Fig. 3.4C & D).

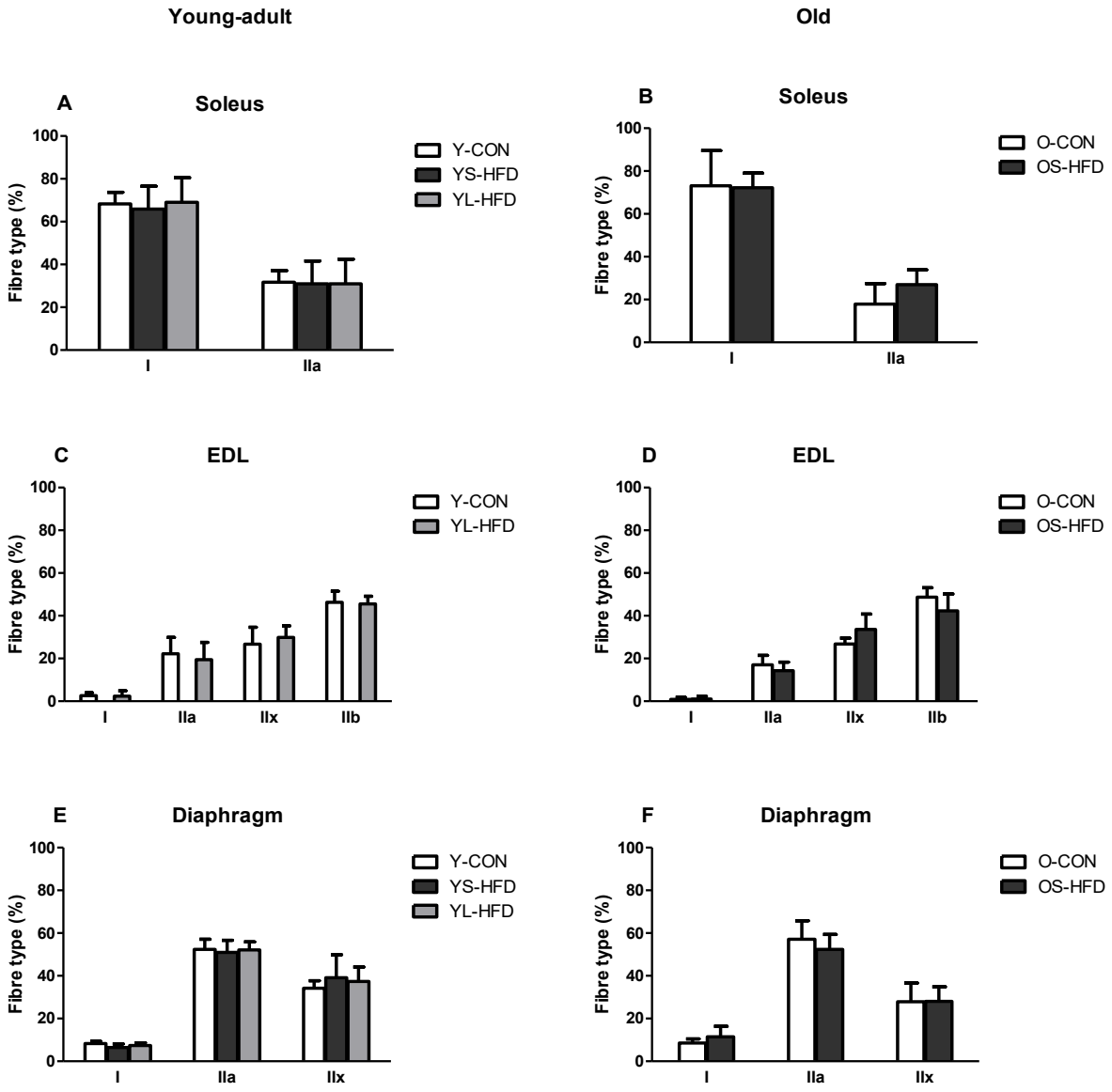


Figure 3.3 Fiber type composition in the (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks of high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20-week-old mice; right: 79-week-old mice. Values are means ± SD (n = 3-7).

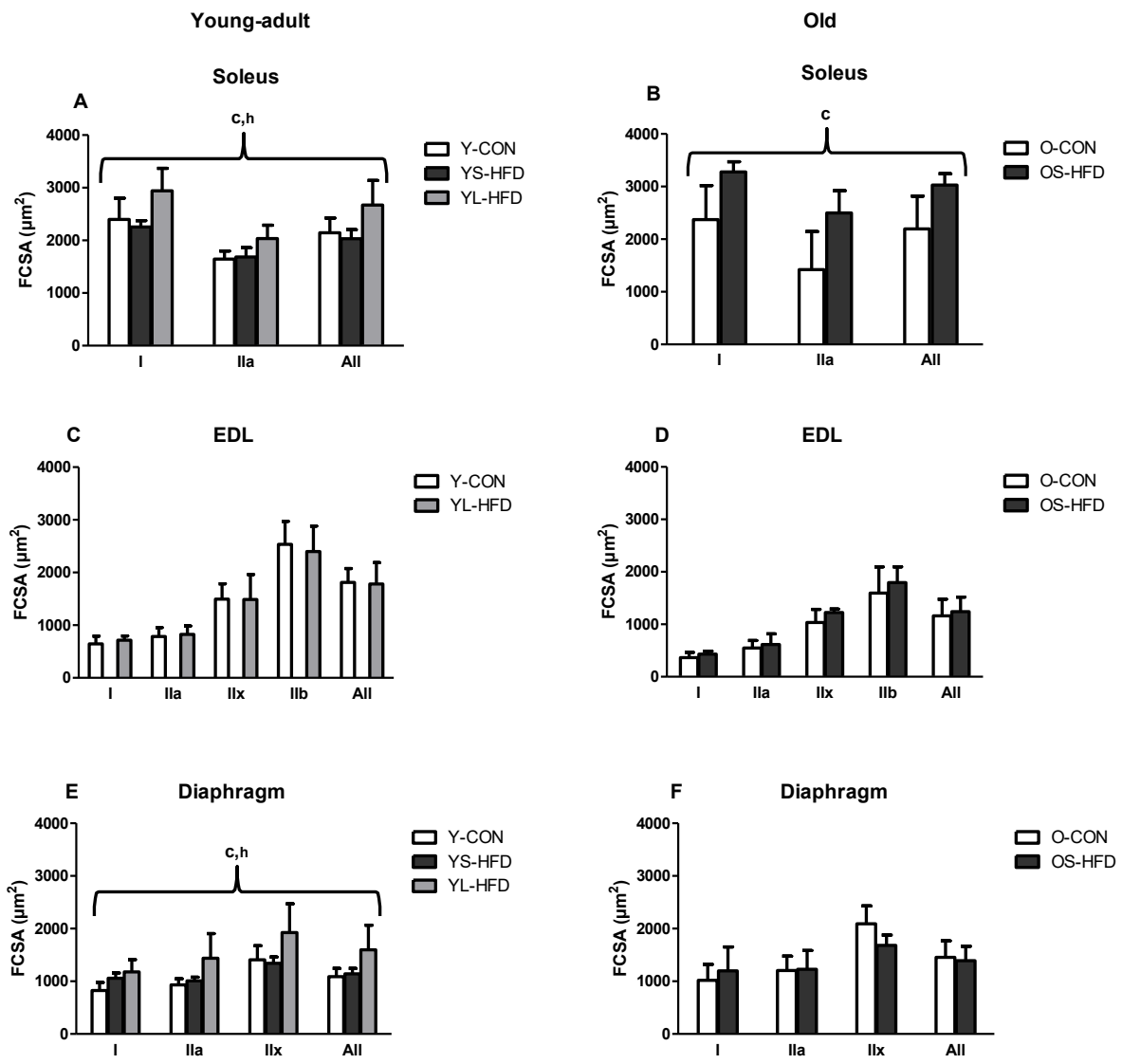


Figure 3.4 Fiber cross-sectional area (FCSA) in the (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20-week-old mice; right: 79-week-old mice. [c] different from CON; [h] different from YS-HFD at $p \leq 0.004$. Values are means ± SD (n = 3 -7).

3.3.4 Succinate dehydrogenase (SDH) activity (SDH-OD)

In young-adult mice, the mass-specific SDH activity, the SDH-OD, was higher in the soleus, EDL and diaphragm muscle from animals in the HFD than CON group, irrespective of duration of HFD ($p < 0.001$; $sp = 0.992$; Fig. 3.5A, C & E), but no significant effect of HFD was seen in the old animals (Fig. 3.5B, D & F). In both young-adult and old mice, the SDH-INT in the EDL was not significantly different between animals fed a HFD or CON diet in (Fig. 3.6C & D). In young-adult mice, the SDH-INT of fibers in the soleus muscle was L-

HFD > HFD > CON (Fig. 3.6A; $p \leq 0.033$; sp = 0.982) and in the diaphragm L-HFD > CON (Fig. 3.6E; $p < 0.001$; sp = 0.928). In the old mice, the HFD soleus also had a higher SDH-INT than CON (Fig. 3.6B; $p = 0.008$; sp = 0.901), but no significant effect of HFD was seen in the diaphragm of the old animals (Fig. 3.6F).

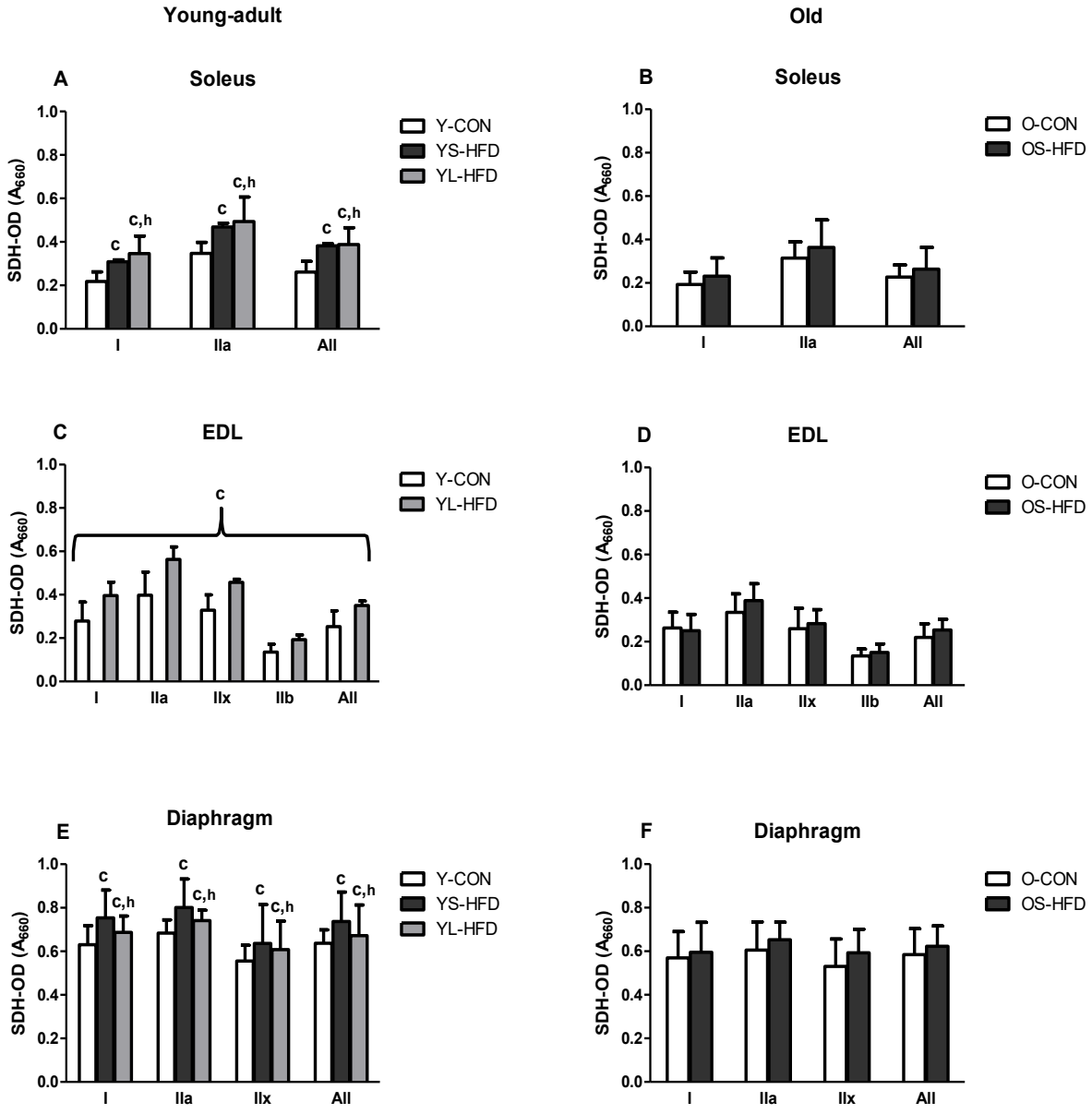


Figure 3.5 Succinate dehydrogenase (SDH) optical density in (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20-week-old mice; right: 79-week-old mice. [c] Different from CON at $p < 0.001$; [h] different from YS-HFD at $p \leq 0.025$. Values are means ± SD (n = 3-7).

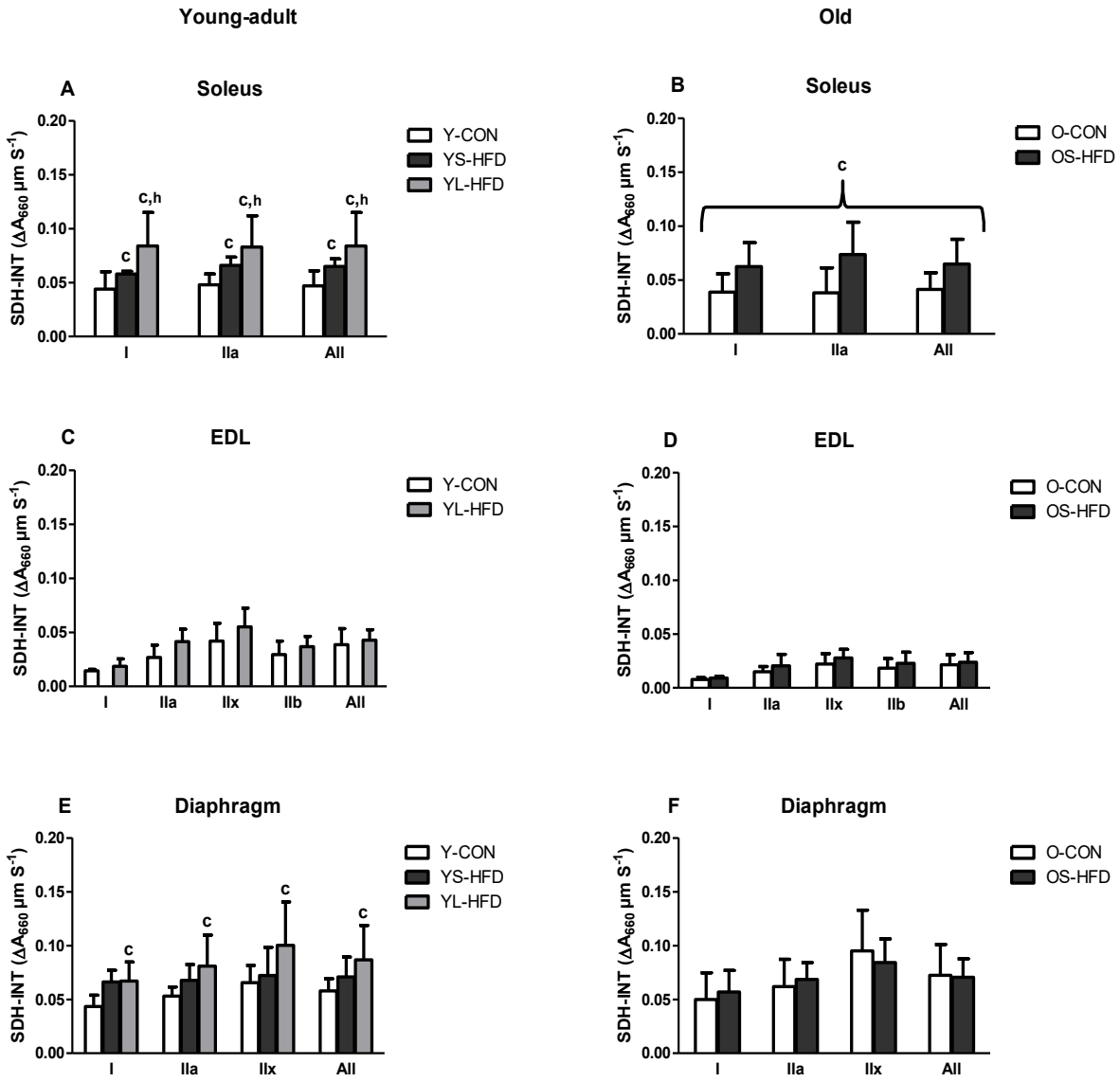


Figure 3.6 Integrated succinate dehydrogenase activity (SDH-INT) in (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20-week- and right 79-week-old mice; ; [h] different from YS-HFD at $p \leq 0.033$. [c] Different from CON at $p \leq 0.008$. Values are means ± SD (n = 3-7).

3.3.5 Muscle capillarization

Indices of global capillary supply. In the young animals 16 weeks, but not 8 weeks HFD, induced a significant increase in the C:F in all three muscles ($p \leq 0.006$; sp = 0.928; Fig. 3.7A). In the old animals, 9 weeks HFD induced an increase in C:F in the soleus muscle only ($p = 0.006$; sp = 0.912; Fig. 3.7B). There were no significant differences in the CD between control animals and animals fed for 8-9 or 16 weeks a HFD in any of the muscles from

young-adult or old animals (Fig. 3.7 C & D). There was also no significant differences in the $Log_R SD$ between CON and HFD in any of the muscles (Fig. 3.7E & F).

Local capillary to fiber ratio (LCFR). In young animals, there was a main effect of the diet on the LCFR ($p < 0.001$, sp = 0.760; Fig. 3.8). However, the muscle * diet interaction ($p = 0.003$; sp = 0.886) indicated that the effect of diet differed between muscles. In the soleus muscle of young mice, the LCFR in animals on a HFD for 8 weeks was lower than CON ($p = 0.045$; sp = 0.594) and was higher in mice on 16 weeks on a HFD than CON and those on 8 weeks HFD (Fig 3.8A; $p \leq 0.005$; sp = 0.562). In the soleus of old mice, 9 weeks on a HFD resulted in a higher LCFR (Fig. 3.8B; $p < 0.001$; sp = 0.991). Post-hoc analysis revealed that in the EDL of both young and old animals the LCFR did not differ significantly between animals on a CON or HFD (Fig 3.8C & D). The LCFR of fibers in the diaphragm was higher in mice on a HFD for 16 weeks than CON ($p = 0.016$; sp = 0.587). There was no significant difference in diaphragm LCFR between CON and 8 weeks HFD in either young or old animals (Fig. 3.8E & F).

Capillary fiber density (CFD). There was no main effect of diet in either young or old animals (Fig. 3.9). However, the diet * muscle interaction in young animals ($p = 0.008$; sp =0.705) was explained by a higher CFD in the diaphragm of young animals fed a HFD for 16 weeks than in control-fed or those fed a HFD for 8 weeks.

3.3.6 Determinants of fiber capillary supply

To assess differences between muscles, fiber types and diet in the matching of oxygen supply (LCFR) and demand (SDH-INT) of a fiber, the LCFR/SDH-INT was calculated as a measure of the supply:demand ratio. The HFD led to a decrease in the LCFR/SDH-INT of muscle fibers ($p = 0.003$), irrespective of duration of HFD, age, fiber type and muscle of origin (Fig. 3.10) as reflected by the absence of significant interactions between diet with age, muscle and type.

In soleus, EDL and diaphragm, the LCFR correlated positively with FCSA in both CON and HFD (Fig. 3.11).

The contribution of different factors to the LCFR (capillary supply of a fiber) was assessed by performing a stepwise linear regression. The factors included in the model were FCSA, SDH-OD, muscle (soleus, EDL and diaphragm) and diet. The primary determinant of LCFR was FCSA ($r_{adj}^2 = 0.670$; $p < 0.001$), which explained most (65.3%) of the variance in LCFR.

Muscle ($r_{adj}^2 = 0.783$; $p < 0.001$), SDH-OD ($r_{adj}^2 = 0.790$; $p < 0.001$) and diet ($r_{adj}^2 = 0.797$; $p < 0.001$) explained an additional 12.9% of the variance in LCFR, with only a 0.7% contribution of diet.

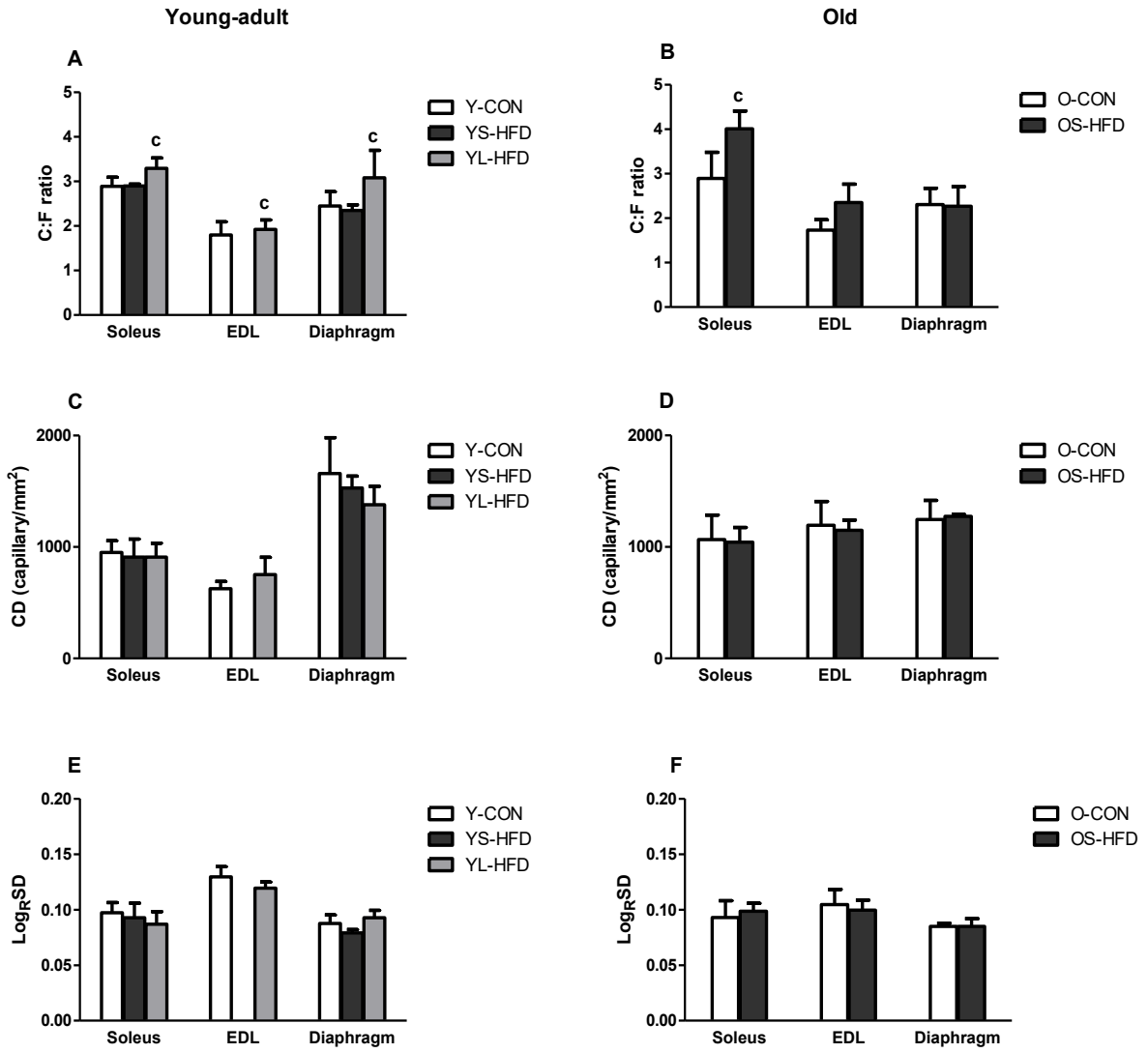


Figure 3.7 A-B) Capillary to fiber ratio (C:F), C-D) capillary density (CD) and E-F) heterogeneity of capillary spacing (Log_RSD) in the soleus, extensor digitorum longus (EDL) and diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20- and right:79-week-old mice. ^{c} different from control at $p \leq 0.006$. Values are means ± SD (n = 3-7).

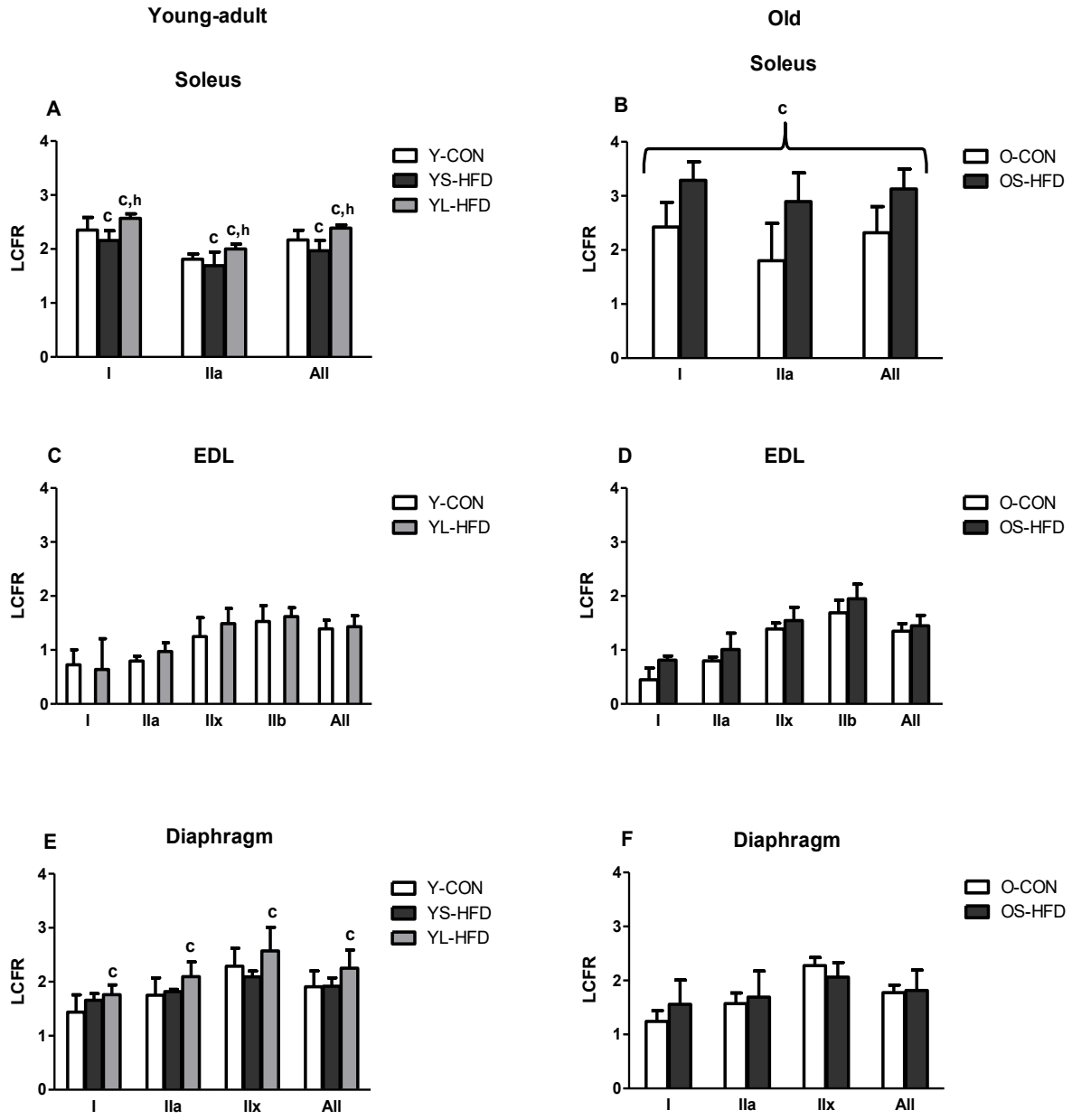


Figure 3.8 Local capillary to fiber ratio (LCFR) in (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20- and right:79-week-old mice. ^{c} different from CON at $p \leq 0.016$; h different from YS-HFD at $p = 0.045$. Values are means ± SD (n = 3-7).

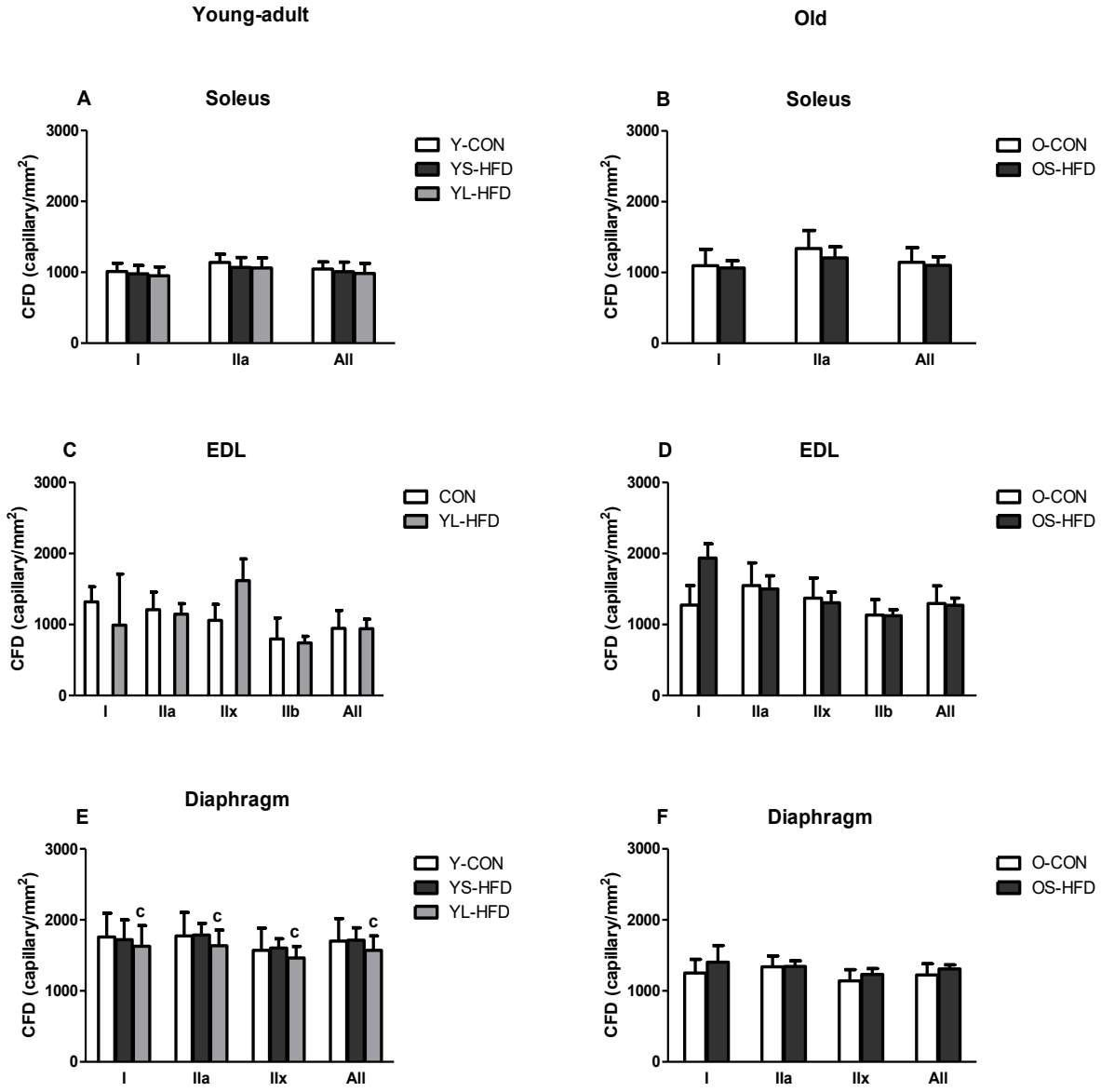


Figure 3.9 Capillary fiber density (CFD) in (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20- and right:79-week-old mice. ^{c} different from CON at $p = 0.003$. Values are means ± SD ($n = 3$-7).

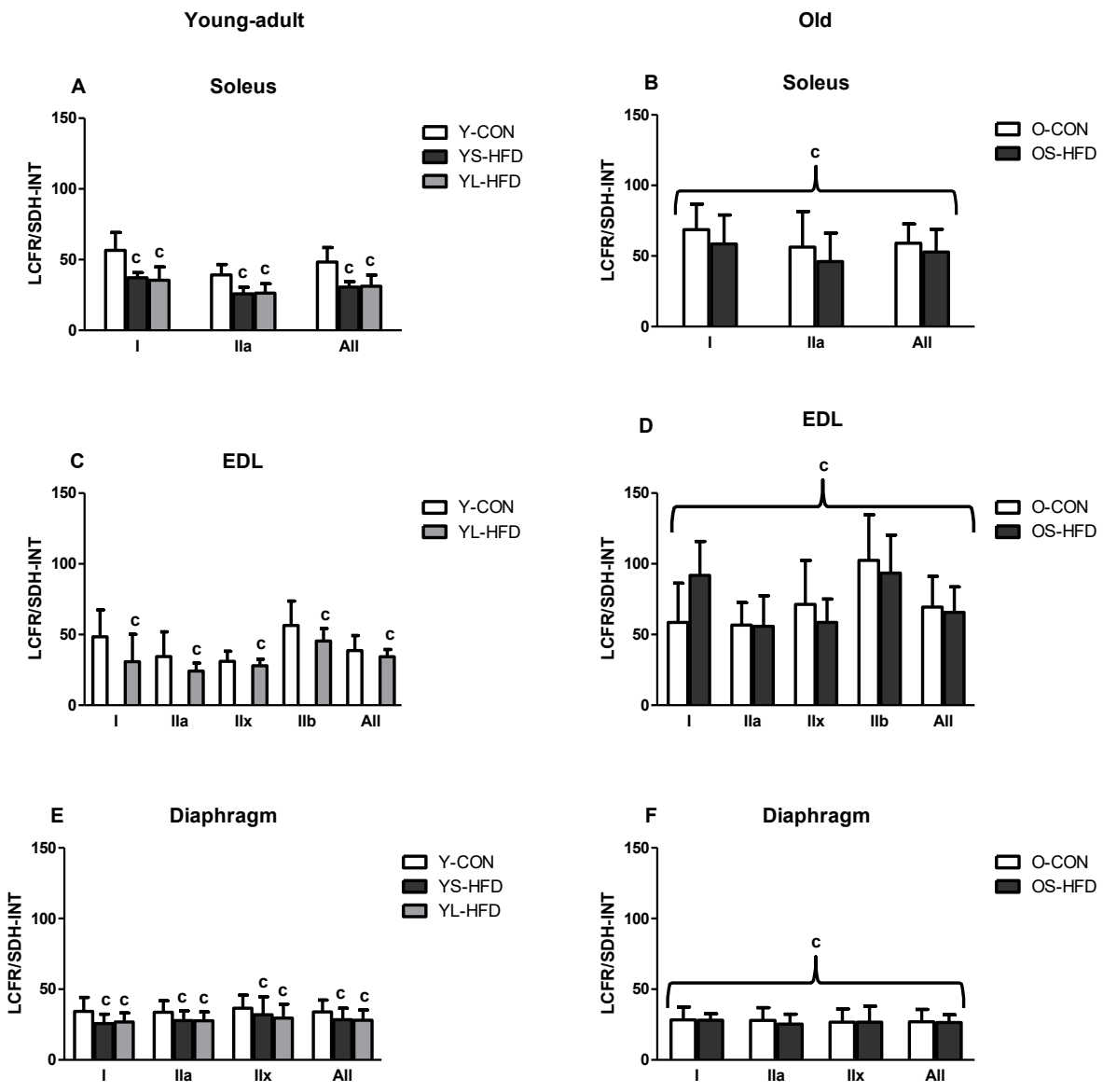


Figure 3.10 Local capillary to fiber ratio (LCFR):Integrated succinate dehydrogenase activity (SDH-INT) in (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20- and right:79-week-old mice. ^{c} main effect of diet $p = 0.003$. Values are means ± SD (n = 3-7).

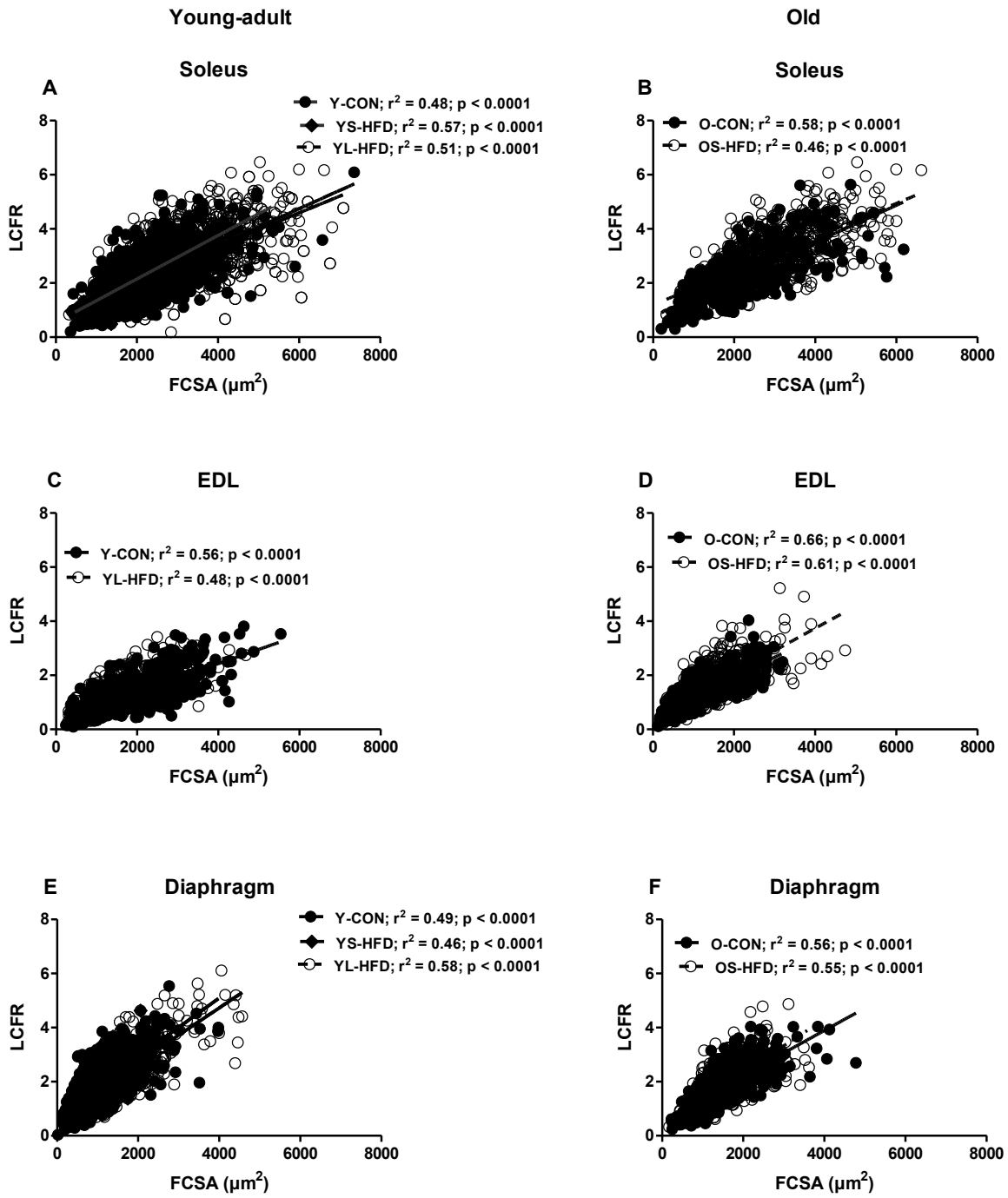


Figure 3.11 Relationship between the local capillary-to-fiber ratio (LCFR) and the fiber cross-sectional area (FCSA) of individual fibers in (A-B) soleus, (C-D) extensor digitorum longus (EDL) and (E-F) diaphragm muscles of control (CON) and 8-9 weeks high fat diet (S-HFD) mice; L-HFD: 16 weeks HFD; left: 20- and right:79-week-old mice (All fibers combined for 20-week old Soleus n = 3673; EDL n = 817; Diaphragm n = 3331; 79-week old Soleus n = 1006; EDL, n = 1159; Diaphragm, n = 1266).

3.4 Discussion

In the present study, we evaluated whether morphological changes induced by a high-fat diet (HFD) differed between the soleus, EDL and diaphragm and whether the adaptations differed between young-adult (20 weeks) and old mice (79 weeks). We observed that although the effect of a HFD did not differ between fiber types, the changes in muscle morphology seemed to be least pronounced in the EDL muscle. While increases in body mass and BMI, and accumulation of intramyocellular lipid (IMCL), an increase in oxidative capacity, angiogenesis and fiber hypertrophy occurred in the diaphragm and soleus muscle, they occurred sooner in old than young mice. The angiogenesis was in general proportional to the HFD-induced increase in fiber size, but signs of a developing mismatch between oxygen supply and demand were reflected by the lower ratio of capillary number: maximal oxygen consumption of a fiber (reflected by the LCFR:SDH-IT ratio) in animals on a HFD. Thus, older animals are more susceptible to HFD-induced changes in muscle morphology that may result in a mismatch between oxygen supply and demand to the working muscles.

Effects of HFD on body weight and intramyocellular lipid levels (IMCL)

The consumption of a HFD resulted in a substantial accumulation of gonadal fat, increased BMI and muscle mass. In line with previous studies (Hegarty et al., 2002; Silvennoinen et al., 2013; Eshima et al., 2017; Baek et al., 2018; Andrich et al., 2018; Kaneko et al., 2011) this was accompanied by elevated IMCL levels of the soleus, diaphragm and EDL of our mice. This HFD-induced increase in body mass, BMI and IMCL occurred after only 9 weeks in old mice, but was only seen after 16 weeks in the young-adult mice, similar to the absence of changes in IMCL after 4 weeks, and elevated IMCL levels after 12 weeks of a HFD seen by others in young-adult mice (Eshima et al., 2017). It should be noted, however, that the BMI and gonadal fat pad mass of the control old mice was higher than that of the young-adult mice, suggesting greater adiposity. As fatty acids are primarily stored in adipocytes and only when in excess are stored elsewhere (Koutsari et al., 2012), their larger adiposity may cause the earlier deposition of fatty acids in the muscle of old mice. Whatever the explanation, this suggests that old mice are more susceptible to the effects of a HFD.

Muscle fiber type composition and fiber cross-sectional areas (FCSA)

Our data showed that a HFD did not induce a change in fiber type composition in any of the muscles, irrespective of age, as has been also observed by others in rodents (Turpin et al., 2009; Shortreed et al., 2009b). Eshima et al. (2017), however, reported an increased

proportion of type IIx fibers concomitant with a decrease of type IIb fibers in the EDL muscle of mice on a 12-week HFD. The discrepancy between their study and our study may be related to the sex and strain of the mice, where we used female CD-1 mice and Eshima et al. (2017) used male C57BL/6J mice. Indeed, it has been found that 52 weeks HFD caused a decrease of type I fibers in the soleus of male, but not female mice (Denies et al., 2014). The absence of significant changes in fiber type composition corresponds with the observation that in both the young-adult (Hurst et al., 2019; Tallis et al., 2017a) and old mice (Hill et al., 2019) there were no significant changes in the velocity of contraction at which peak power occurred or differences in fatigue resistance.

Corroborating previous observations (Turpin et al., 2009; Eshima et al., 2017; Shortreed et al., 2009b; Bott et al., 2017), we observed an increase in soleus and diaphragm FCSA of young-adult mice on a HFD for 16, but not on those on a HFD for 8 weeks. In old mice, the FCSA was already elevated in the soleus after 9 weeks HFD, indicating that the effects of HFD on soleus FCSA occurred earlier in old than young-adult mice, again supporting the notion that muscles of old mice are more susceptible to obesity or a HFD.

The hypertrophy in the postural soleus muscle may be an adaptation to the increased loading resulting from an elevated body mass. Indeed, in both young-adult and old mice the soleus muscle mass:body mass ratio did not change significantly after HFD. This may also explain why we did not observe a significant change in the FCSA of fibers in the EDL, which does not play a significant antigravity role. In line with this, it has been found that unloading by hindlimb suspension results in greater atrophy in the soleus than EDL muscle (Stevens et al., 2000). Nevertheless, there was no commensurate increase in absolute isometric force in the muscles of mice on a HFD (Tallis et al., 2017a; Hill et al., 2019; Hurst et al., 2019), which may be a consequence of an increased proportion of the cytoplasmic volume occupied by IMCL. Indeed, only after 12 weeks, but not after 4 weeks on a HFD the specific tension of muscles was reduced (Eshima et al., 2017; Hurst et al., 2019; Tallis et al., 2017a) in young-adult, but surprisingly not in old muscles (Hill et al., 2019).

In the diaphragm, the FCSA increased with HFD in the young-adult mice only, but not in the old mice. It should be noted, however, that this effect on the FCSA in the diaphragm of the young-adult mice was only seen after 16 weeks on a HFD, and it is thus possible that such an increase in FCSA would also occur with longer duration of a HFD in the old mice. It is possible that the increased FCSA is compensatory hypertrophy to maintain the maximal force

and power generating capacity of the diaphragm in the face of a decreased force and power generating capacity per muscle mass previously observed in the same animals after a HFD and with ageing (Hill et al., 2019; Hurst et al., 2019).

In summary, HFD did not induce changes in fiber type composition, and any increases in FCSA were muscle specific - showing no change in the EDL - occurred earlier in old than young-adult mice, and were most likely a secondary adaptation to the increased loading due to an increase in body mass, rather than a direct effect of a HFD.

Oxidative capacity

Here we showed that a HFD caused an increase in the oxidative capacity (reflected by the SDH-OD) in muscles from young-adult, but not old mice. The increase in skeletal muscle oxidative capacity with HFD has been previously reported in young-adult rodents (Hancock et al., 2008; Miller et al., 1984; Turner et al., 2007; Thomas et al., 2014; Eshima et al., 2017; Sikder et al., 2018; Li et al., 2016; Garcia-Roves et al., 2007). Interestingly, it has been reported that the expression of enzymes in the citric acid cycle, β-oxidation and respiratory chain is comparable to standard diet after 2 weeks HFD, but similarly elevated after 8 and 16 weeks of a HFD (Sadler et al., 2012). Such HFD-induced changes in the expression of oxidative enzymes correspond to our observation of similarly elevated SDH-OD in young-adult mice on a HFD for 8 and 16 weeks. It is possible that in the young animals this elevated oxidative capacity, even in the fast EDL muscle, and associated capacity for β-oxidation may have prevented the early accumulation of IMCL in the young animals, something not seen in the old animals, where indeed IMCL accumulation already occurred after 9 weeks HFD.

The increase in SDH-OD (without an increase in IMCL) after 8 weeks of a HFD was somewhat less after 16 weeks in the diaphragm. Such a decrease may be due to lower physical activity levels in response to a HFD (Moretto et al., 2017) that would particularly reduce the activity of the respiratory muscles. It maybe that lower physical activity levels in old than young mice (Ingram, 2000; Tallis et al., 2017b) may also underlie the absence of an increase in oxidative capacity in the muscles from old mice. Another possibility is that the increase in muscle oxidative capacity in young-adult mice is at least partly mediated by activation of peroxisome proliferator-activated receptor α (PPARα) that has been shown to induce an increased expression of genes involved in β-oxidation in neonatal cardiomyocytes (van der Lee et al., 2000). The absence of, or attenuated increase in oxidative capacity in the

old mice may then be related to a reduced expression of PPARα, something observed in the heart of old mice (Bonda et al., 2017). Whatever the explanation, these data indicate that while a HFD induces an increase in muscle oxidative capacity in young-adult mice, this is not the case in old mice.

Capillarization

Above we have discussed that in young-adult mice a HFD induced an increase in muscle oxidative capacity, and as seen by others also an increase in β-oxidation (Sadler et al., 2012) and therefore probably a shift from glucose to fatty acid metabolism. Given that per ATP 8% more oxygen is needed during fatty acid than glucose oxidation, one may argue that the muscles of young-adult mice on a HFD have a larger oxygen demand, something opposite to the pathological cardiac hypertrophy that is associated with a shift from fatty acid to glucose metabolism to overcome energy starvation (van Bilsen et al., 2004). It has therefore been suggested that to match oxygen supply with increased oxygen demand, a HFD promotes angiogenesis in addition to mitochondrial biogenesis, something reported by others (Silvennoinen et al., 2013) and in the present study as reflected by the elevated C:F and LCFR.

The absence of HFD-induced angiogenesis (no significant rise in LCFR) in the EDL and diaphragm of the old mice with no change in oxidative capacity (reflected by similar SDH-OD) appears to support the notion that HFD-induced angiogenesis may serve to ensure an adequate oxygen supply in the young-adult mice on a HFD. However, this increased demand for oxygen cannot be the sole explanation, as the increased oxidative capacity in the EDL of young-adult mice was not accompanied with angiogenesis, and in the soleus of old mice, there was angiogenesis without a significant rise in oxidative capacity. Thus, rather than a coupling between changes in oxidative capacity and capillarization, these HFD-induced changes appear to be uncoupled.

The uncoupling between changes in capillary supply and oxidative capacity of a fiber confirms our previous observation that the oxidative capacity does not determine the capillary supply to a fiber, but rather fiber size (Bosutti et al., 2015). Our data support this relationship as in all cases where an increase in fiber size occurred, there was also angiogenesis, to such an extent that the overall capillary density and the capillary density per fiber (CFD) did not differ significantly between animals on a control diet and a HFD, except for a reduction in the

diaphragm from young-adult mice. The significance of fiber size and the small contribution of oxidative capacity for the capillary supply to a fiber was also reflected by a stepwise regression that showed that 67% of the variation in the capillary supply to a fiber was explicable by fiber size, with an additional 11% by muscle of origin, and only 0.7% by oxidative capacity (SDH-OD). Similar to what we have observed during ageing (Messa et al., 2019; Barnouin et al., 2017), the relationship between capillary supply with fiber size, muscle of origin and oxidative capacity was essentially unaltered by HFD (0.7% of the variation explained). This suggests that other functions of the microcirculation, such as removal of heat and waste products, and substrate delivery are more important than oxygen delivery.

Another factor that has not been considered in studies of HFD-induced obesity is the heterogeneity of capillary spacing, reflected by the logarithmic standard deviation of the capillary supply areas (Log_DSD) (Hoofd et al., 1985). Increased heterogeneity of the capillary spacing plays a role in the oxygenation of the tissue (Degens et al., 2006; Barnouin et al., 2017). The similar Log_RSD in the muscles shows that angiogenesis following a HFD does not occur at random, but rather maintains the distribution of capillaries to preserve the potential for adequate intramuscular oxygenation.

To investigate the relationship between supply and demand further we estimated the maximal oxygen demand of a fiber as the integrated SDH activity (FCSA * SDH-OD) and calculated the capillary supply (LCFR) to demand ratio (LCFR/SDH-INT) for each fiber. Following a HFD, the capillary supply to oxygen demand decreased in all muscles. This decreased ratio suggests a developing oxygen supply-demand mismatch during a HFD, similar to that seen in cardiac hypertrophy in rats (Des Tombe et al., 2002), suggesting the capillary supply becomes in deficit to oxygen capacity following a high-fat feeding. It is possible that after extended periods of HFD even capillary rarefaction occurs, something seen in muscles of obese people, that potentially could further aggravate the mismatch between oxygen supply and demand (Gomes et al., 2017).

Conclusion

The data of the present study show that the muscles of old mice are more susceptible to HFD-induced changes in morphology than young-adult mice. The adaptations are muscle specific, with an increase in fiber size in the soleus, but no change in the EDL. The increase in oxidative capacity is uncoupled from the HFD-induced angiogenesis but is explicable by

increases in fiber size. It, therefore, appears that many of the HFD-induced changes in muscle morphology, except the rise in intramyocellular lipids, are a consequence of additional loading of the muscle, rather than a direct effect of a HFD. It remains to be seen how longer durations of HFD and obesity affect muscle morphology, as caspase activation will over time result in muscle wasting (Kob et al., 2015b) and muscle function shows already signs of impairments (Hill et al., 2019; Hurst et al., 2019).

Limitations of study II

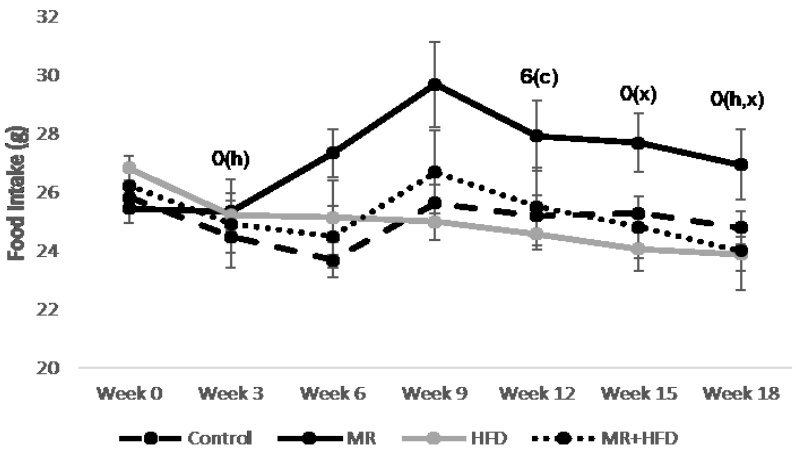
Dietary fat intake often has been associated with the increase in adiposity. In humans, it has been reported that high-fat diets (≥ 30% of energy from fat) can lead to obesity (Jequier, 2002; J. O. Hill et al., 2000; French and Robinson, 2003; Bray and Popkin, 1998; Schrauwen and Westerterp, 2000; Smilowitz et al., 2010). The percentage of high-fat diets usually used for studying obesity in the rodents are within the range of 30-78% of total energy intake (Buettner et al., 2007) and we have used 63.7% of total energy intake to generate obese mice, which are much higher than the levels that humans routinely consume. It remains questionable to whether experimental use of these diets with very high levels of fat effectively models the situation of human obesity. Thus, if rodent models can be made more closely to mimic what happens in humans then it is likely that the insights from the present study would be enhanced.

About 20 years ago the company Research Diets created a series of 3 diets known as the DIO series. These had, respectively 10%, 45% and 60% fat by calories. While a normal rodent diet contains about 10% fat, both 45 and 60% fat diets are high-fat for rodents. Mice on the 45% fat diet become obese. However, mice on the 60% fat diet become more obese, and do so more rapidly, which is advantageous since it reduces the time that the animals need to be housed, thereby reducing caging costs. Therefore, we have used the 63.7% fat rodent diet as a matter of economics and convenience. The typical American or European diet comprises about 36–40% fat by energy, therefore a tolerable high-fat human diet might contain 50–60% of energy as fat (Speakman et al., 2019). Thus, the 63.7% fat rodent diet might present a much greater distortion of the fat content of a normal rodent chow. However, a key question remains to whether there is any difference in the resultant obesity, physiology and metabolism of the mice fed high (c. 45%) vs. very high (c. 60%) fat diets. The very high fat diets might generate a more exaggerated metabolic response than high-fat diets, but the difference is relatively insignificant. In fact, it has been shown that the magnitude of obesity

and impacts on glucose homoeostasis and insulin resistance increase with increasing fat content up to around 60% fat (Akahashi et al., 1999; Hu et al., 2018) but some studies have failed to detect such differences (Mundy et al., 2007; Morrison et al., 2010). A comparison of metabolite profiles of low-fat (10%) vs. high-fat diet (45% fat) vs. very high-fat diet (60% fat) fed mice revealed that 80 metabolites were altered in the lungs of high-fat fed mice relative to the control diet (Showalter et al., 2018). Nevertheless, only 35 of these 80 changed metabolites were common across the two high-fat diets, showing these diets produced rather different metabolic responses. This does indicate that the impact of high vs. very high diets is often small, but it can occasionally be much larger.

Laboratory rodents generally comprise 20% or more protein which is higher than the average protein intake (15%) of humans throughout life (Fulgoni, 2008). Hu et al. (2018) studied the effects of 29 different diets that varied in protein, fat and sucrose content in 5 strains of mice. These diets mean to model the American diet in terms of the fatty acid composition and carbohydrate composition. It was found that the dietary fat, but not the protein or carbohydrate, was responsible for increased adiposity.

Given the restrictions imposed at Coventry University, diets were selected based on metabolizable energy. Because of the nature of the HFD protocol, we were unable to measure differences in the energy intake of the young-adults and early-ageing CD-1 mice. Nevetheless, the main focus was to achieve a change in adiposity, which we have achieved. Energy intake may vary considerably from one individual to the next due to body size, body weight and the levels of physical activity. In the experimental study II, we did not measure the total energy intake in diet by different groups of CD-1 mice. This may be a confounding variable that might increase variation in the results found on the effects of a HFD on skeletal muscle morphology.



Although we did not measure the caloric intake in different mice, which is one of the main limitations of study II, an as yet unpublished study has shown that despite a similar food intake a HFD (see figure above) can still induce an increase in body mass and BMI in mice (see table below; data for young animals highlighted).

	YOUNG BM (g) Wk 0	BM (g) Wk 18	BSA (cm^2)	BMI ($g \cdot cm^{-2}$)	**OLD** BM (g) Wk 0	BM (g) Wk 18	BSA (cm^2)	BMI ($g \cdot cm^{-2}$)
Control	30.1±1.3	31.5±0.6	88.6±1.0	0.302±0.004	35.8±0.7	37.2±1.3	96.5±1.2	0.318±0.008
MR	29.1±0.5	27.3 ± 1.7^{0}	82.0 ± 3.1^{c}	0.265 ± 0.012^{c}	37.2±1.2	28.2 ± 0.6^{0}	81.8 ± 3.1^{c}	0.269 ± 0.008^{c}
HFD	31.4±0.7	$44.5\pm2.1^{0,c,m,x}$	$108.6\pm3.2^{c,m,x}$	$0.402\pm0.015^{c,m,x}$	39.0±2.2	$42.8\pm2.6^{0,m,x}$	$105.8\pm2.2^{c,m,x}$	$0.372\pm0.011^{c,m,x}$
MR+HFD	31.3±0.6	29.0 ± 3.3^{0}	85.1±3.6	0.272 ± 0.018^{c}	37.6±2.3	29.9 ± 1.3^{0}	88.1±2.1	0.284 ± 0.008^{c}

Body mass (BM), Body surface area (BSA) and body mass index (BMI) of young and old mice before and after being 18 weeks on a control, methionine restricted (MR), high fat diet (HFD), or MR+HFD diet. $^{c, m, x}$: significantly different from control, MR, MR+HFD group respectively at $p \leq 0.041$. ^{0}: significantly different from week 0 at $p \leq 0.004$. Data is presented as mean ± SEM.

The experimental setup of study II was mainly designed to generate adiposity and to detect HFD-induced adaptation in the muscles of young-adult and early-ageing CD-1 mice, which were achieved. A 9-week HFD was used to induce a significant gain in weight and adipose tissue in older mice. Obesity in humans is normally developed and present over several years of poor dietary choices and leading a sedentary lifestyle. Examining the effects that obesity has on the morphology of skeletal muscle during feeding over a larger proportion of animal's lifespan would thus mimic the human situation more. In addition, further work should consider examining skeletal muscle morphological changes following a varied range of feeding periods. It is clear from the evidence that the muscle contractile properties are likely

to change, depending on duration of feeding. Such studies would be important in determining the muscle-specific onset of obesity-associated changes in skeletal muscle morphology and the potentially more severe implications of feeding diets longer than that used in study II.

Summary of study II

Lipids accumulate in skeletal muscle when energy intake exceeds the storage capacity of adipose tissue and when fatty acid oxidation is less than the delivery of fatty acids to the muscle cell. Current evidence suggests that part of the development of insulin resistance, which is a major risk factor for metabolic diseases, may be caused by lipid accumulation in skeletal muscle that leads to insulin resistance of muscle fibres (Unger et al., 2010; Addison et al., 2014). In addition, insulin-resistant individuals with a family history of type 2 diabetes have lower mitochondrial content and oxidative capacity in their skeletal muscle fibres (Petersen et al., 2004), suggesting that reduced mitochondrial content, mitochondrial function and reduced capacity for fatty acid oxidation in skeletal muscle are important contributors to the development of insulin resistance and type 2 diabetes. Ageing exacerbates obesity-induced problems by reducing skeletal muscle mitochondrial density and function that may be caused by oxidative damage of DNA and/or an age-related reduction in physical activity (Short et al., 2005). However, the synergistic effects of ageing and obesity are poorly understood. A number of animal studies have reported the effects of different duration of HFD on muscle morphology, but no systematic comparison of the effect of duration of the HFD has been made. In the experimental study II, the effects of a HFD of different duration on skeletal muscle morphology of 20- and 79-week-old female CD-1 mouse soleus, EDL and diaphragm, respectively were compared with age-matched lean controls. The HFD resulted in a significant increase in body mass and gonadal fat pad mass compared to the control group.

Previous studies have indicated a heavy reliance on the inbred C57BL/6J mouse strain which is more susceptible than the CD-1 mouse? to the obesity-promoting effects of a HFD (Lee et al., 1995; Surwit et al., 1997; Alexander et al., 2006). Gao et al. (2015) have reported that the use of the C57BL/6J strain lacks a full representation of a heterogeneous population when studying the physiological effects of dietary-induced obesity, further vindicating the usage of the CD-1 strain in this chapter.

In addition to metabolic improvements, there are also studies that suggest that regular physical activity attenuates the loss of motor neurons and facilitates reinnervation (Mosole et al., 2014; Power et al., 2010). It is, therefore, vital to understand the effects of regular

physical training on the morphology of skeletal muscle and how it is affected by ageing. This aspect will be considered in the experimental study III.

Age-related skeletal muscle morphological changes of human and rodent models

Introduction

In humans, aged-associated progressive decline in muscle mass and strength starts at the age of 40-60 (Faulkner et al., 2007; Porter et al., 1995). At the age of 80, the muscle force generating capacity is reduced by 40% compared to that at the age of 20-30 (Doherty et al., 1993). As mentioned previously, this gradual decline in muscle function and muscle mass has been shown to contribute significantly to the increased incidence of falls and decreased quality of life in old age (Mcphee et al., 2016; Musich et al., 2018). In order to maintain muscle function in older age, it is prerequisite to understand the mechanisms and causes of aged-related muscle wasting. In addition, muscle wasting is aggravated by a reduction in specific tension, the force generating capacity per cross-sectional area of the remaining muscle tissue (Frontera et al., 2000). However, it is challenging to quantify the physiological cross-sectional area of a human muscle *in vivo*, accurately. This highlights the importance of models investigating sarcopenia. Rodent models, in particularly mouse models, have been extensively used to enhance our understanding of age-associated changes in human skeletal muscle (Tallis et al., 2014; Hill et al., 2018). However, to what extent age-related morphological changes observed in rodents resemble those in humans have not been explored thoroughly.

Roles of rodent models of muscle aging

The use of aging rodents as models to study age-related morphological changes in human skeletal muscle has some obvious advantages. Human longitudinal studies require a long follow-up period. This makes it difficult to study muscle aging compared to the relatively short life span of rodents (only 2-3 years). In addition, it is also ethically possible to perform invasive procedures in rodents that are not possible in humans (Cartee, 1995). Furthermore, environmental factors and diets can be tightly controlled, and activity can be accurately monitored in laboratory animals, which is virtually unachievable in humans (Alway et al., 2005). Finally, terminal experiments can be performed in rodents, allowing one to dissect whole muscles, which provide the opportunity to study skeletal muscle morphology and molecular mechanism in whole muscle in comparison to human studies where at best a small biopsy can be analysed. It may be argued that the experimental control of rodent models

compromises the validity and relevance of these models to humans (Alway et al., 2005). Nevertheless, it is this possibility to control several factors that help to obtain a better understanding of the extent at which various factors contribute to the observed variation in the rate of muscle ageing between people (Degens & Korhonen, 2012).

When comparing human and rodent aging, it is vital to consider how a given age of a rodent compares to that of humans. For this purpose, Ballak and collaborators have introduced the 'relative age', defined as a percentage of the mean life span (MLS) of humans and rodents For both humans and rodents, the authors reported average life expectancy at 100% MLS which allows to calculate relative ages from reported absolute ages in months and years (Ballak et al 2014a). The mean life span in developed countries has been reported to 80 years (Smith, 1993) while the mean life span of C57BL/6 has been estimated to 26.7 months (Blackwell et al., 1995; Sheard and Anderson, 2012). Based on of these values of MLS, 20- and 79-weeks old CD-1 mice correspond to approximately (16-20 years) and (60-65 years) humans.

Age-related changes in muscle strength

Most human studies that investigated the age-related changes in muscle morphology and function have focused on the quadriceps muscles while in rodents mostly muscles of the triceps surae complex have been studied. This may make direct comparisons between rodents and humans difficult as thigh and calf muscles may show different age-associated changes. However, reports of human studies (Davis et al., 1986; Vandervoort and McComas, 1986) indicated that age-associated changes in the force generating capacity of muscle triceps surae had a similar decline (~ 40% between 20 - 30 years and 70 years and older) as that observed in the muscle quadriceps femoris (Frontera et al., 2000; Young et al., 1985). Remarkably, in both group of muscles the decline in force generating capacity was negligible before the sixth decade of life (Doherty et al., 1993; Vandervoort and McComas, 1986). Within the same animal, effects of ageing showed considerable reductions of both the soleus (36 - 50%) and vastus lateralis (58-60%) mass. These data indicate that in both humans and rodents the quadriceps femoris and triceps surae show similar age-associated reductions in force generating capacity and muscle mass. Thus, the use of triceps complex in rodent models does not preclude a fair comparison with the age-related changes in the human quadriceps complex.

Studies that compared force generating capacity in humans and rodents reported that both human and rodent muscle skeletal muscles exhibit peak force at similar relative mean life span (Brooks and Faulkner, 1988; Brown and Hasser, 1996; Degens and Alway, 2003). However, Ballak et al. (2014a) indicated that in mice and rats the reduction in muscle strength appears to occur at relatively late stage of life compared to that for humans.

Age-related changes in muscle mass

As stated earlier, the loss in muscle strength with increasing age is largely due to a decrease in muscle mass. Direct measurement of muscle mass in human skeletal muscle is unattainable. Thus, most studies assessed anatomical cross-sectional area (ACSA) of muscles in humans used computed tomography or MRI as an estimate of muscle size (Klitgaard et al., 1990b; Overend et al., 1992; Rice et al., 1989; Young et al., 1985). Vastus lateralis is commonly used in human studies due to its accessibility. It is, therefore, important to compare the decline in muscle mass and the relative threshold age (losing <10% muscle mass) of the vastus lateralis. For mice, an age-related decrease of only ~ 15% was observed in muscle mass at 100% mean life span (Ballak et al., 2014a). This age-associated loss of muscle mass was much lower compared to humans. In addition, the relative age at which mice start losing muscle mass (~90% MLS) was higher than that for human (~75% MLS). Thus, it appears that, in both rodents and humans, there is a threshold age beyond which muscle wasting and weakness develops. In mice, it seems that the reduction in muscle mass occurs relatively later in life than in humans. More data on middle-aged humans and rodents are needed to better understand the initial stages of age-related muscle deterioration.

Age-related loss of muscle fibre number

Rodent studies (Sheard and Anderson, 2012; Zerba et al., 1990b) showed that in mice the effects of ageing on muscle fibre number become substantial only after an age of 90% mean life span (> 24 months). However, it appears that human skeletal muscles (Lexell et al., 1986; Lexell et al., 1988) lose relatively more muscle fibres, when considering all muscle fibres, compared to rodents. It is likely that similar mechanisms of neuronal death and loss of motor units cause this loss of fibres in both human and rodent muscles.

Skeletal muscle morphological changes of human and rodent models

Rodents and humans are considerably different with respect to body size, life span and metabolic rate, which might make direct comparisons difficult. However, for the proper translation of knowledge gained in rodents to humans, it is important to establish this

comparison. CD-1 mice at an age of 79 weeks correspond to an age of 60-65 year old people may be well suitable to study early signs of sarcopenia, including the occurrence of a reduction in specific tension (Hill et al., 2018). Ballak et al. (2014a) reported that in mice, ageing related reductions in maximal muscle force and mass were much lower than those found for humans, while specific tension seems to be affected almost equally. This suggests that in mice the ageing related muscle wasting occur at a higher relative age. Thus, in both humans and mice, the decline of force generating capacity seems affected by a loss in muscle mass and specific tension, but the contribution of these may vary. According to Ballak et al. (2014a) compared to humans, the age-related effects of sacorpenia in mice appear only late in life. However, it could be the mean life span of the mice was underestimated as it has been demonstrated to increase over the years. Additionally, old healthy mice show almost no age-related muscle weakness until morbidities and concurrent deterioration start to kick in, after which death follows quickly. In contrast, the human health care system may cure or pamper old and sick individuals, who otherwise would have died. Thus, even with the best intentions, the number of aged people with impaired quality of life and physical fitness grows, which exposes them to (additional) age-related muscle weakness, compared to rodents.

Life expectancy depends on a number of factors including genetic and environmental factors such as nutrition, physical activity and stress which may all vary between humans and rodents. Housing conditions have improved over time, particularly with the introduction of specific pathogen free (SPF) barrier facilities that have led to an increased life expectancy of laboratory rodents (especially C57BL mice) throughout the last decades (Ballak et al 2014a). The impact for muscle aging research is that since that time older and healthier rodents can be tested with a smaller chance of measuring effects of co-morbidities.

Laboratory rodents are generally supplied with standard industrial food. These diets generally comprise 20% or more protein which is higher than the average protein intake (15%) of humans throughout life (Fulgoni, 2008). Hu et al. (2018) studied the effects of 29 different diets that varied in protein, fat and sucrose content in 5 strains of mice. These diets mean to model the American diet in terms of the fatty acid composition and carbohydrate composition. It was found that the dietary fat, but not the protein or carbohydrate, was responsible for increased adiposity. However, whether the life-time intake of proteins differs between humans and rodents and whether this actually has a different effect on life span remains unclear.

The amount and duration of regular physical activity may also affect the process of sarcopenia (Degens & Korhonen, 2012). Throughout aging, physical activity of humans is substantially reduced. During daily life, the intensity of energy spend is considerably reduced in old (90 yrs, ~115% MLS) compared to young (~25 yrs, 30% MLS) (Westerterp, 2000). An age-associated decline in physical activity has also been indicated in laboratory rodents (Ingram, 2000). For 13-month-old C57BL/6 mice the frequency of ambulatory movements was only half compared to that of 5-months-olds. Subsequently, this remained unchanged up to 25 months (94% MLS) of age (Lhotellier and Cohen-Salmon, 1989). It appears that the age-related decline in physical activity is rather similar for humans and rodents and as such the degree of aging related to inactivity does not explain the differences in magnitude of sarcopenia. Thus, it is important to give considerations to factors that influence life expectancy and the rate of muscle aging may differ between humans and rodents.

The main questions remain whether knowledge about age-related morphological changes in mice could be translated to humans, and if so how they relate to each other. Diaphragm muscle changes associated with sarcopenia are likely implicated in a variety of pathologies in humans including pneumonia and respiratory distress syndrome. One of the underlying factors affecting these diseases is the atrophy of type IIx and/or type IIb fibres which decreases the capacity of the body to perform explosive behaviours required for airway clearance. In addition, selective hypertrophy of the diaphragm type IIx fibres found in study I indicates that there is an increased ability to perform respiratory behaviours during early ageing corresponding to 60 - 65 years in humans. The atrophy of EDL fibres in early ageing populations found in CD-1 mice may be translated in humans by the increase adverse outcomes that coincide with increasing age such as increased risks of falls and fractures. In study II, in mice there was an elevated fat mass load in the diaphragm during early ageing without a change in the mitochondrial activity. In both rodents and humans, this may potentially contribute to elevate respiratory disease risk associated with the negative cycle of obesity in humans. To counteract sarcopenia it is important to know the mechanisms and causes of muscle aging, particularly during early ageing. The present data suggest that interventions should, at least during the early stages of sarcopenia, aim to attenuate the age-related decline in muscle morphology. This could be achieved by regular physical training that has been demonstrated to increase specific force (Erskine et al., 2011). Since the impact of physical exercise may decrease with increasing age (Degens, 2012; Slivka et al., 2018), it is essential to start regular physical training before sarcopenia takes its toll.

CHAPTER 4

Absence of an ageing-related increase in fibre type grouping in master athletes and non-athletes

Results presented in this chapter are based on an article published in the Scandinanvian Journal of Medicine and Science in Sports : **Guy A.M. Messa**, Mathew Piasecki, Jörn Rittweger, Jamie S. McPhee, Erika Koltai, Zsolt Radak, Bostjan Simunic, Ari Heinonen, Harri Suominen, Marko T. Korhonen, Hans Degens (2020)

Abstract

Background

The ageing-related loss of muscle mass may be partly attributable to motor neuron loss and motor unit remodelling, where cycles of denervation and reinnervation may result in fibre type grouping in older muscles. The purpose of this study was to examine fibre type grouping in athletes and non-athletes of a wide age range (19-85 years) and to evaluate to which extent any observed grouping is explicable by the fibre type composition of the muscle. Since regular physical activity may stimulate reinnervation, we hypothesised that fibre groups are larger in master athletes than in age-matched non-athletes.

Methods

Fibre type grouping was assessed in *m. vastus lateralis* biopsies from 22 young (19-27 years) and 35 healthy older (66-82 years) non-athletes, and 14 young (20-29 years), 51 middle-aged (38-65 years) and 31 older (66-85 years) athletes. An 'enclosed fibre' was any muscle fibre of a particular type surrounded by fibres of the same type only. A fibre type group was defined as a group of fibres with at least one enclosed fibre.

Results

Only type II fibre cross-sectional area (FCSA) showed an age-related decline that was greater in athletes ($p < 0.001$) than in non-athletes ($p = 0.012$). There was no significant age-related effect on fibre group size or group number in athletes or non-athletes, and the observed grouping was similar to that expected from the fibre type composition of the muscle.

Conclusions

At face value these observations do 1) neither show evidence for an age-related loss and remodelling of motor units nor that 2) improved reinnervation with regular physical activity, but 3) histological examination may not reveal the full extent of ageing-related motor unit remodelling.

4.1 Introduction

Ageing is associated with progressive muscle wasting and weakness, a condition referred to as sarcopenia, where almost 30% of the mass and more than 35% of the force generating capacity may be lost by the age of 70 years (McPhee et al., 2018). Consequently, older persons suffer functional and mobility limitations leading to increased fall risk, reduced quality of life and loss of independence (Musich et al., 2018). With people older than 65 years representing the fastest growing segment, and particularly those over 85, in the western populations, it becomes ever more important to expand knowledge on ageing muscle to develop effective strategies to reverse or attenuate the ageing-related decline of muscle mass and function to delay the onset of functional limitations (McPhee et al., 2016). The gradual loss of muscle mass with advanced age is attributed to both atrophy of particularly type II fibres (Andersen, 2003; Barnouin et al., 2017) and loss of fibres (Lexell et al., 1988; Wilkinson et al., 2018; McPhee et al., 2018).

A motor unit consists of a single motor neuron (MN) and all of the muscle fibres that it innervates. In large muscles, such as the *m. vastus lateralis*, a single motor neuron can innervate hundreds or even thousands of fibres. The loss of muscle fibres in aged muscles is thought to be at least partly attributable to loss of motor neurons with consequent denervation of their associated fibres and ultimately disappearance if they are not reinnervated (Larsson et al., 2019; Power et al., 2013; Piasecki et al., 2016a). Many of the fibres that have become denervated because of motor neuron loss may, however, be reinnervated by the remaining motor neurons, which may result in fibre type grouping (Kanda and Hashizume, 1989; Lexell and Downham, 1991).

Levels of physical activity decrease with age (Ingram, 2000) and like ageing, results in muscle atrophy (Degens and Alway, 2006). The question thus arises to what extent muscle wasting and weakness in old age are due to reduced physical activity levels. Master athletes maintain high levels of physical activity (Degens et al., 2013; Hannam et al., 2017) and are thus considered a good model to disentangle the effects of ageing *per se* from reduced levels of physical activity on skeletal muscle (Rittweger et al., 2009; Degens et al., 2013; Harridge and Lazarus, 2017).

Some studies reported no ageing-related loss of motor units in master athletes (Power et al., 2010), or an attenuated loss even in octogenarians (Power et al., 2016; Drey et al., 2016),

while others found that master athletes do suffer similar motor unit loss as seen in non-athletes (Piasecki et al., 2016c; Piasecki et al., 2019). Several studies interpreted increased motor unit size (Piasecki et al., 2019; Piasecki et al., 2016a) and larger fibre type groups (Mosole et al., 2014; Carraro et al., 2017) as evidence for improved reinnervation in master athletes, but others found smaller motor unit size, suggested to be representative of less collateral reinnervation in master athletes (Power et al., 2016). Thus, the evidence is equivocal as to whether regular physical activity protects against ageing-related motor neuron loss and facilitates reinnervation.

The reports on fibre type grouping in master athletes studied only old master athletes and did not compare them with young- and middle-aged master athletes, nor did they consider the potential impact of fibre type composition on the observed fibre type grouping. This is important because fibre type composition can vary markedly between individuals and grouping is more likely for those with a high proportion of one single fibre type than in those with similar proportions of type 1 and type 2 fibres. Therefore, the objective of this study was to assess fibre type grouping in athletes and non-athletes of a wide age range (19-85 years). In addressing this objective we considered the extent to which any observed grouping is explicable by the fibre type composition of the muscle so that the chance occurrence of groups can be separated from the possible exercise-mediated fibre type grouping. Based on previous observations on the size of fibre groups (Mosole et al., 2014; Carraro et al., 2017), and the loss of motor units (Piasecki et al., 2016c; Piasecki et al., 2019) in master athletes, we hypothesised that both the fibre group size and number of groups increases with increasing age in master athletes beyond that expected from muscle fibre type composition. As it has been suggested that master athletes may have better reinnervation capacity than non-athletes (Mosole et al., 2014; Piasecki et al., 2019), we hypothesised that the group size is larger in master athletes than non-athletes.

4.2 Methods

4.2.1 Subjects

The study population consisted of 22 young (19-27 yr; men n = 14; women n = 8) and 35 healthy older (66-82 yr; men n = 27; women n = 8) non-athletes who participated in the MYOAGE study (McPhee et al., 2013; Barnouin et al., 2017) and master athletes. The master athletes were divided into three groups: 20-29 yr (men n = 14), 38 - 65 yr (men n = 44;

women n = 7) and 66-85 yr (men n = 32; women n = 3). Master athletes were recruited at the European Veteran Athletics Championship 2008 (Ljubljana, Slovenia), World Master Athletics Championships in 2009 (Lahti, Finland), the European Veterans Athletics Championships in 2010 (Nyíregyháza, Hungary), or by means of personal letters from among the members of Finnish track and field organizations as described in Korhonen et al. (2006). The event specialities ranged from sprint and power events to middle- and long-distance running events (200m, 400m, 110m hurdles, long jump, hammer, javelin, 1500m and 5000m). The studies were approved by the ethical committee of the Manchester Metropolitan University (UK), the Republic of Slovenia National Medical Ethics Committee (Slovenia), and the ethics committees of the University of Jyväskylä (Finland) and the Semmelweis Institute (Budapest, Hungary) and have, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All participants gave written informed consent.

4.2.2 Anthropometry and muscle size

For each participant the body mass and height was measured and the body mass index (BMI: $kg \cdot m^{-2}$) calculated. In 100 athletes, the thickness of the *m. vastus lateralis* was determined while lying on a bed at 50% thigh length with a 5-cm linear-array 7.5-MHz ultrasound probe as described previously (Korhonen et al., 2006).

4.2.3 Biopsy sampling and histochemistry

Biopsies were obtained from the middle portion of the *m. vastus lateralis* (VL) at 40% of the distance from the patella to greater trochanter under aseptic conditions by using either a conchotome or Bergström needle after local anesthesia with 1-2% lidocaine. The biopsy was placed on cork with Optimum Cutting Temperature compound (Scigen® Gardena) and immediately frozen in isopentane cooled in liquid nitrogen, or with vigorous shaking in liquid nitrogen, and stored at −80°C for histochemistry.

Serial 10-µm cross-sections were cut on a cryostat (Leica CM 3050S) at -21°C and stained for myofibrillar ATPase after acid (pH 4.30) preincubation as described by Brook and Kaiser (1970). Type I fibres stained dark, type II fibres light and type I/II fibres intermediate (Fig. 1A).

4.2.4 Morphometry

The stained cross-sections were photographed under a light microscope (Carl Zeiss Vision GmbH, Aalen, Germany) at 10x objective with a digital camera (Zeiss Axio Cam MRc, Göttingen, Germany). The contours of each fibre were drawn using a digitizing program (Program Btablet, BaLoH Software, Ooij, The Netherlands), and the co-ordinates of the outlines stored for further analysis with AnaTis (BaLoH software, Ooij, NL). In each biopsy, 58-718 complete fibres were analysed. The fibre type proportion (expressed as fibre number percentage) and the fibre cross-sectional area (FCSA) of each fibre were assessed. The variation in FCSA was given as the standard deviation of the FCSA (SD FCSA). The shape factor (circularity) of the fibre was calculated as: $perimeter^2/(4\pi \times FCSA)$ (Barnouin et al., 2017) where a circular fibre has a value of 1 and increasing values indicate increasing deviation from circularity (irregularities).

4.2.5 Analysis of fibre type grouping

To assess the extent of fibre type grouping in a cross-section, the method of Jennekens et al. (1971) was used. An 'enclosed fibre' is any muscle fibre of a given type surrounded by fibres of the same type only (Jennekens et al., 1971; Lexell et al., 1984). A fibre type group was defined as a group of fibres with at least one enclosed fibre, similar to that used by Mosole et al. (2014). In each cross-section, the number of enclosed fibres for each type was counted manually. The prevalence of enclosed fibres (%), reflecting fibre type grouping, was calculated as: $100\% \times n_{enclosed}/n_{total}$, where '$n_{enclosed}$' is the number of enclosed fibres of a given type and 'n_{total}' the total number of muscle fibres of the same type in a region of interest (Jennekens et al., 1971). An 'enclosing fibre' was defined as a fibre that surrounds an 'enclosed' fibre. The remaining fibres that were neither 'enclosed' nor 'enclosing' fibres were called 'remaining' fibres.

To determine the extent to which the muscle fibres were enclosed by chance, a mathematical model was applied (Johnson et al., 1973) that assumed a random spatial arrangement of the fibres of the two main histochemical types (type I and type II fibres). Additionally, it was assumed that the proportion of type I fibres is constant throughout a cross-section. The number of neighbours for each fibre in a cross-section were counted. The prevalence of the expected fibre type grouping (%) was calculated as follows:

$$\%\text{Fibres of a given type enclosed by chance} = 100 \times P^{n+1}$$

‘P’ the proportion of a given fibre type in the cross-section and ‘n’ the number of fibres surrounding a fibre of a particular type in the cross-section. Only very occasionally enclosing fibres were on the edge of the region of interest and also considered enclosing fibres.

4.2.6 Statistical analyses

All data were analysed with IBM SPSS version 25. The Kolmogorov Smirnov test showed that all data had a normal distribution.

For height, body mass, BMI, muscle thickness and fibre type composition a two-way ANOVA was applied with as factors sex and group (young (YC) and old non-athletes (OC), and young (YA), middle-aged (MA) and old (OA) athletes). For FCSA, % enclosed fibres (grouping) and the shape factor a repeated-measures ANOVA was used with as within-factor fibre type and between-factors sex and group. To assess whether the fibre type grouping was more than expected by chance we performed a similar analysis, but with the addition of expected grouping as a within factor. To assess whether the size of enclosed, enclosing and remaining fibres differed from each other and between groups, a repeated-measures ANOVA was performed with as within-factor 1) the condition of the fibre (three levels: enclosed, enclosing, remaining) and as between-factors 2) fibre type (as in some cases only type I or type II grouping was present), sex and group. In the case of significant age effects or interactions, a Bonferonni *post-hoc* analysis was performed to identify the significant differences between groups. A measure of statistical power (sp) for each p-value was included. Unless otherwise specified, all data are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Participant characteristics

In the figures, data from endurance athletes are illustrated where indicated, but not included in the statistical analyses as there were only 5 endurance athletes in the population.

Physical characteristics are shown in Table 1. Women were shorter than men ($p < 0.001$; sp = 0.979) and had a lower body mass ($p = 0.001$; sp = 0.953). It can be seen that athletes were in general taller than non-athletes ($p < 0.05$; sp = 0.975), and the OA were shorter than the YA and MA ($p \leq 0.002$; sp = 0.985). The body mass was lowest in the YC and OA ($p < 0.015$; sp = 0.957). The BMI of OC was larger than that of YC and all athlete groups ($p < 0.05$; sp =

0.714). Vastus lateralis muscle cross-sectional area correlated negatively with age in the athletes ($r^2 = 0.29$, $p < 0.001$).

Table 4.1 Participant characteristics and *m. vastus lateralis* thickness of male and female athletes and non-athletes in different age groups

		YC	OC	YA	MA	OA	Effects (p-values)		
N Men/women		**N = 22 14/8**	**N = 35 27/8**	**N = 14 14/0**	**N =51 44/7**	**N = 35 32/3**	**Sex**	**Group**	**Sex x Group**
Age (yr)		22.0 (2.7)	72.9 (4.0)	23.9 (3.0)	52.3 (8.3)	71.8 (4.9)			
Height (m)	**M**	1.72 (0.07)	1.69 (0.08)	1.77 (0.05)[1,2]	1.77 (0.06)[1,2]	1.71 (0.05)[2,3,4]	< 0.001	< 0.001	0.354
	W	1.66 (0.05)	1.61 (0.07)		1.73 (0.07)	1.63 (0.03)			
Mass (kg)	**M**	69.2 (13.4)	77.7 (15.6)[1]	76.5 (9.9)[1]	76.5 (9.5)[1]	68.6 (6.6)[2,3,4]	< 0.001	0.002	0.091
	W	61.3 (3.9)	63.2 (8.8)		72.7 (14.9)	51.0 (4.6)			
BMI ($kg \cdot m^{-2}$)	**M**	23.2 (3.2)	26.8 (3.6)[1]	24.3 (3.1)[2]	24.3 (3.1)[1,2]	23.5 (1.9)[2]	< 0.001	0.003	0.074
	W	22.3 (1.8)	24.3 (1.5)		24.1 (3.3)	19 (1.3)			
Muscle thickness (cm)	**M**			2.70 (0.27)	2.18 (0.35)[3]	1.86 (0.40)[3,4]	NA		NA
Grouping	**M**	18/22 (82%)	31/35 (89%)	13/14 (93%)	44/51 (86%)	34/35 (97%)			

BMI: body mass index. M = men; W = women. Young (YC) and older (OC) non-athletes, young athletes (YA), middle-aged athletes (MA) and older master athletes (OA). Muscle thickness was only available for male athletes (6 young, 35 middle-aged and 26 older master athletes). Grouping indicates the number of muscles in the group with grouping. [1]different from young non-athletes at $p \leq 0.029$; [2] different from old non-athletes at $p < 0.05$; [3] different from young athletes at $p \leq 0.002$; [4] different from middle-aged athletes at $p \leq 0.002$; NA: not applicable Data are expressed as mean ± SD.

4.3.2 Fibre type composition

An example of the myosin ATPase staining for an 85-year-old man is shown in Fig. 4.1A. The proportion of hybrid fibres (intermediate staining intensity) represented less than 1% of the fibre population and they were excluded from analysis. There was no ageing-related difference in fibre type composition (Fig. 4.1B) in either men, women, athletes and non-athletes.

4.3.3 Muscle fibre cross-sectional area and fibre size variation

Fibre cross-sectional area (FCSA). Muscle cross-sectional area divided by FCSA provides a rough estimate of fibre number. In a sub-group of male power athletes for whom both muscle cross-sectional area (calculated from ultrasound obtained thickness) and FCSA were available, the ratio did not differ significantly between YA, MA and OA and was not correlated with age (Fig. 4.2). Figure 4.3 shows the FCSA. For all fibres combined, the FCSA was larger in muscles from men than from women ($p < 0.001$; $sp = 0.997$). A sex × fibre type interaction ($p = 0.002$; $sp = 0.933$) for FCSA was reflected by the larger FCSA for type II than type I in men ($p < 0.001$; $sp = 0.997$), while in women type I fibres were larger than type II fibres ($p = 0.022$; $sp = 0.143$). There was a significant main effect of group (YC, OC, YA, MA, OA) on FCSA of pooled fibres ($p = 0.001$; $sp = 0.881$), but the group x fibre type interaction ($p = 0.001$; $sp = 0.985$) indicated that the effects of group on FCSA differed between fibre types. Indeed, the FCSA of type I fibres did not show a significant association with age in both non-athletes (Fig. 4.3A) and athletes (Fig. 4.3C), but there was a progressive age-related decrement in the FCSA of type II fibres for both non-athletes ($p = 0.029$; Fig. 4.3B) and athletes ($p \leq 0.015$; Fig. 4.3D). The absolute age-related decrement in type II FCSA was larger in athletes than in non-athletes (Fig. 4.3B vs. 4.3D). This was further supported by the observation that while YA had larger fibres than YC ($p < 0.005$; $sp = 0.881$), there was no significant difference in FCSA between OA and OC (Fig. 4.3; 4.9A, B).

Fibre size variation. The fibre size variation, reflected by the standard deviation of the fibre cross-sectional area (SD FCSA) did not significantly correlate with age (Fig. 4.4). There was a main effect of fibre type on SD FCSA ($p = 0.014$; $sp = 0.942$), but the sex x fibre type interaction ($p =0.002$; $sp = 0.991$) was reflected by the higher SD FCSA for type I than type II in women ($p < 0.001$; $sp = 0.949$), but no significant difference between types in men.

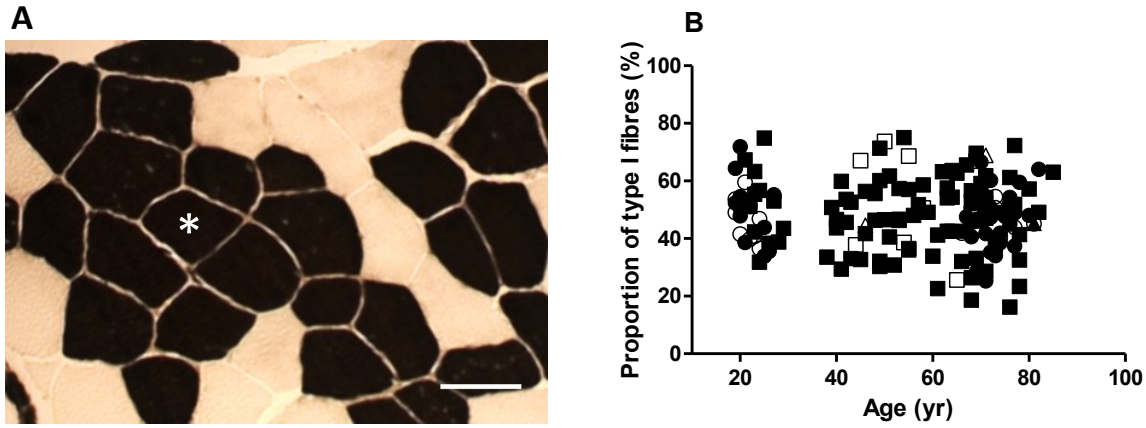


Figure 4.1 (A) Example of a vastus lateralis muscle biopsy cross-section obtained from an 85-year-old man and stained for myosin ATPase after preincubation at pH 4.30. Type I fibres were stained black and type II stained light. *: an enclosed type I fibre. Scale bar = 50 µm. **(B)** The relationship between age and proportion of type I fibres in male (●) and female (○) control, male (■) and female (□) power, and male (▲) and female (△) endurance master athletes (n = 157).

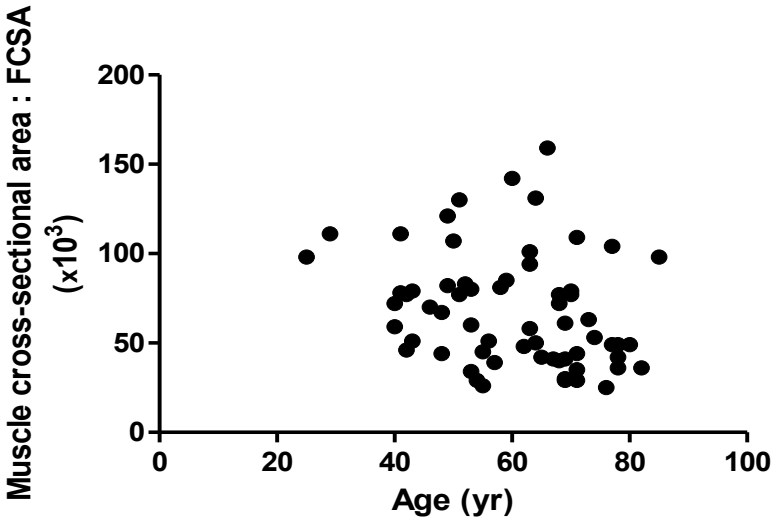


Figure 4.2 Relationship between age and the ratio of muscle cross-sectional area : fibre cross-sectional area (FCSA) in male power master athletes (n = 67).

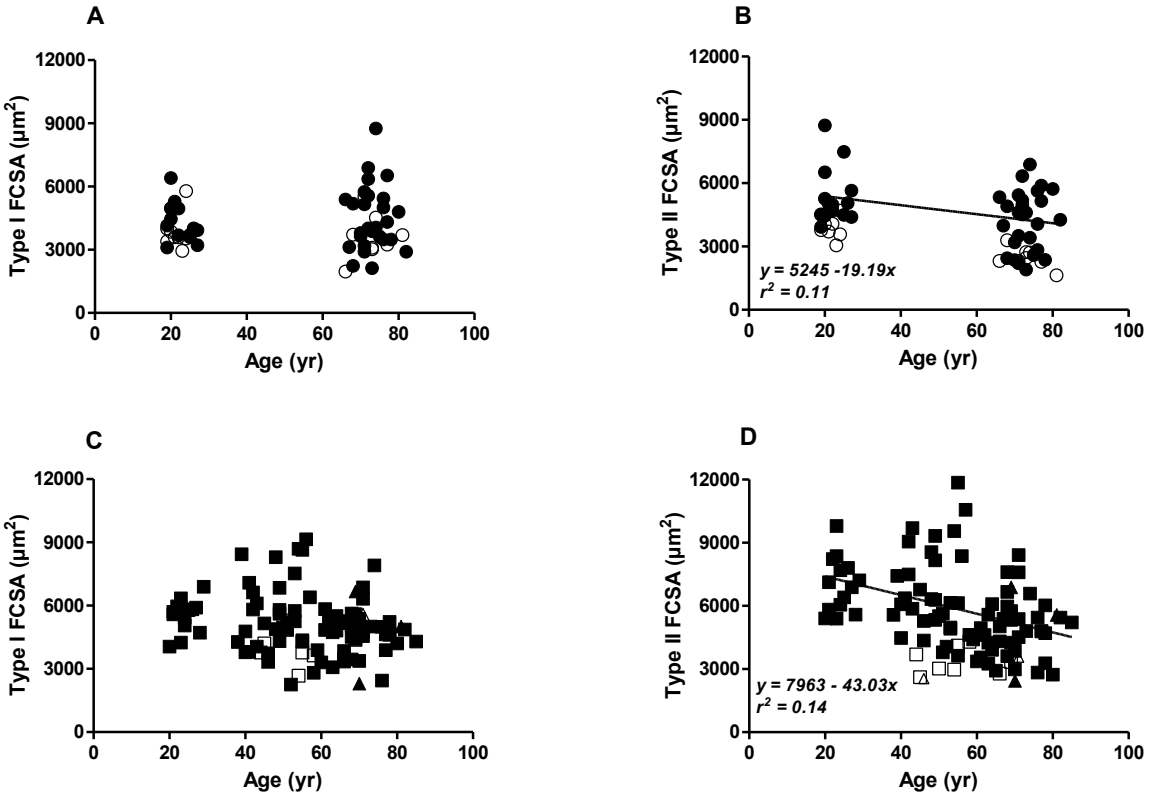


Figure 4.3 Relationship between age and fibre cross-sectional area (FCSA) of type I **(A)**, type II **(B)** fibres in male (●) and female (○) control (n = 57). **(C)** and **(D)** indicate the same relationship in male (■) and female (□) power master athletes, and male (▲) and female (△) endurance master athletes (n = 100).

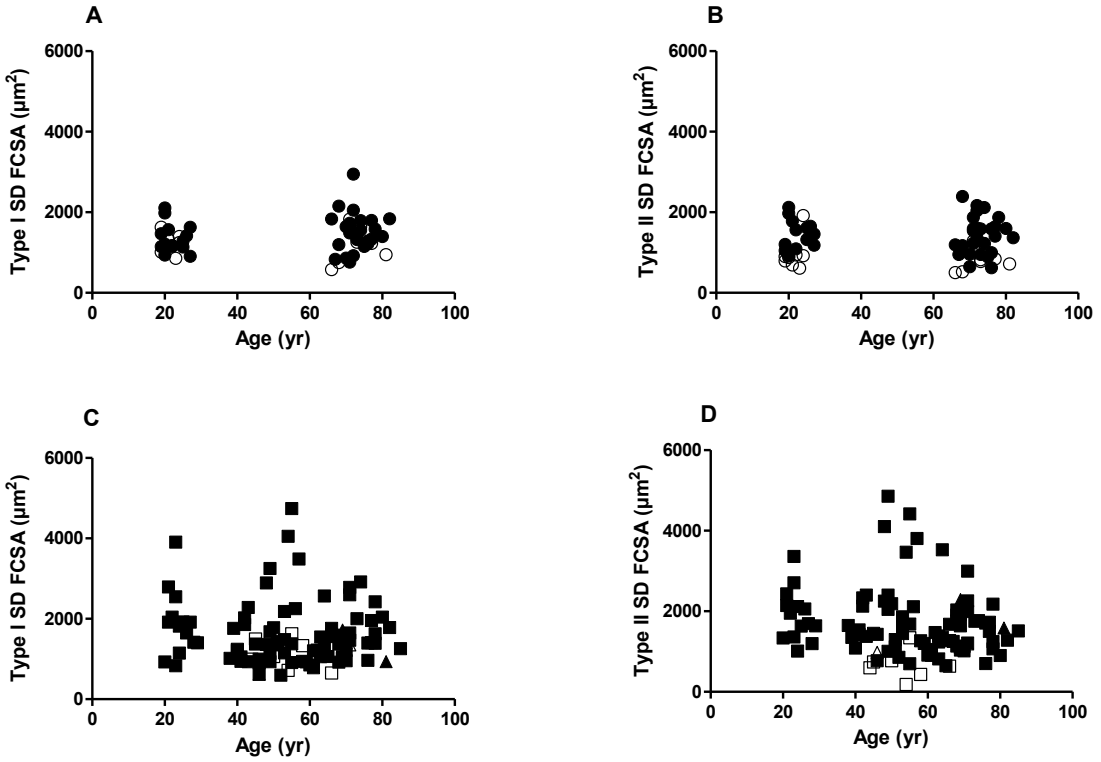


Figure 4.4 Relationship between age and the standard deviation of the fibre cross-sectional area (SD FCSA) of type I **(A)** and type II **(B)** fibres in male (●) and female (○) control (n = 57). **(C)** and **(D)** indicate the same relationship in male (■) and female (□) power, and male (▲) and female (△) endurance master athletes (n = 100).

4.3.4 Fibre type grouping

Number of groups. No significant difference was observed in the proportion of enclosed fibres between men and women, or between type I and type II fibres. There was also no significant age-related increase in the observed proportion of enclosed fibres in athletes and non-athletes of either type I (Fig. 4.5A) or type II (Fig. 4.5B) fibres, with at any age participants showing no grouping (Table 4.1).

There was an exponential correlation between the expected proportion of enclosed fibres and fibre type proportion (Fig. 4.6A). In figure 4.6B, the regression analyses showed similar linear correlations between the observed and expected proportion of enclosed fibres for type I and type II fibres that were close to the line of identity ($p < 0.001$).

The expected grouping did not differ significantly between men and women or between type I and type II fibres. Similar to the observed grouping, also the prevalence of the expected

fibre type grouping showed no significant age-related increase (Fig. 4.7A & B), and there was no significant difference between the observed and the expected grouping (Fig. 4.7A & B).

The fibre group size (Fig. 4.8A & B) and number of fibre groups per 1000 fibres (Fig. 4.8C & D) did not differ significantly between groups, men and women, or between type I and type II fibres.

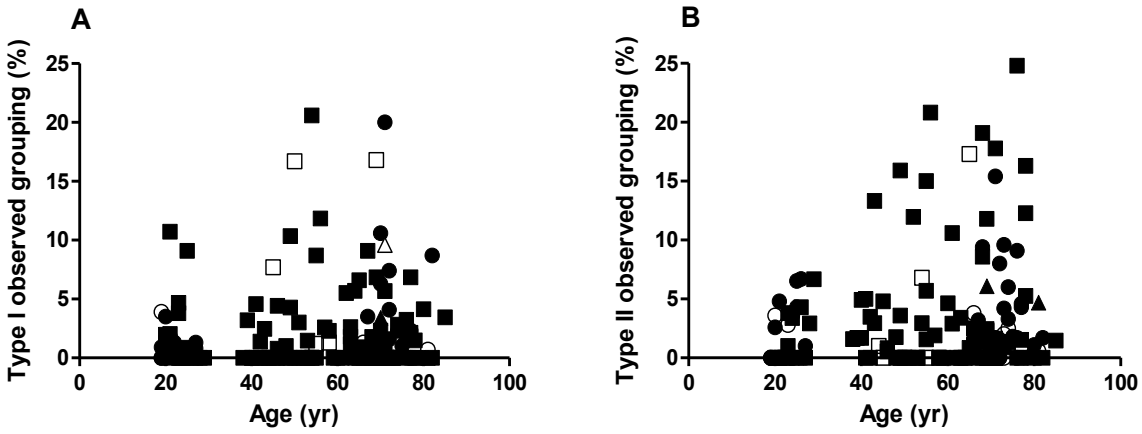


Figure 4.5 Relationship between age and observed prevalence of grouping of **(A)** type I and **(B)** type II fibres in the vastus lateralis muscle of male (●) and female (○) non-athletes, male (■) and female (□) power master athletes, male (▲) and female (△) endurance master athletes for all subjects combined (n = 157).

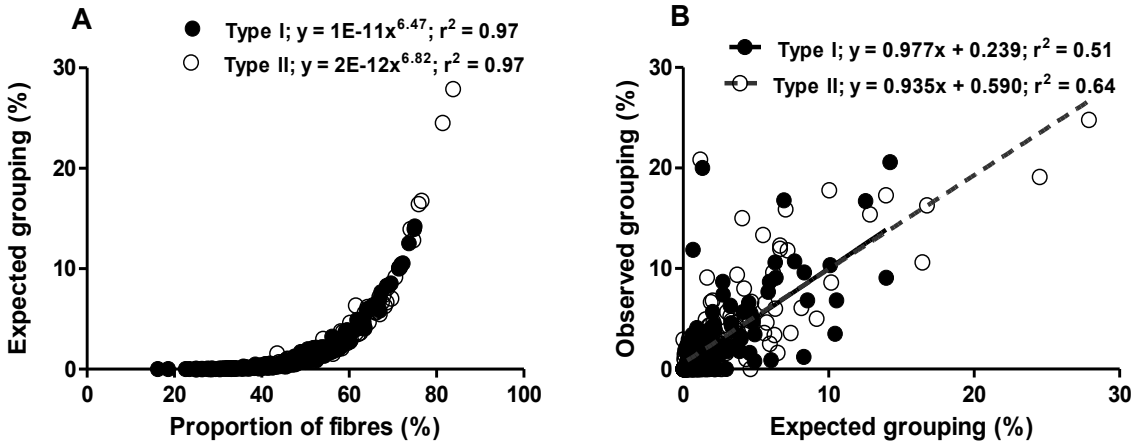


Figure 4.6 **(A)** Exponential correlation between the prevalence of expected grouping of type I and type II fibres for the vastus lateralis muscle and fibre type proportions for all subjects combined. **(B)** Relationship between observed and expected grouping of type I and type II fibres for all subjects combined (n = 157). It can be seen that the regression lines for type I (solid line) and II (dashed line) fibres are similar and close to the line of identity.

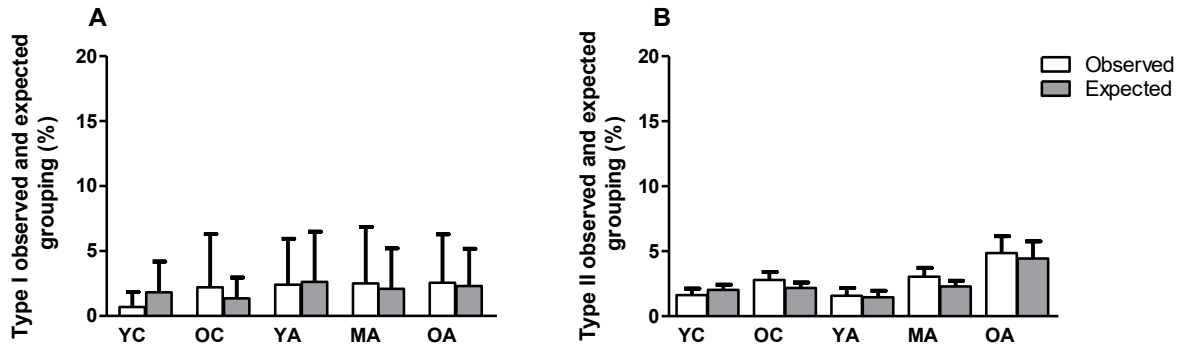


Figure 4.7 Observed and expected grouping for **(A)** Type I and **(B)** Type II fibres in the vastus lateralis muscle of young (YC: n = 22) and healthy older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older master athletes (OA: n = 35). Values are means ± SD.

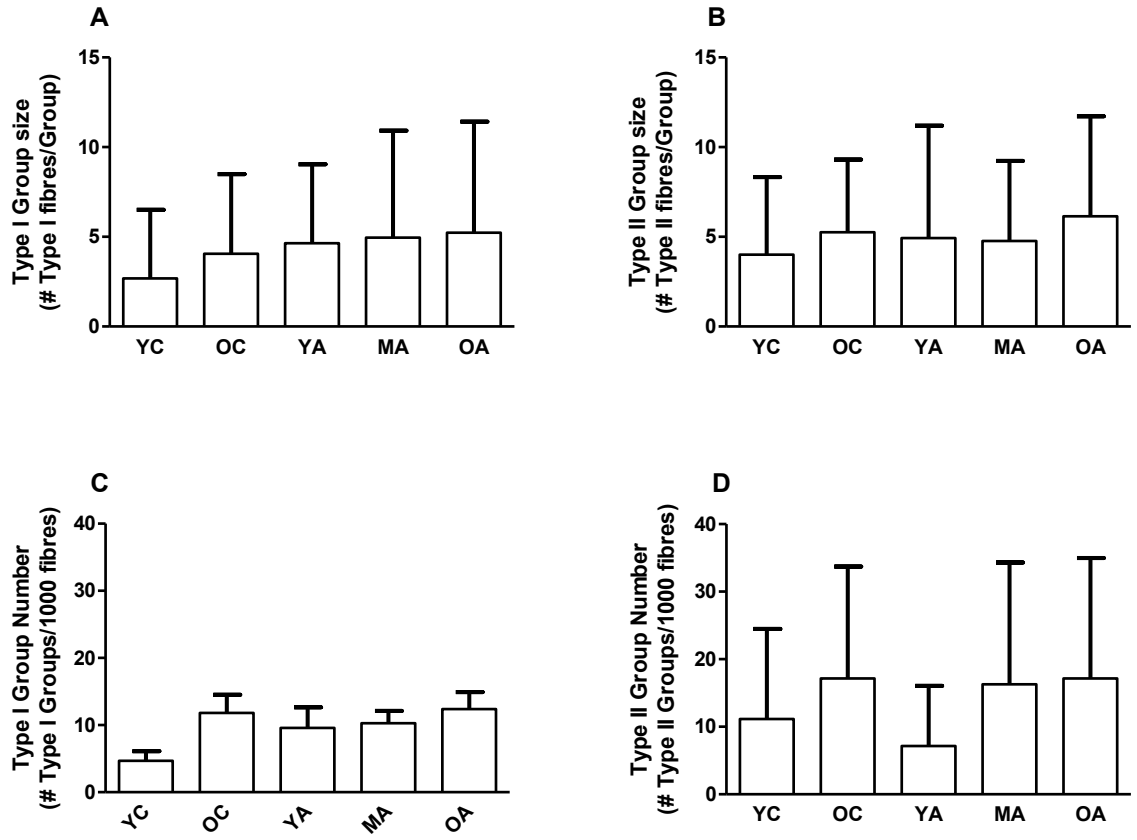


Figure 4.8 **(A)** and **(B)** show fibre group size and **(C)** and **(D)** indicate number of fibre groups in the vastus lateralis muscle of young (YC: n = 22) and healthy older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older master athletes (OA: n = 35). Values are means ± SD.

4.3.5 Characteristics of grouped fibres

There was no significant difference in FCSA between the enclosed, enclosing and remaining type I (Fig. 4.9A) or type II (Fig. 4.9B) fibres.

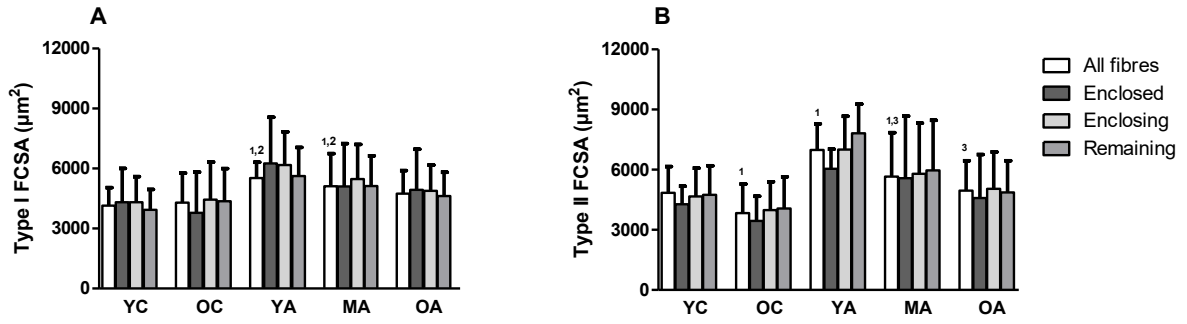


Figure 4.9 Fibre cross-sectional area (FCSA) of **(A)** type I and **(B)** type II fibres, respectively in the vastus lateralis muscle of young (YC: n = 22) and older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older athletes (OA: n = 35). Enclosed fibres: fibres surrounded by fibres of the same type only; enclosing fibres: fibres that surround an enclosed fibre; remaining fibres: fibres that are neither an enclosing nor an enclosed fibre. [1] different from YC at $p \leq 0.045$; [2] different from OC at $p \leq 0.004$; [3] different from YA at $p \leq 0.015$. Data are expressed as mean ± SD.

The circularity or roundness of a fibre is reflected by the shape factor. Although there were main effects of type ($p < 0.001$; sp = 0.999) and group (YC, OC, YA, MA, OA)) ($p = 0.001$; sp = 0.938), there were also sex x fibre type ($p = 0.002$; sp = 0.867) and group x fibre type ($p = 0.004$; sp = 0.982) interactions. Post-hoc comparison showed that in both men and women the shape factor was larger in type II than type I fibres ($p \leq 0.006$; sp = 0.989), with no significant sex differences in the shape factor in fibres of each type (Fig. 4.10A-D).

The group x type interaction ($p = 0.004$; sp = 0.982) was reflected by an absence of significant differences between groups in the shape factor of type I fibres (Fig. 4.11A), while for type II fibres (Fig. 4.10B) the shape factor was higher in OC than YC ($p < 0.001$; sp = 0.938). For type II fibres only, the OC had a larger shape factor than any of the athlete groups ($p \leq 0.025$; sp = 0.921), and while OA had a higher shape factor than MA ($p = 0.007$; sp = 0.941), it was not significantly different from that in YA.

There was a significant group x condition interaction ($p=0.022$; sp = 0.867). Post-hoc tests showed that in the YC, YA, MA and OA there were no significant differences in shape factor between the enclosed, enclosing and remaining fibres (Fig. 4.11A & B). In the OC, the

enclosed fibres had a larger shape factor than the enclosing and remaining fibres (p = 0.044; sp = 0.879).

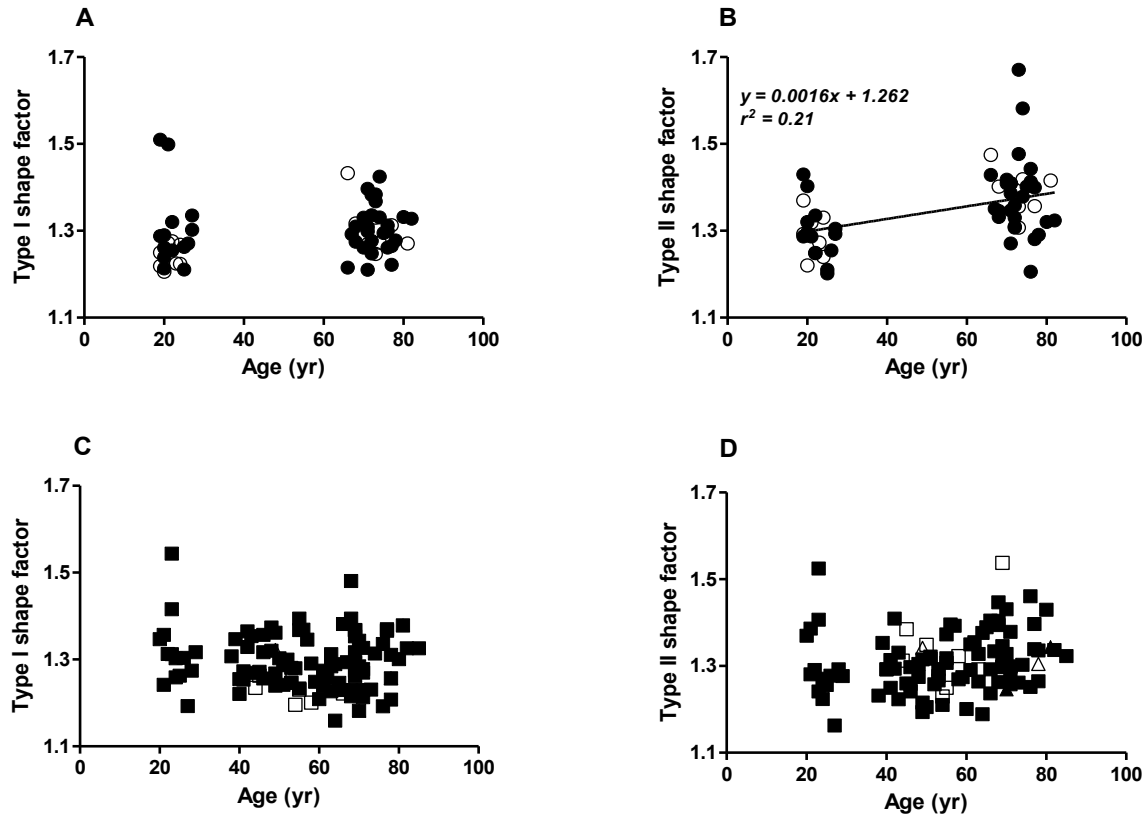


Figure 4.10 Relationship between age and shape factor of **(A)** type I and **(B)** type II fibres in male (●) and female (○) non-athletes (n = 57). **(C)** and **(D)** indicate the same relationship in male (■) and female (□) power master athletes, and male (▲) and female (△) endurance master athletes (n = 100). Regression line is indicated for the shape factor of type II fibres in the control group.

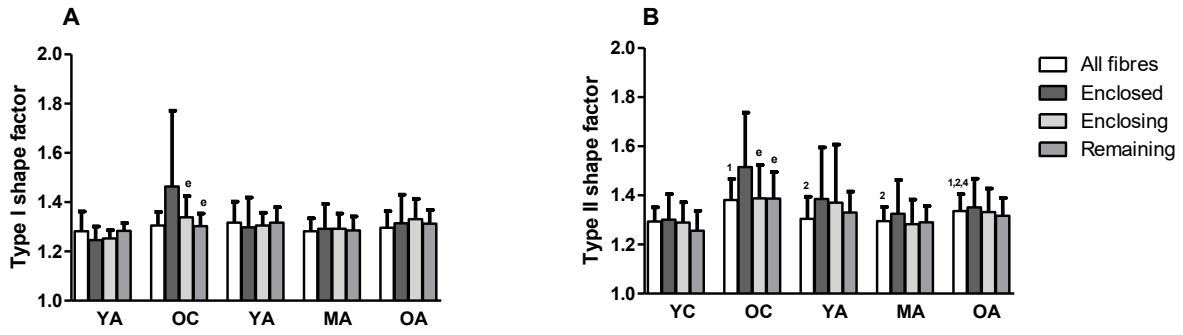


Figure 4.11 The shape factor of **(A)** type I and **(B)** type II fibres in the vastus lateralis muscle of young (YC: n = 22) and older (OC: n = 35) non-athletes, and young (YA: n = 14), middle-aged (MA: n = 51) and older (OA: n = 35) athletes. [e] different from enclosed fibres within the same group at $p \leq 0.044$. [1] different from YC at $p \leq 0.018$; [2] different from OC at $p \leq 0.025$; [4] different from MA at $p < 0.007$. Values are means ± SD.

4.4 Discussion

The main observation of the present cross-sectional study was that in contrast to our expectations and despite indications of preferential type II fibre atrophy in both athletes and non-athletes, there was no age-related increases in the number and size of fibre type groups in either controls or master athletes. The observed proportion of enclosed fibres, indicative of grouping, was explicable by the fibre type composition of the muscle, which showed no significant age-related differences. These histological observations and the similar muscle cross-sectional area to fibre size ratio in young, middle-aged and old athletes do not provide evidence for the common concepts of 1) age-related motor unit remodelling, 2) improved reinnervation with regular physical activity and, 3) at least in athletes, suggests that there may be no loss of muscle fibres. However, the age-related motor unit loss and enlargement in motor unit size previously reported with electromyographic analyses may be reconciled with these histological observations if denervation and reinnervation following motor neuron loss does not necessarily lead to fibre type grouping.

Fibre type composition. In line with previous observations (Lexell et al., 1983b; Grimby et al., 1984; Lexell et al., 1988; Rantanen et al., 1994; Korhonen et al., 2006; Pollock et al., 2018; Barnouin et al., 2017), the proportion of type I and type II fibres in the *m. vastus lateralis* in both athletes and non-athletes was unaltered with age. It has been reported that older muscles have a higher percentage of hybrid fibres than young muscles (those co-expressing both slow and fast myosin heavy chain (MHC) isoforms) (Klitgaard et al., 1990a), but in this study the proportion of hybrid fibres was negligible in muscles from both old and young-adult athletes and non-athletes.

Muscle fibre cross-sectional area (FCSA). We observed that in athletes there was an age-related decrease in the size (thickness) of the *m. vastus lateralis*. The absence of a significant age-related decline in the estimated number of fibres (muscle cross-sectional area divided by FCSA) in the *m. vastus lateralis* of athletes suggests that the observed muscle atrophy is explicable by muscle fibre atrophy. This is in contrast to previous reports suggesting a similar contribution of fibre atrophy and fibre loss to the age-related atrophy in non-athletes (McPhee et al., 2018; Lexell et al., 1988) but in line with a previous study showing no age-related loss of fibres (Nilwik et al., 2013). In rat plantaris muscle it has been reported that the ageing-related loss of fibres is attenuated by intensive running exercise (Suwa et al., 2016) and strength training (Klitgaard et al., 1989). The discrepancy may therefore be related to differences in physical activity patterns, with higher levels of physical activity of the non-sarcopenic participants (Nilwik et al., 2013) and our athletes than that of the recreationally-active sarcopenic older people in our previous work (McPhee et al., 2018). Another possible explanation is that ultrasound underestimates the actual difference in muscle size between young and old when compared with MRI work (McPhee et al., 2018).

Whatever the cause of the discrepancy, we and previous studies in untrained (Lexell et al., 1988; Coggan et al., 1992b; Andersen, 2003; Deschenes, 2004; Verdijk et al., 2007; Nilwik et al., 2013; Barnouin et al., 2017; McPhee et al., 2018) and trained humans (Proctor et al., 1995; Korhonen et al., 2006; Faulkner et al., 2008; Dreyer et al., 2006; Porter et al., 1995) found that the FCSA of type II fibres was significantly reduced with increasing age, while the size of type I fibres was unaffected. In fact, the absolute slope of the ageing-related decrease in type II FCSA was higher in athletes than non-athletes and explains that while young athletes had larger muscle fibres than young non-athletes, no such difference in FCSA was seen between old athletes and non-athletes despite the high activity levels of OA. Thus, in contrast to previous suggestions that regular physical activity preserves muscle mass and morphology (Narici et al., 2004; Mosole et al., 2014; Carraro et al., 2017; McKendry et al., 2019) the absolute age-related gains in type II FCSA by regular physical activity were diminished in old age, but the relative gains were most likely similar.

Fibre type grouping

Number of groups. The common assumption is that the ageing-related loss of motor neurons (Tomlinson and Irving, 1977) is associated with motor unit remodelling that will be apparent as an increase in the number and size of fibre type groups (Hepple and Rice, 2016; Lexell,

1997). While many studies do report an age-related increase in the prevalence of grouping in both animal (Aare et al., 2016) and human muscle (Lexell et al., 1986; Lexell and Downham, 1991), and even more so in older athletes (Mosole et al., 2014; Zampieri et al., 2015), our data showed no age-related increase in the proportion of enclosed fibres in either non-athletes or athletes. Our study does not stand alone, since others have also reported no age-related change in the prevalence of groups in the human *m. vastus lateralis* (Kelly et al., 2018).

Although in theory part of the discrepancy between studies may be due to differences in the sarcopenic state of the muscle, no differences in motor unit numbers have been observed in pre-sarcopenic, sarcopenic and severe sarcopenic groups (Gilmore et al., 2017). Another possibility is the age of the participants where the likelihood of grouping has been reported to appear after the age of 70 years (Lexell, 1997). We contend, however, that the discrepancy between our observations and those in many, but not all, of the previous studies is attributable to not taking into account the chance occurrence of fibre type grouping due to the fibre type composition. This is an important consideration as we illustrate an exponential increase in the expected grouping of a fibre of a given type with increasing proportion of that particular type (Fig. 5), where the same pattern occurred when we plotted the data of Johnson et al. (1973). Another study reported that 58% of the variance of grouping is explained by fibre type composition (Kelly et al., 2018). In this context, it is interesting to note that the more pronounced type I grouping in master athletes was associated with a larger proportion of type I fibres than in age-matched non-athletes (Mosole et al., 2014; Zampieri et al., 2015). In fact, Mosole et al. (2014) show that there is a positive relationship between the proportion of type I fibres and the observed type I grouping, and hence the observed grouping can be explained by differences in fibre type composition between athletes and non-athletes. In further support of this explanation, we found no significant difference between the expected and observed grouping in young and old athletes, and non-athletes. The absence of an ageing-related change in fibre type composition and number of groups challenges the concept of an ageing-related denervation-reinnervation process that is thought to lead to fibre type grouping.

Fibre group size. It is thought that denervation-reinnervation is not only reflected by an increased prevalence of fibre type grouping, but also by an increase in the motor unit size, which may also be reflected in the fibre group sizes. Indeed, previous electromyographic studies have shown an age-related increase in the size of motor units (Piasecki et al., 2016c; Piasecki et al., 2019) and group size in histology (Kelly et al., 2018). It has been suggested

that the larger group number (Zampieri et al., 2015) and group size (Mosole et al., 2014) in master athletes than age-matched non-athletes is indicative for a better reinnervation capacity. Yet, in our study, we did not see evidence for an ageing-related increase in group size in either athletes or non-athletes. While we did not investigate this relationship, the chance of a large group size increases with increasing proportion of a given fibre type. This can readily be seen in the extreme situation of a muscle consisting of only type I fibres that would present as one extremely large group. Thus, the absence of any differences in fibre group size between any of the groups is explicable by the similar fibre type composition between ages and training status. Thus, similar to the absence of an age-related increase in the number of fibre type groups, also the data on group size do not confirm the current concept of an age-related denervation-reinnervation process that is suggested to lead to grouping.

Characteristic of grouped fibres. The development of fibre type grouping over time is suggested to be a consequence of reinnervation of denervated fibres by adjacent axons from remaining motor neurons. In this scenario, the adopted fibre(s) may change type as they become reinnervated by a motor neuron that innervates fibres of a different type. In line with such a concept, it has been shown that many grouped type I fibres show characteristics, presumably reminiscent, of type II fibres (Kelly et al., 2018). One of these characteristics was the larger size of grouped than non-grouped type I fibres (Kelly et al., 2018). We, however, did not find any significant differences in size of type I or type II fibres between grouped and non-grouped fibres. Fibre type- and size-selective denervation-reinnervation therefore appears unlikely to us, and the difference in phenotype seen by Kelly and colleagues (2018) may be more a reflection of the continuum of fibre type phenotypes (Staron and Pette, 1993) rather than being a legacy of denervation-reinnervation cycles.

As grouped fibres may be the result of denervation-reinnervation, one might argue that in particular these fibres may show signs of denervation, or being in the process of reinnervation. It has been suggested that angulated fibres - that can appear flattened, crushed or crescent-shaped - are reflective of such a phenomenon, and they have particularly been reported in muscles of older people (Hepple and Rice, 2016; Lexell, 1997). In the present study, we calculated the shape factor (1 indicates a perfect circle and values > 1 increasing deviation from circularity) to indicate abnormality of fibre morphology. In line with previous observations (Barnouin et al., 2017; Kirkeby and Garbarsch, 2000), our data showed that in non-athletes, but not in athletes, the shape factor of type II fibres increased with increasing

age, whereas the shape factor of type I fibres remained unaffected. The cause of such a preferential increase in angularity of type II fibres is uncertain, but has been suggested to be a first sign of denervation (Rowan et al., 2012; Purves-Smith et al., 2012) or reorganisation of motor units (Kirkeby and Garbarsch, 2000; Andersen, 2003). We consider this unlikely as the absence of significant differences in shape factor between grouped and non-grouped type II fibres suggests that it affects all type II fibres indiscriminately. Further support for another cause than denervation comes from the observation that type II fibres were more angular than type I fibres, even in the young athletes and non-athletes, and the absence of an increase in angularity in denervated rat muscles (Paudyal et al., 2018). Overall, the ageing-related increase in angularity of type II fibres may be due to disuse (Kouyoumdjian, 1993), rather than a denervation-reinnervation process.

In context

Overall, the absence of an ageing-related increase in the number and size of fibre type groups challenges the common idea that much of the ageing-related muscle wasting is attributable to an ongoing denervation-reinnervation process. The support for such a process is significant, and reflected by an age-related loss of fibres in both human (Lexell et al., 1988; McPhee et al., 2018) and rodent muscles (Lushaj et al., 2008). This is, however, not unequivocal, as others report no significant ageing-related decrease in fibre number in both human (Nilwik et al., 2013) and rodent muscles (Ballak et al., 2014b). The major limitation of all of these studies is that they are cross-sectional designs. Nevertheless, the ageing-related reductions in axons in nerves (Larsson et al., 2019) and loss of motor neurons (Tomlinson and Irving, 1977) do suggest that motor neuron loss does indeed occur. Electromyographic studies also suggest almost invariably that motor unit loss occurs during ageing as well as an increase in individual motor unit sizes in both athletes and non-athletes (Drey et al., 2016; Galea, 1996; Mccomas et al., 1971; Piasecki et al., 2016a; Piasecki et al., 2019; Power et al., 2010; Power et al., 2016), the latter suggestive of reinnervation. The question thus arises how such an apparent discrepancy between our data and the overwhelming evidence for motor unit remodelling during ageing can be reconciled.

Perhaps most important is that an ongoing denervation-reinnervation process is not necessarily unidirectional (e.g. only type II fibres being denervated and reinnervated by a type I motor neuron), and in that instance the fibre type composition remains unaltered. Grouping may then still occur when neighbouring axons reinnervate the denervated fibres,

which then would result in clusters even when the fibre type composition is unaltered. However, glycogen depletion studies on the distribution of motor unit fibres in ageing rats have shown that an increase in the number of fibres per motor unit is accompanied by an increase in the motor unit territory, without evidence of fibre type clustering (Edstrom and Larsson, 1987). This then would reconcile our observations of an absence of fibre type grouping and yet an increase in motor unit size seen in electromyographic studies. If so, histological examination alone may not be sufficient to assess ageing-related motor unit remodelling. Many studies have drawn conclusions concerning fibre type grouping in master athletes (Mosole et al., 2014; Kelly et al., 2018). However, the considerations above and our observation that the fibre type grouping is similar to that expected from fibre type composition indicate that perhaps we need to be careful with drawing firm conclusions from muscle biopsies on age-related motor unit remodelling, unless pathological grouping is evident.

Another important consideration is that almost all, if not all, reports of motor unit loss in old age are cross-sectional and show a large variation in the number of motor units within a muscle between people of the same age (Galea, 1996; Piasecki et al., 2016b). It could be that part of the age-related reduction in motor units and motor neurons in cross-sectional studies is not presenting a real loss, but rather a lower number of motor units and motor neurons at birth due to differences in lifestyle (e.g. pre/post-natal diet) over the past decades. In fact, in piglets it has been shown that limited intrauterine protein supply has both a negative effect on myogenesis and muscle growth potential (Rehfeldt et al., 2012) and in rats, the motor neuron survival during embryonic development appears dependent on the number of muscle fibres available for innervation (Habgood et al., 1984). If this is the explanation then there is perhaps no age-related motor unit loss and in this context it is interesting to note that in cross-sectional study both motor unit size and number did not differ between middle-aged and old people as reflected by an unchanged myelinated axons and muscle fibre number in the intrinsic laryngeal muscles (Santo Neto and Marques, 2008).

Conclusion

In the present study, we found no evidence for an age-related increase in fibre type grouping, and no difference in grouping between athletes or non-athletes. The prevalence of fibre type grouping that was observed was similar to that expected based on the fibre type composition

of the muscles. Older age was, however, associated with a smaller fibre cross-sectional area of type II fibres, and this age-related decrement was in absolute terms even more pronounced in master athletes. These findings do not support the common notion of 1) an ageing-related motor unit remodelling 2) nor that reinnervation is enhanced with prolonged physical training. The ageing-related motor unit loss and increase in motor unit size often seen with electromyographic analyses may be reconciled with these histological observations if denervation and reinnervation following motor neuron loss does not necessarily lead to fibre type grouping.

Limitations of study III

This experimental study should be commended for the number of participants tested which is always challenging for this type of research, associated with the time constraints and recruitment challenges associated with testing more individuals. However, this is indeed a cross-sectional study and that has some limitations. One of the big challenges faced in this area of research is why are the older people still active? Is it because they are genetically better suited to doing an activity and is this related to the fact that they may have a muscle fibre composition that makes them better suited to it. In the present study we did, however, not see significant differences either in the athletes or non-athletes, nor between athletes and non-athletes. In fact, it has recently been shown that even older people who had never been active can reach the same performance levels as master athletes who have exercised many years (Piasecki et al., 2019a). Nevertheless, longitudinal studies elucidating the association between fibre type proportion and fibre type grouping are required to better understand these important relationships. This would aid to fully characterize fibre type grouping changes that occur over time in skeletal muscle of different individuals.

Summary of study III

In addition to ageing and diet intake, physical inactivity leads to muscle atrophy (Degens and Alway, 2006). However, it is not known to what extent muscle wasting and weakness in old age are due to decreased physical activity levels. Master athletes maintain high physical activity levels (Degens et al., 2013; Hannam et al., 2017) and thus are considered a good model to disentangle the effects of ageing *per se* from reduced levels of skeletal muscle physical activity (Rittweger et al., 2009; Degens et al., 2013; Harridge and Lazarus, 2017).

Many studies have associated increased motor unit size (Piasecki et al., 2019; 2016a) and large fibre type groups (Mosole et al., 2014; Carraro et al., 2017) which was interpreted as

indicative for improved reinnervation in master athletes. Others, however, have indicated small motor unit size, suggested to be representative of less collateral reinnervation in master athletes (Power et al., 2016). Thus, evidence is equivocal to whether regular physical activity attenuates ageing-related motor neuron loss and enhances reinnervation. In the experimental study III, fibre type grouping was assessed in athletes and non-athletes of a wide age range (19-85 years). Previous studies on fibre type grouping in master athletes considered only old master athletes and did not compare them with young- and middle-aged master athletes, or age-matched non-athletes, nor did they take into consideration the potential impact of fibre type proportions on the observed fibre type grouping. This is important since fibre type composition varies between individuals and fibre type grouping is more likely for those with a high proportion of one single fibre type compared with those with similar proportions of type I and type II fibres.

CHAPTER 5

General discussion

5.1 Discussion

Age-related loss of muscle mass is a major cause of a reduced independence in older individuals. It is also associated with increased fall risk and related injuries, greatly reducing the quality of life of the older persons resulting in a burden on the healthcare system. The function of older skeletal muscle is influenced by several factors, including dietary habits and physical activity. Therefore, the first aim of the present study was to investigate in a mouse model the effects of early ageing on skeletal muscle morphology. More specifically, we compared the capillary supply to a fibre in relation to type, size, oxidative capacity and IMCL in a postural slow oxidative muscle (the soleus), a muscle that is intermittently active (the fast, more glycolytic EDL) and a muscle that is constantly active (the diaphragm, highly oxidative with a mixed fibre type composition) between young-adult (20 weeks old) and early ageing mice (79 weeks old). We hypothesized that capillary rarefaction and a decrease in oxidative capacity occur during early-ageing with some modest atrophy that are more aggravated in locomotor than in the respiratory muscles. The results of study I provide novel evidence that during early ageing there is no significant loss of muscle mass in the hind limb mouse muscles. The EDL fibres had atrophied and remarkably, the diaphragm hypertrophied with the absence of changes in the number of capillaries supplying a fibre or their oxidative capacity. Thus, it appears that early ageing exerts differing effects in respiratory and limb muscles, where fibre atrophy is not necessarily preceded by capillary rarefaction and reductions in oxidative capacity.

In study II, we comprehensively investigated whether the effects of a HFD on the morphological changes varied between the soleus, EDL and diaphragm muscles and whether the adaptations in young-adult (20 weeks old) are different compared to early-ageing mice (79 weeks old). We hypothesized that a HFD leads to an increase IMCL in all muscles. As a HFD is known to increase fatty acid oxidation in skeletal muscle, we further hypothesized that this lead to increased oxygen demand and thus increased capillarisation and oxidative capacity in the diaphragm while the locomotory muscles will show a decrease in oxidative capacity and capillarisation. Moreover, morphological changes induced by a HFD will increase with the duration of feeding and be more pronounced at old age. We showed that while an accumulation of IMCL, an increase in oxidative capacity, angiogenesis and fibre hypertrophy occurred in the diaphragm and soleus muscle, the muscles of old mice were more susceptible to HFD-induced changes in morphology than those of young-adult mice.

There is a considerable interest in understanding fibre type grouping that is thought to arise from an ongoing denervation-reinnervation process. It is thought that life-long physical exercise enhances the reinnervation of denervated fibre. A larger size of fibre groups (Mosole et al., 2014; Carraro et al., 2017) and a loss of motor units (Piasecki et al., 2019; Piasecki et al., 2016b) have been reported in master athletes. Based on these observations, in study III we hypothesized that both fibre group size and number of groups increase with increasing age in master athletes beyond that expected from muscle fibre type composition. A novel aspect of this study was that, contrary to previous reports, not only was fibre type grouping examined in old master athletes and compared with young- and middle-aged master athletes, but also the potential impact of fibre type composition on the observed fibre type grouping was considered. In fact, fibre type composition can differ markedly between individuals, and grouping is expected to be increasingly prevalent in muscles with an increasing proportion of one single fibre type. Data from study III show that neither the number nor the size of fibre type groups differed between young and older individuals in either controls or master athletes. In addition, the prevalence of observed fibre grouping, as reflected in the number of enclosed fibres in the samples, was explicable by the fibre type composition of the muscle, which also exhibited no significant age-related differences. Thus, if anything, histological observations in the current study offer no support to the idea of age-associated motor unit remodelling or improved reinnervation with regular physical activity.

Morphological alterations in mouse skeletal muscles during early ageing are muscle specific (I)

It is well-known that ageing is accompanied with numerous skeletal muscle morphological changes from adulthood to old age. It is however, difficult if not impossible, to simultaneously evaluate in humans to what extent ageing affects fibre size, fibre number, oxidative capacity, capillarization and the levels of IMCL, some of the main morphological characteristics of skeletal muscle. Nevertheless, this can be studied systematically in a murine model. We used 20- and 79-week-old mice as models for young-adult and early-ageing mice, respectively to study age-related changes in muscle morphology (I). Muscle morphological alterations during early ageing may be masked by maturational changes when not fully-matured animals are used as the control group (Ballak et al., 2014b). Therefore, 20-week-old CD-1 mice were chosen in this study as the fully matured young-adult group to minimalize bias of maturation and 79-week-old mice representing early ageing, since they previously show a decline in specific tension, but without loss of muscle mass (Hill et al., 2018). In this

study, the effects of early ageing on muscle morphology were examined in soleus (slow twitch), EDL (fast twitch) and diaphragm (mixed fibre type) muscles. By determining the muscle morphological indices of functional status, the present work is the first to extensively offer a better understanding of the early-ageing-related muscle-specific morphological alterations in mouse skeletal muscle.

Our data, along with other reports (Greising et al., 2013; Omairi et al., 2016) showed that the EDL muscle is composed primarily of type IIb fibres, whereas the diaphragm has a majority of type IIa fibres and the soleus contains primarily type I fibres. No significant age-related differences in the proportion of type I, IIx and IIb fibres were seen in all the muscles studied.

Perhaps the most striking finding of study I was the atrophy of the EDL and hypertrophy of the diaphragm muscle fibres with increasing age. The latter observation is at odds with results of other studies reporting a decrease of diaphragm fibres at advanced age (Greising et al., 2013). Discrepancy between these studies may be due to differences in age groups and strains of the animals used. We compared 20- with 79-week-old mice and Greising et al. (2013) compared 20- with 100-week-old mice. Nevertheless, the diaphragm may hypertrophy during ageing to adapt to the increased cost of breathing because of the ageing-related reduction in lung compliance (Sharma and Goodwin, 2006). Thus, these novel results suggest that the fibre size increase during ageing in the respiratory muscles is opposite to the decreases in fast limb muscles, and that early-ageing morphological changes are more associated with muscle functional demands than fibre type composition.

Studies in humans (Houmard et al., 1998; Pastoris et al., 2000) and animals (Bass et al., 1975; Holloszy et al., 1991) have shown some evidence that the effects of ageing on oxidative capacity differs between muscles. In this thesis, the oxidative capacity of the fibres was determined using succinate dehydrogenase activity (SDH) staining. We found that the oxidative capacity of the fibres was higher in the diaphragm muscle than in the limb muscles. The diaphragm is the primary inspiratory muscle, being continuously active throughout life, in contrast to limb muscles, and this may explain the higher oxidative capacity in the diaphragm. There was no age-related change in oxidative capacity of the fibres in the soleus and the diaphragm. However, in the EDL the decrease in the integrated SDH activity per fibre in early ageing was attributable to both a decrease in the mass-specific oxidative capacity (SDH-OD) and fibre cross-sectional area. Muscle capillarization, as measured by the capillary to fibre ratio (C:F), was greater in soleus than in EDL and diaphragm. This

observation is in accordance with a previous study on muscle capillarisation (Murakami et al., 2010).

It has been suggested that capillary loss may preceded the age-related fibre atrophy (Larsson et al., 2019), but in our study we found no age-associated changes in C:F in the muscles, indicating no capillary loss during early ageing. In addition, the enhanced capillary density (CD) in the EDL during early ageing was associated with a decrease in the fibre size, while in the diaphragm the reduced CD was explicable by an increase in FCSA during early ageing, with no signs of angiogenesis or capillary rarefaction, respectively.

It has also been reported that not only the C:F and fibre area influence tissue oxygenation but also the spatial distribution of capillaries, where a heterogeneous distribution of capillaries has a negative impact on tissue oxygenation (Degens et al., 2006; Degens et al., 1994a; Goldman et al., 2006). In study I, we found an age-related reduction in the heterogeneity of capillary spacing, reflected by the logarithmic standard deviation of the capillary supply radius (LogRSD) (Hoofd et al., 1985; Degens et al., 1993b; Barnouin et al., 2017), which suggests a more homogenous distribution of capillaries. Although the heterogeneity of capillary spacing increased linearly with an increase in fibre size variation, we did not find any variation in the fibre size during early ageing in the CD-1 mice used. From these observations, it was proposed that the constraint of capillary positioning at the fibre periphery is one determinant of the heterogeneity of capillary spacing (Degens et al., 2009), and that the positioning of capillaries during early ageing is not at random (Degens et al., 2006), but rather such to maintain adequate muscle oxygenation.

The local capillary to fibre ratio (LCFR) from any of the muscles expressed was positively related to FSCA, consistent with previous observations (Degens et al., 1992; Bosutti et al., 2015; Barnouin et al., 2017). This suggests that the capillary supply to a muscle fibre is more related to fibre size than the oxidative capacity of the fibre. The relationship between supply and demand was further estimated by determining the maximal oxygen demand of a fibre as the integrated SDH activity (FCSA * SDH-OD) and calculating the capillary supply (LCFR) to demand ratio (LCFR/SDH-INT) for each fibre. Here, we found that during early ageing, the capillary supply becomes even more in excess to oxidative capacity, particularly in the soleus muscle, supporting the notion that the oxidative capacity is not the main determinant of the capillary supply to a fibre, but other functions, such as removal of heat and waste products, and substrate delivery are more important.

The impact of a high-fat diet in mice is dependent on duration and age, and differs between muscles (II)

In study II, we provide novel evidence that skeletal muscles of young-adult (20 weeks) and early ageing (79 weeks old) female CD-1 mice fed a HFD for 8, 9 or 16 weeks undergo muscle-specific morphological adaptations. There was, however, no significant effect of a HFD on fibre type composition. While the EDL displayed no FCSA change following a HFD, in the the FCSA of the soleus was increased. This is consistent with a study reporting that HFD led to increased soleus muscle fibre areas, but no change in fibre type proportions or area in the EDL (Turpin et al., 2009). These differences may be the result of an elevated activity in the calf muscles with no such increased loading in the dorsiflexors of relatively sedentary rodents.

Several studies have indicated that HFD induced mitochondrial biogenesis and was associated with an increased activity of mitochondrial enzymes and fatty acid oxidation capacity in the skeletal muscle of mice and rats. We did find something similar in young, but not in the old mice following a HFD consumption. The increased oxidative capacity seen in young mice fed a HFD could represent an early adaptation to the elevated IMCL levels typical of the HFD. In fact, there was an increase in the levels of IMCL with HFD, albeit in a fibre type and muscle specific manner. It has been reported that 4 weeks of high-fat or high-sugar diet in young mice (10 weeks old) was associated with some metabolic alterations that could reflect the onset of deleterious processes in pre-diabetic skeletal muscle (Bonnard et al., 2008). The increase in oxidative capacity in response to a HFD in young animals may also be an early attempt to enhance β-oxidation. In addition to increase oxidation capacity, it has been reported that HFD can stimulate angiogenesis in order to match oxygen supply with increase oxygen demand (Silvennoinen et al., 2013). In line with this suggestion, we found that HFD increased capillarisation in the soleus and diaphragm of young-adult mice, as reflected by the increase in both C:F and LCFR. The absence of a significant increase in LCFR in the EDL and diaphragm of the old mice with no changes in oxidative capacity suggests that HFD-induced angiogenesis serves to ensure an adequate oxygen supply in the young-adult mice on a HFD. Nevertheless, the increased oxidative capacity in the young EDL mice was not accompanied by angiogenesis, and in the old soleus mice, the angiogenesis was without a significant increase in oxidative capacity.

These observations imply that there is an uncoupling between changes in capillary supply and oxidative capacity of the fibres, and similar to study I the oxidative capacity does not determine the capillary supply to a fibre, but rather fibre size (Bosutti et al., 2015). There was no significant difference in overall muscle capillary density and the capillary density per fibre between mice on a standard diet and those on a HFD, except for a decrease in the diaphragm from young-adult mice. Also, the similar heterogeneity of capillary spacing, reflected by the logarithmic standard deviation of the capillary supply areas (LogDSD), implies that angiogenesis caused by a HFD is not a random process, but rather preserves the distribution of capillaries to maintain the potential for adequate intramuscular oxygenation. Overall, study II provides a comprehensive analysis of skeletal muscle morphology following 8, 9 and 16 weeks of HFD in young-adult and early-ageing mice. Following a HFD, there were increases in body mass and BMI, an accumulation of intramyocellular lipid, an increase in oxidative capacity, angiogenesis and fiber hypertrophy in the diaphragm and soleus muscle, and the response in the old animals occurred earlier than in young animals.

Absence of an ageing-related increase in fibre type grouping in master athletes and non-athletes (III)

In study III, we assessed in biopsies of the vastus lateralis the fibre type composition using the myofibrillar ATPase staining. In each sample, fibres were counted and classified as type I or type II. The prevalence of fibre type grouping was determined based on the number of enclosed fibres in muscle specimens (Jennekens et al., 1971), an enclosed fibre being a fibre that is completely surrounded by fibres of the same histochemical type. It is clear that the number of enclosed fibres of any one type will rise as the proportion of that fibre type in the sample rises. On this account, the expected number of enclosed fibres was also calculated for a complete range of fibre type proportions, using a mathematical model that assumed that the arrangement of individual fibres conformed to a hexagonal lattice and that the spatial distribution of type I and type II fibres was random (Johnson et al., 1973).

Consistent with previous publications (Lexell et al., 1983a; Grimby et al., 1984; Lexell et al., 1988; Rantanen et al., 1994; Korhonen et al., 2006; Pollock et al., 2018; Barnouin et al., 2017), we showed that increasing age was not accompanied by altered fibre type proportions of the vastus lateralis muscle. However, there was an age-related decrease in type II FCSA while the size of the type I fibres remained unaffected. These observations were consistent to

what has been previous reported in untrained (Barnouin et al., 2017; McPhee et al., 2018) and trained individuals (Korhonen et al., 2006; Faulkner et al., 2008).

Perhaps the most significant finding of study III was the absence of age-related increase in the prevalence of fibre type grouping in either non-athletes or athletes, observations also reported by others (Kelly et al., 2018). However, discrepancies in study results exist and could be explained by multiple factors including the age of the participants, where the chance of having enclosed fibres has been shown to increase beyond the age of 70 years (Lexell, 1997). Another factor contributing to the discrepancy between our observations and those of others in the assessment of fibre grouping is that the fibre type proportion of the muscle examined has not been taken into account in other studies. As mentioned previously, the prevalence of enclosed fibres will increase as the proportion of that fibre type in the cross-section rises. In this regard, Kelly et al. (2018) reported that about 58% of the variance of grouping is explained by fibre type composition. Moreover, a positive correlation has been reported between the proportion of type I fibres and the observed type I grouping (Mosole et al., 2014), and thus the observed grouping in those studies may well be explained by differences in fibre type composition between athletes and non-athletes. In line with this observation, in the current study no significant difference between the expected and observed number of enclosed fibres in young and old athletes, and non-athletes was found. Thus, the absence of changes in fibre type composition and prevalence of grouping with increasing age does not support the accepted concept of an ageing-related denervation-reinnervation process that is thought to lead to fibre type grouping, although it should be recognised that a fibre does not need to be completely enclosed following some level of reinnervation.

The ageing process is thought to not only cause an increase in the prevalence of fibre type grouping through denervation-reinnervation, but also to increase the size of the surviving motor units, which may be reflected by fibre group sizes. For example, the larger group number (Zampieri et al., 2015) and group size (Mosole et al., 2014) in master athletes than age-matched non-athletes is thought to indicate a better reinnervation capacity, even in octogenarian female master athletes (Sonjak et al., 2019). These observations are at odds with the results of our study, which showed no ageing-associated increase in group size in either athletes or non-athletes. Typically, it is expected that the size of a group increases with increasing proportion of a particular fibre type, so that a muscle comprising only type I fibres would present one extremely large group. Therefore, it is concluded that the lack of any differences in fibre group size between any of the groups is ascribed by the similar fibre type

composition between ages and training status. Here again, the data on group size contrasts the existing concept of an age-related denervation-reinnervation process that is suggested to contribute to fibre grouping.

Despite the accepted concept that denervation-reinnervation events in advanced age lead to fibre type grouping, that is also thought to be associated with loss of fibres in both human (Lexell et al., 1988; McPhee et al., 2018) and rodent muscles (Lushaj et al., 2008), other studies have reported no significant ageing-related reduction in fibre number in both human (Nilwik et al., 2013) and rodent muscles (Ballak et al., 2014b). Yet, electromyographic studies suggest almost invariably that motor unit loss occurs during ageing and an increase in individual motor unit sizes in both athletes and non-athletes (Drey et al., 2016; Galea, 1996; Mccomas et al., 1971; Piasecki et al., 2016a; Piasecki et al., 2019; Power et al., 2010; Power et al., 2016), the latter suggestive of reinnervation. There is thus an apparent discrepancy between our data and the overwhelming evidence for age-related motor unit remodelling that somehow needs to be reconciled.

One explanation for the apparent discrepancy is that an ongoing denervation-reinnervation process is not necessarily unidirectional (e.g. only type II fibres being denervated and reinnervated by a type I motor neuron), and therefore even during ongoing denervation-reinnervation the fibre type composition may remain unchanged. In such a situation, fibre grouping may still be generated when nearby axons reinnervate the denervated fibres, which then would lead to clusters even when the fibre type composition is unchanged. However, reports of glycogen depletion on the distribution of motor unit fibres in ageing rats have indicated that an increase in fibre numbers per motor unit is accompanied by an increase in the motor unit territory, without signs of fibre type clustering (Edstrom and Larsson, 1987). This then helps to reconcile our observations of a lack of fibre type grouping and yet an increase in motor unit size observed in electromyographic studies. In this case, histological examination alone may have a limit to investigate ageing-related motor unit remodelling.

It must also be noted that the majority, if not all, age-related studies of motor unit loss are cross-sectional and display a large variation in the motor unit numbers within a muscle between individuals of the same age (Galea, 1996; Piasecki et al., 2016b). It is possible that the age-related decrease in motor units and motor neurons in cross-sectional studies is not showing a real loss, but instead a lower number of motor units and motor neurons at birth due to differences in lifestyle over the past decades. For example, it has been shown in piglets that

limited intrauterine protein supply has both a negative effect on myogenesis and muscle growth potential (Rehfeldt et al., 2012) and in rats, the motor neuron survival during embryonic development is dependent on the number of muscle fibres available for innervation (Habgood et al., 1984). If this is the case, there is perhaps no age-linked motor unit loss. With this in mind, it is worth to note that in one cross-sectional study both motor unit size and number did not differ between middle-aged and old people as revealed by an unaltered number of myelinated axons and muscle fibres in the intrinsic laryngeal muscles (Santo Neto and Marques, 2008).

In line with a concept of reinnervation of denervated fibres by adjacent axons from remaining motor neurons that leads to fibre grouping, (Kelly et al., 2018) reported that grouped type I fibres display characteristics presumably indicative of type II fibres. For example, it was found that grouped type I fibres had larger size compared to non-grouped type I fibres (Kelly et al., 2018). However, our data did not show any significant differences in size of type I or type II fibres between grouped and non-grouped fibres. We argue that the difference in phenotype indicated by Kelly et al. (2018), rather than being a result of denervation-reinnervation process, may reflect more the continuum of fibre type phenotypes (Staron and Pette, 1993).

Some studies have suggested that muscle fibres, particularly in older individuals, change morphology as a result of denervation and reinnervation process (Hepple and Rice, 2016; Lexell, 1997). We addressed this issue by calculating the shape factor (1 indicates a perfect circle and values > 1 increasing deviation from circularity) to indicate abnormality of fibre morphology. In line with previous studies (Barnouin et al., 2017; Kirkeby and Garbarsch, 2000), we showed that in non-athletes, but not in athletes, the shape factor of type II fibres increased with increasing age, while the shape factor of type I fibres remained unchanged. Although the increased angularity of type II fibres has been attributed to a denervation (Rowan et al., 2012; Purves-Smith et al., 2012) or reorganisation of motor units (Kirkeby and Garbarsch, 2000; Andersen, 2003), we argue that this unlikely since our data indicate the absence of significant differences in shape factor between grouped and non-grouped type II fibres, showing that all type II fibres were affected indiscriminately. Thus, we attribute the ageing-associated increase in angularity of type II fibres to disuse (Kouyoumdjian, 1993), rather than a denervation-reinnervation process.

5.2 Summary and conclusions

The main findings in this thesis were that:

In early-ageing mice there was no significant loss of hind limb muscle mass, but an atrophy of EDL muscle fibres, and hypertrophy of muscle fibres in the diaphragm without alterations in the number of capillary to fibre or oxidative capacity. These findings suggest that the effects of early ageing on respiratory and limb muscles are different, where fibre atrophy is not necessarily accompanied with, or preceded by, capillary rarefaction and reductions in oxidative capacity. Yet, regardless of age and muscle type, the number of capillaries supplying a fibre was determined by the fibre size with no significant contribution of oxidative capacity (Study I).

Early-ageing mice on a HFD are more susceptible to muscle morphological changes than young-adult mice. Remarkably, no changes were detected in fibre type composition, and EDL muscle was less susceptible to HFD-induced morphological changes than diaphragm and soleus. While the response occurred sooner in old mice than young mice, we showed that HFD caused an increased in body mass and BMI, IMCL, oxidative capacity, angiogenesis and fibre hypertrophy in diaphragm and soleus. Moreover, we found that angiogenesis was in general proportional to the HFD-induced muscle hypertrophy. Interestingly, signs of a developing mismatch between oxygen supply and demand was apparent in animals fed a HFD, as reflected by the lower ratio of capillary number: maximal oxygen consumption of a fibre in the diaphragm. Thus, in older animals, a HFD produced muscle morphological changes, which may lead to a mismatch between oxygen supply and demand to the working muscles (Study II).

The preferentially atrophy of type II fibres in both athletes and non-athletes was not accompanied by an age-associated increased in the number and size of fibre type groups in either controls or master athletes. Moreover, the prevalence of the observed grouping, which showed no age-associated differences, was explicable by the fibre type composition of the muscle. Our findings provide no evidence of an ageing-related motor unit remodelling nor that improvement of the reinnervation of denervated fibres with prolonged physical training (Study III).

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