Structure-function relationship and plasticity in old mouse muscle

By
Samson Ben Ballak, MSc.

[1]*School of Healthcare Science, Manchester Metropolitan University, Manchester, United Kingdom.*

[2]*Laboratory for Myology, MOVE research institute amsterdam, Faculty of Human Movement Sciences, VU University Amsterdam, Amsterdam, The Netherlands.*

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy
March 2015

Acknowledgements

I am really grateful that I have been able to complete this thesis, but it would not have been possible without the help of a lot of people. I would like to thank all of those people, with special thanks to a couple of them in particular.

First of all, Tinelies. When I began working on the project, you were already working hard to learn and develop the techniques necessary for the operations. Your knowledge and skills have been essential for the success of this project. During the first year of my PhD, we had 3 intensive days of operations per week. I realise how hard this was for you, and so I want to sincerely thank you for your commitment during that time.

Hans, the first time we met, I knew my PhD and its supervision was in great Han(d)s. I do not know anyone so loyal and committed to his students as you are. I will never forget the times that I was working behind my desk and you would come by and say that you had about 15 minutes between 2 meetings to have a coffee and a talk about the project, illustrating your willingness to help every second you could. Even though you are busy and sometimes forced into tasks you would rather not do, you still make as much time for your students as you can. Hans, we will see each other in a bit! Although I don't know where a bit is, I know it will be soon! Thank you for all the time and energy you have spent on me!

Skype meetings until 1 a.m. that's Richard! The man who works around the clock and barely seems to sleep. Richard, I do not know how you manage this, but your commitment to your work is incredible. You approached every meeting with such enthusiasm for the research and your boundless energy was really contagious! After every meeting I was so inspired that I just wanted to work until 1 a.m. to read and put the smallest detail of our research together. Thank you for your energy and dozens of feedbacks on my manuscripts! After every feedback, the manuscript would become even better and better than I could imagine. Thanks!

Arnold, you were the man to guard the red line. Where both Hans, Richard and I went into depth on every little question, you were pragmatic and clear on what to do. Without you we

would have still been busy adding a comma or a point in line 3 at the end of section 4 of page 3. Arnold, Richard, Hans; the three of you together were the perfect supervisory team; the perfect mix of pragmatic thinking, energy, positivity, commitment and loyalty. I learned so much from you, thank you!

Glenn, Guus and Carla, I know I asked you loads of really small things. Questions like whether I used the right solution, whether there would be dry ice, if there was any particular antibody left, or how the microscope worked. But all those little questions made a whole lot of help together. Thank you for that!

Pete and Moi, my computer team. Wow, you showed me what the future looks like! Everything is possible, as long as you are a programmer! We will only become more dependent on people like you and I am so happy that both of you were willing to help me with the capillaries and fibres.

I also want to thank all students who were involved with my project, in particular Eva, Evelien, Frank and Joshua who worked really hard on fiber CSA's and SDH measurements. The work you did was great and I hope were encouraged to pursue your own PhD-track!

Kat and Sander. We were the first generation of MMU Move-Agers, that will bind us forever! Sander my move-age twin-brother. Dutch, the Amsterdam-Manchester combination, even similar timed mobility periods and both our girlfriends are Kim's! You are the sunshine at work. Every morning at the MMU I came in to work, the coffee was (about) ready and we would discuss all kind of mostly sports related topics. How I miss those talks and coffee! Sander I really hope that we (and our Kim's) will see each other a lot even without the Move-Age program! Kat, where should I start! With San, Sam and the Kim's? Or the fact that you are such a kind person and you had to put up with Sander and me for 3 years! I do not know how you did that! In those 3 years, you have become really dear to me! Thanks for the nice times! We will keep in touch for sure, I promise!

Move-Agers, I am so glad that I was and will always remain a part of the Move-Age group. The annual meetings, the start-up courses, we had so many nice times. However, unfortunately we also had to experience the loss of one of us. I think this will unite us forever. Knowing all of you, from all kinds of different backgrounds and cultures has honestly enriched my life.

Colleagues at MMU, I know I could complain a lot about the weather and the holes in the roads. But I have really enjoyed my time in Manchester and that is mostly because of the nice working environment! Thanks for that.

I would also like to thank Armin van Buuren for producing music that got me through weeks, months of repetitive tasks, with a smile on my face!

Furthermore, I would like to thank my parents for always supporting me and giving me the opportunity to develop as a person. There is no way that I would have accomplished this without the unconditional support from you.

Dub, my real twin-brother. No matter how hard we try to be different, we will always be similar in things we achieve. It is always nice to see you start things that I have not done (yet). Or too see you accomplish things I have not done, because then I know I can do it too. When you started your PhD I knew I could do it too, and it feels great that we are finishing around the same time and can even literally connect the front pages of such an important landmark in our lives (the front pages of our theses share similar fragments).

Kim, last but certainly not least. I know my two year leave to Manchester was harder for you than for me. It was not easy, but by letting me do this, you have shown what a beautiful person you are. You have supported me to accomplish something great in the last 3 years. Therefore, I will try to support you as much as I can coming years so you can achieve the goals you have in your work. I love you!

Table of Contents

List of figures

List of tables

List of abbreviations

% CT	Percentage of connective tissue
ACSA	Anatomical cross-sectional area
Akt	also known as PKB: Protein kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
C:F	Capillary to fiber ratio
Ca^{2+}	Calcium ions
CD	Capillary density
CFD	Capillary fibre density (of a given fibre)
COX-4	Cytochrome c oxidase 4
CSA	Cross-sectional area
DAF	Number of domains overlapping a given fibre
DAPI	4',6-diamidino-2-phenylindole
eEF2	Eukaryotic elongation factor 2
EGTA	Ethylene glycol tetraacetic acid
$FADH_2$	Reduced flavin adenine dinucleotide
FCSA	Muscle fibre cross-sectional area
FI	Fatigue index
Flk-1	VEGF receptor
Fmax	Maximal isometric force
Fspec	Specific muscle force
HIF-1α	Hypoxia-inducible factor 1-alpha
Id2	Inhibitor of differentiation protein 2
IGF-1	Insulin-like growth factor

IL-4	Interleukin-4
K^+	Kalium ions
$\square_o$	Optimal length
LCFR	Local capillary to fibre ratio
Log_rSD	Logarithmic standard deviation of the domain radii
MGF	Mechanical growth factor
MHC	Myosin heavy chain
MRF	Myogenic regulatory factors
MRTD	Maximal rate of force development
mTOR	Mammalian target of rapamycin
MyoD	Protein involved in myogenic differentiation
NADH	Reduced nicotinamide adenine dinucleotide
PAS	Periodic acid-Schiff
PBST	Phosphate buffered saline tween
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGMS	Plantaris, gastrocnemius medialis and soleus muscles
Pi	Inorganic phosphate
R50	Half relaxation time
ROS	Reactive oxygen species
SC	Satellite cell
SDH	Succinate dehydrogenase
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximum rate of oxygen uptake
WGA	Wheat germ agglutinin

Publications and grants

Articles in peer-reviewed journals:

- '*Aging related changes in determinants of muscle force generating capacity: A comparison of muscle aging in men and male rodents'.* **Ballak SB**, Degens H, de Haan A, Jaspers RT. Aging Research Reviews 2014 Mar; 14: 43-55.

- '*Plantaris muscle weakness in old mice: relative contributions of changes in specific force, muscle mass, myofiber cross-sectional area, and number'*. **Ballak SB,** Degens H, Busé-Pot T, de Haan A, Jaspers RT. AGE 2014 36: 9726.

- '*Blunted hypertrophic response in old mouse muscle is associated with a lower satellite cell density and is not alleviated by resveratrol'*. **Ballak SB,** Jaspers RT, Deldicque L, Chalil S, Peters EL, de Haan A, Degens H. Experimental gerontology 2015 62 23-31.

- *Validation of a new semi-automated technique to evaluate muscle capillarization'.* **Ballak SB**, Yap MH, Harding PJ, Degens H. Advances in Experimental Medicine and Biology (ACCEPTED)

Articles submitted:

- *'Blunted angiogenesis and hypertrophy are associated with increased fatigue resistance and unchanged aerobic capacity in old overloaded mouse muscle'.* **Ballak SB**, Jaspers RT, Busé-Pot T, Harding PJ, Yap MH, Deldicque L, de Haan A, Degens H.

Abstracts and conference communications:

- *'Contributions of lower muscle mass and specific force to muscle weakness in old mice'*. **Ballak SB**, Degens H, Busé-Pot T, de Haan A, Jaspers RT. 2013 IUPS, Birmingham, Poster communications, 1008P.
- *'Increased mass rather than specific tension causes the increased force generating capacity in hypertrophied adult and old mouse plantaris muscle'*.**Ballak SB**, Degens H, Busé-Pot T, de Haan A, Jaspers RT. EMC, Amsterdam, J Muscle Res Cell Motil (2014) 35:55-63.
- ***'****Validation of a new semi-automated technique to evaluate muscle capillarisation'*. **Ballak SB**, Metcalfe J, Yap MH, Harding PJ, Degens H. 2014 ISOTT, London.
- *'Lower satellite cell content partly explains blunted hypertrophic response in old mice, but is not alleviated by resveratrol'*. **Ballak SB,** Jaspers RT, Peters EL, de Haan A, Degens H. 2014 ECSS, Amsterdam.

Academic prizes/Grants

- Best oral presentation 3th MOVE-Age conference. Amsterdam July, 2014: '*Muscle ageing in a mice model'*.
- Best oral presentation MMU Research day. Manchester May, 2013: '*Age-related impaired muscle function in male mice'*.
- Skill Development Award, awarded by the MMU and Physiological Society travel grant for ISOTT 2014 conference in London.

Abstract

Age-related muscle wasting and weakness (sarcopenia) ultimately lead to a dependent lifestyle. The relative contribution of myofibre loss, atrophy and reduced myofibre function to the age-related decline in muscle function is, however, unknown. Resistance exercise is an effective means to enhance muscle strength via muscle hypertrophy, but the hypertrophic response may be attenuated at old age. Therefore, the overall aim of this PhD-project was to examine the factors that contribute to the impaired muscle function and attenuated hypertrophic response in old age. To investigate this we assessed plantaris muscle function *in vivo* in adult (9-month-old) and old mice (25-month-old). An additional group of old mice received 0.4% resveratrol. To determine the hypertrophic response we overloaded one plantaris muscle, while the contra-lateral muscle served as internal control.

This thesis identified a reduction in specific force, rather than myofiber atrophy and myofiber loss, as the main contributor to the age-related muscle weakness in a model representing the onset of sarcopenia. The reduced specific force was partly explained by an increase in intramuscular connective tissue. At the same time, it was observed that aged muscle also developed hypertrophy in response to chronic overload, although less so than in adult muscle. The blunted hypertrophic response in old muscle was associated with a lower SC density and impaired angiogenesis, but there was no evidence for a lower SC proliferation capacity. Resveratrol did not ameliorate muscle weakness or rescue the hypertrophic response, but even reduced the number of SCs in hypertrophic muscles. Finally, a literature study illustrated that effects of ageing on determinants of muscle force showed qualitatively the same patterns as observed in men. This overview showed that to translate data gained in rodent models of muscle ageing to humans, it is important to correct for relative ages.

In conclusion, although with increasing age loss of muscle mass and function do take place, the capacity of muscle to adapt to overload, although weakened, remains until old age. This plasticity of muscles provides hope that exercise interventions are effective into old age and may be implemented to attenuate or even reverse the increased dependent life style and hence improve the quality of life in aged people. The side effect would be a decreased burden on healthcare systems.

Statement:
All Western blots analysis, mentioned in chapter 4 and 6 were not performed by the PhD-student, but by the team in Leuven, Belgium.

Chapter 1

General introduction

1 Introduction

In Western societies the proportion of people older than 65 years is increasing at the fastest pace ever (United Nations, 2011) and will most likely result in increased age-related health care problems. Loss of independence, inability to perform daily tasks, increased number of falls and bone fractures are examples of these problems. These issues have a major impact on the quality of life of the older person and their treatment and care also places a burden on healthcare.

Sarcopenia is one of the major factors causing a decrease in independence in old age. Sarcopenia is Greek for “poverty of flesh”. It indicates the loss of muscle mass and concomitant loss in muscle function with age (Marzetti et al., 2013; Roseberg, 1989). The age-related loss of muscle mass is not only accompanied with a loss of muscle strength, but also with a reduction in endurance capacity. A better understanding of the aetiology of muscle ageing will help in the design of interventions to a) minimise the age-related reduction in the quality of life and b) lower the social and economic impact of the greying of society.

The force generating capacity of a muscle is determined by the quantity (mass) and quality (specific force) of the muscle. Muscle mass is determined by the total number and cross-sectional area (CSA) of the individual fibres, while specific force is determined by the force normalized to the CSA of the muscle. During ageing both the quantity and the quality of the muscle are negatively affected. However, to date the relative contributions of loss of fibres, fibre atrophy and reduction in muscle specific force to the age-related reduction in muscle force is not known.

Not only muscle strength, but also muscle endurance capacity declines with increasing age. The two major factors determining muscle endurance or fatigue resistance are mitochondrial function and the capillary network (Degens and Veerkamp, 1994). An age-

related decline in both factors also contributes to the age-related decrease in maximal oxygen consumption, which can be as much as 10% per decade (Heath et al., 1981).

The age-related reduction in physical activity is thought to be one of the strongest, but not the sole, contributor to sarcopenia (Degens and Alway, 2006). Also, chronic low-grade systemic inflammation (Degens, 2010) and impaired mitochondrial function (Degens and Veerkamp, 1994) may impair muscle function and cause the decrease in muscle mass with increasing age. Resistance training is an effective means to counteract the age-related deterioration in muscle mass and function (Leenders et al., 2013), though the effect of resistance exercise seems to be somewhat attenuated in old, compared to adult human muscle (Kosek et al., 2006). The cause of the attenuated hypertrophic response is not well understood. It could be that either the loss of muscle fibres or the reduced ability to hypertrophy per muscle fibre decreases the absolute gain in muscle volume after a hypertrophic stimulus.

In addition to training, administration of anti-oxidants may attenuate muscle deterioration during ageing. Resveratrol is a polyphenol, which has been shown to reduce the oxidative stress and inflammation in aged mice muscle (Jackson et al., 2011). Administration of resveratrol may potentially improve muscle function (Lagouge et al., 2006), counteract mitochondrial impairment and even age-related muscle wasting (Marzetti et al., 2011). However, the effects of resveratrol on the determinants of muscle force generating capacity and endurance have not been studied extensively yet.

The purpose of this thesis is to better understand the determinants that underlie the reduced muscle force and endurance in old age, and how a hypertrophic stimulus and supplementation with an anti-oxidant like resveratrol could potentially influence these determinants. Thereto, there were the following objectives: (1) to quantify the contribution of muscle fibre loss, fibre atrophy and reduction in specific tension to the age-related loss in

muscle force, (2) to assess whether, and if so, why the hypertrophic response in old mouse muscle is attenuated, (3) to test whether the age-related loss of muscle mass and blunted hypertrophic response can be rescued by supplementation with the ant-inflammatory anti-oxidant resveratrol and (4) to assess the impact of ageing, overload and resveratrol on muscle oxygenation.

The insights gained may help in the design of exercise programs and diet to improve muscle fitness in old people, by e.g. combining resistance exercise with anti-oxidant supplementation. Thus, ultimately this work would help old people to age healthier, remain independent longer and thereby reduce the burden on healthcare systems.

The following sections will first discuss the determinants of muscle force and secondly the impact of age on each of these determinants. Then the effects of overload and resveratrol on muscle contractile function of old muscle will be discussed. In addition, the impact of ageing on determinants of muscle endurance and oxygenation will be discussed.

2 Normal skeletal muscle function, physiology and morphology

The main function of skeletal muscle is to enable locomotion and maintain body posture. A muscle contracts after it receives an action potential. This action potential, except from reflexes, originates in the brain and travels to the motoneuron. The action potential travels through the neuron until it reaches the neuro-muscular junctions of the fibres it innervates where it, through various chemical events at the cell surface, causes the release of Ca^{2+} from the sarcoplasmic reticulum. The consequent rise in the intracellular [Ca^{2+}] ultimately causes the contraction of the muscle fibres. The motoneuron activates all its innervating fibres and works as a unit, hence the term motor unit for the motoneuron and the fibres it innervates. The number of motor units within one muscle varies with muscle size and function.

2.1 The muscle fibre

A muscle consists of muscle fibres (a muscle cell is referred to as a muscle fibre) which are made up of myofibrils, which in turn are built up of thousands of sarcomeres in series. The major constituents of the sarcomere are the filaments actin and myosin. When the intracellular Ca^{2+} concentration is elevated, the myofilaments myosin and actin start to interact with each other, forming crossbridges between myosin and actin. Every time a myosin-head hydrolyses ATP, the energy currency of the cell, the myosin-head undergoes a mechanical power stroke, causing, depending on the resistance, actin to slide along the myosin filament and/or the development of force by the myosin-head.

The muscle fibre is multi-nucleated. Inside the cell every nucleus is thought to be responsible for maintenance of a small part of that cell, its myonuclear domain (Van der Meer et al., 2011a). During muscle growth and repair new myonuclei are required to maintain the nuclear domain size. These myonuclei can be derived from satellite cells (SCs), the stem cells of muscles. The SCs remain quiescent until activation, after which the cells proliferate and differentiate to form new myonuclei. This process occurs after an anabolic stimulus or during repair of an injured muscle (Hawke and Garry, 2001).

2.2 Muscle fibre types

There is a whole range of different muscle fibre types expressing different kinds of myosin heavy chains (MHCs); in muscles from adult rodents one can distinguish type I, IIA, IIX and IIB myosin heavy chain. Type I fibres are slow but have a high fatigue resistance. On the other end, type IIB fibres are fast contracting, but have a poor fatigue resistance, while type IIA and IIX are intermediate fibre types. Note that in human muscle the predominant types are type I, IIA and IIX. In older literature the type IIX fibre in humans is often referred

to as IIB, but comparisons with rodent muscles showed that human IIB fibres express MHCs similar to rodent IIX (Jones et al., 2004). Differences in contractile shortening velocity are largely caused by the different MHC isoforms. A fibre with IIB MHC contracts the fastest and a fibre containing the type I MHC the slowest (Degens and Larsson, 2007). Differences in fatigue resistance are mostly attributable to a higher mitochondrial content in the slower compared to the faster muscle fibres. Muscles involved in control of balance or posture usually have a slower phenotype, while muscles needed to generate force quickly, have a faster phenotype. Two calf muscles illustrate this perfectly: in mice, but less clearly so in men, the *m. soleus* is mainly involved in maintenance of posture and is a slow muscle, while the *m. gastrocnemius* is mainly used for locomotion and predominately contains fast type II muscle fibres (Augusto et al., 2004).

2.3 Muscle fatigue

Fatigue is defined here as the inability of muscle to sustain a given force or power output. When power output of a muscle during a series of repeated contractions is maintained the rate of ATP synthesis equals the rate of ATP hydrolysis (Brooks and Faulkner, 1991). If power output declines during a series of repeated contractions it may be that the cell is not able to generate the ATP required for the given power output. There are different systems and levels of organisation where fatigue may originate, ranging from problems in the motor cortex to problems in the excitation-contraction coupling (Fig. 1.2).

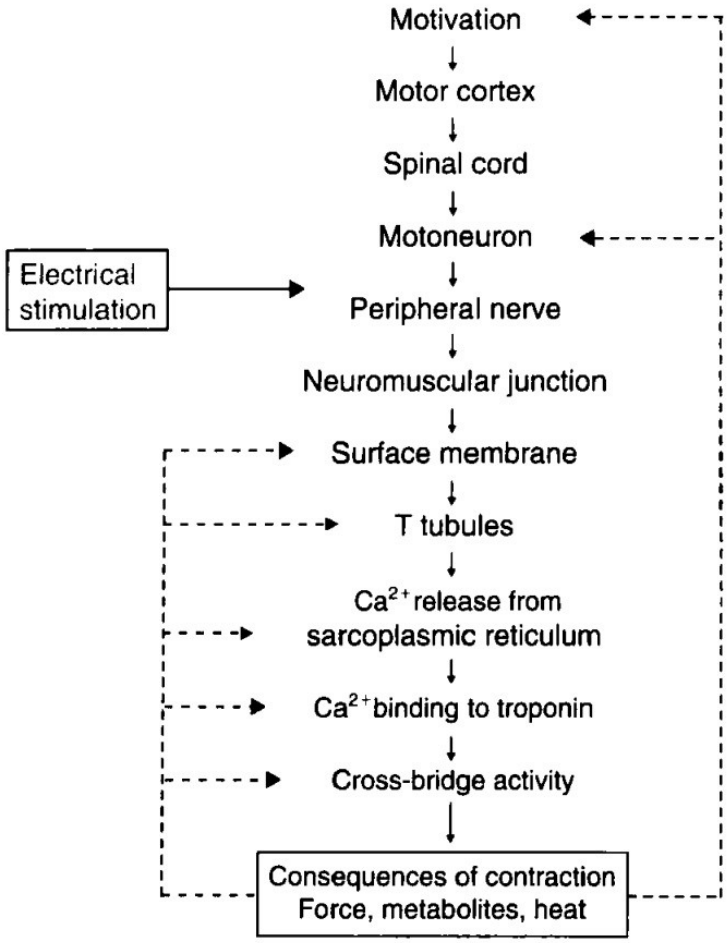


Figure 1.1 Possible sites of fatigue. From Jones et al. 2004, Skeletal muscle from molecules to movement, Elsevier.

While the CSA and type of the muscle fibre determine its power output, endurance is largely determined by the mitochondrial content of the fibre. Mitochondria are the powerhouses of the cell, where ATP is formed through oxidation of different substrates. For the oxidative breakdown of substrates to generate ATP the mitochondria require oxygen and produce carbon dioxide and water. More specifically, glucose is broken down into pyruvate and via acetyl-CoA it enters the citric acid cycle, in which NADH and FADH2 are formed and subsequently oxidised in the electron transport chain to form ATP. Fat is broken down in the β-oxidation, producing acetyl-CoA which then also enters the citric acid cycle for further oxidation. The more mitochondria there are in a fibre the larger the capacity of that fibre to

generate ATP aerobically. One enzyme that is often studied to histochemically obtain an indication of the mitochondrial content of a fibre is succinate dehydrogenase (SDH). SDH is a good marker for mitochondrial function as it is involved both in the citric acid cycle and the electron transport chain and the intensity of the staining of a fibre in a given period is proportional to its VO_{2max} (van der Laarse et al., 1989).

The aerobic generation of ATP requires an adequate supply of oxygen, removal of carbon dioxide, other waste products and heat. This exchange between the fibres and the blood takes place in the capillaries. Muscles are therefore supplied with a dense capillary network and at the level of the individual fibres there is a strong correlation between the number of capillaries around a muscle fibre, its type of MHC, its CSA and the metabolic activity of surrounding fibres (Degens et al., 1992; Wust et al., 2009a). One of the causes of muscle fatigue is an inadequate supply of ATP to maintain a given task and an insufficient capillary supply and/or oxidative capacity can thus cause a limitation of the endurance performance of a muscle (Degens and Veerkamp, 1994).

Different protocols have been used to study muscle fatigue in rodents. Muscle fatigue measured during a 4-min protocol with 330-ms contractions at 30 Hz every second leads to a rapid decline in force (Degens and Alway, 2003). This protocol has been used successfully to classify the different types of motor units (Burke et al., 1973) and appears to correlate well with the aerobic capacity to supply the muscle with enough energy and age-related changes in those elements. Note, however, that a limitation in the aerobic supply of ATP is not the sole factor that leads to the development of fatigue, but also accumulation of Pi in the cytosol (Jones, 2010) and sarcoplasmic reticulum (Fryer et al., 1995), impaired Ca^{2+} release from the sarcoplasmic reticulum (Allen et al., 2008) and accumulation of K^+ in the T-tubuli (Jones, 1996). However, many of these causes of muscle fatigue can also be improved by enhancing the oxidative capacity, mitochondrial function and/or capillary supply.

3 Effects of ageing on determinants of muscle force generating capacity

Ageing affects a muscle in many ways. First of all, muscle power decreases with age. Muscle power is the product of muscle force and velocity. While the force generating capacity is largely determined by the number of sarcomeres arranged in parallel, the maximal shortening velocity is determined by the number arranged in series and the MHC composition of the fibre. The latter has been reported to shift to a slower profile during ageing (Degens and Alway, 2006; Larsson and Ansved, 1995). With ageing there is a progressive reduction in the force generating capacity approximately amounting up to ~50% when comparing healthy adult (~30yrs) and old (~80yrs) humans (Doherty, 2003; Porter et al., 1995), further progressing beyond the 8th decade (Murray et al., 1980; Vandervoort and McComas, 1986).

Many studies have examined the age-related reduction in muscle mass in humans. However, direct measurement of muscle mass in humans is not possible. Therefore, most studies have measured the anatomical CSA (ACSA) of the muscle and found it to be ~40% smaller in ~80-yr- than ~30-yr-old humans (Lexell et al., 1988). This decay starts at an age of 50-60 yrs and continues progressively in more advanced ages. How these changes relate to changes in muscle force generating capacity is not well known. In addition, what causes the age-related reduction in CSA is still a subject of investigation.

3.1 Fibre loss and fibre atrophy

The age-related muscle wasting is attributable to a loss of muscle fibres and atrophy of the remaining muscle fibres (Faulkner et al., 2007b; Larsson and Ansved, 1995; Lexell et al., 1988). The human *m. vastus lateralis* has been studied extensively for the effect of age on FCSA (Lexell, 1995; Lexell et al., 1988; Nilwik et al., 2013; Porter et al., 1995) showing consistently that in old subjects fast type II fibres show 20-50% atrophy, while slow type I

fibres remain largely unaffected compared to adult subjects. In addition to fibre atrophy some studies observe an almost 50% age-related decline in the number of muscle fibres (Lexell et al., 1988). This is not unequivocal, however, as others report almost no loss of muscle fibres (Klein et al., 2003; Nilwik et al., 2013). Why such differences exist remains to be elucidated, but it may be related to the age of the old group used and differences in activity levels between studies.

3.2 Muscle specific force

While the loss of muscle mass contributes significantly to muscle weakness in old age, this is aggravated by an age-related reduction in specific force of the muscle, the force generating capacity per CSA of the remaining muscle tissue (Degens et al., 2009a; Frontera et al., 2000a; Frontera et al., 2000b; Larsson et al., 1997b; Morse et al., 2005). Several factors may contribute to this decline in the quality of the muscle, such as a preferential atrophy of type II fibres, an increased antagonist co-activation, a decreased agonist activation (Macaluso et al., 2002) and/or the inclusion of non-functional (denervated) fibres (Urbanchek et al., 2001). Besides these factors also the specific tension of the fibres themselves may be reduced (Degens and Larsson, 2007).

In summary, it is clear that the loss of muscle strength in old age is due to a combination of loss of muscle fibres, muscle fibre atrophy and a reduction in the specific tension of individual fibres. Most studies investigate changes in determinants separately, not collectively. Therefore, the relative contribution of the changes in the different determinants of muscle force generating capacity remains to be determined and a model to elucidate the relative contribution of each of these factors to muscle weakness in old will be informative.

4 Effects of ageing on determinants of muscle fatigue resistance

4.1 Effects of age on muscle endurance capacity

An age-related earlier onset of muscle fatigue (Faulkner and Brooks, 1995; Pagala et al., 1998) can have several causes: 1) For an old smaller and weaker muscle, any repeated workload at a given absolute intensity will be closer to the maximal power output than in a young larger muscle and thus result in the earlier development of fatigue. 2) Since the endurance capacity of a muscle is largely dependent on the ability of a muscle to meet its metabolic demand, a decrease in the aerobic capacity would also contribute to an earlier onset of muscle fatigue in old age. It is thus important in this context that a decline in respiratory chain activity or function in human skeletal muscle has been found with increasing age (Konopka and Sreekumaran Nair, 2013; Marzetti et al., 2013). 3) The transmission of the action potential and the excitation-contraction coupling may be altered by ageing and limit muscle performance during ongoing contractile activity. 4) A reduced blood flow or capillary density (CD) in old muscle may cause an earlier onset of muscle fatigue. Indeed some studies reported an age-related decrease in the capillarisation of the muscle (Degens et al., 1993b; Hepple et al., 1997), but not all (Proctor et al., 1995).

Some however, report an improved fatigue resistance of old muscle (Harris, 2005). At first this may be counterintuitive, but when it is considered that fibre atrophy occurs mainly in fast muscle fibres, the fraction in the muscle consisting of slow, type I fibres will increase. Also an age-related increase in the proportion of slow fibres, if existing (Ballak et al., 2014b), may explain the increased fatigue resistance in old muscle (Callahan and Kent-Braun, 2011). Another factor explaining this discrepancy may be the type of contraction, the applied contraction velocity and the reporting of data absolute or relative to the maximal force (Avin and Law, 2011). More research is needed to elucidate these conflicting results. To test which factors contribute to fatigue resistance with increasing age, here the capillary supply and mitochondrial function will be related to sustained endurance capacity.

4.2 Causes of age-related muscle wasting and weakness

An age-related increased oxidative stress, systemic low-grade inflammation (Degens, 2010) and glycation (Nishikawa et al., 2000), a decreased IGF-1 expression (Sattler, 2013) and decreased daily physical activity levels (Degens and Korhonen, 2012) are all factors that contribute to the age-related muscle weakness. An increase in oxidative stress may lead to impaired mitochondrial function (Conley et al., 2007; Kayani et al., 2010b) and enhanced glycation of proteins (Nishikawa et al., 2000). Glycation of the myosin molecule leads to a slowing at which it can propel actin in an *in vitro* motility assay (Ramamurthy et al., 2001), while peroxide reduces both the force and velocity and thereby the power generating capacity of muscle fibres (Gilliver et al., 2010a). Impaired mitochondrial function would also require more oxygen for a given amount of ATP to be generated and may thus reduce the mechanical efficiency and hence cause an earlier onset of fatigue in old age (Degens and Veerkamp, 1994). IGF-1 serum levels are shown to decrease in aged rodents (Velasco et al., 1998) and humans (O'Connor et al., 1998). Since it is essential for muscle mass maintenance and anabolic signaling (Clemmons, 2009), an age-related drop in IGF-1 levels may result in a decreased muscle mass and muscle maintenance. In addition, an age-related decline in physical activity may be a major determinant, influencing all abovementioned factors, that contributes to age-related muscle wasting and weakness (Degens and Korhonen, 2012). Together this shows that age-related muscle wasting is multifactorial and complex.

5 Effects of overload/training on determinants of muscle force during overload

Resistance exercise is widely used to increase muscle mass (hypertrophy) and muscle strength, also in the elderly (Fiatarone et al., 1990; Leenders et al., 2013). However, the absolute increase in muscle mass or strength seems to be attenuated compared to young adults

in humans (Kosek et al., 2006; Slivka et al., 2008) and in rodents (Blough and Linderman, 2000b; Degens and Alway, 2003). For instance, in weight-lifting master athletes there is a progressive loss of power during ageing despite their maintained intense training program (Pearson et al., 2002). Since the activation of the AKT/mTOR pathway was not decreased in old muscle (Mayhew et al., 2009), a diminished SC activation and subsequent accretion of myonuclei may underlie the blunted hypertrophic response seen in aged muscle. As it was observed that subjects with the most SCs pre-training (Petrella et al., 2008) accreted the most new myonuclei and had the largest degree of hypertrophy (Petrella et al., 2006), an age-related decrease in SC number (Chakkalakal et al., 2012) may impair the hypertrophic response in old age. Therefore, it was studied whether hypertrophy was blunted in aged muscle and whether this was related to a reduced SC density.

To answer the abovementioned questions, we used an overload model of the *m. plantaris* in mice in which its synergistic muscles (i.e. *m. gastrocnemius and m. soleus*) were denervated. This model has as a benefit that the *m. plantaris* hypertrophies, with a concomitant proportional increase in maximal muscle force. In addition, fatigue resistance has been reported to increase with 25% even in 33-month-old rats (Degens and Alway, 2003). Muscle hypertrophy is mainly a product of an increase in FCSA. However, with the increase in FCSA capillaries will be pushed apart, thereby increasing the diffusion distances from the capillaries to the mitochondria (Degens, 2012). There is an inverse relationship between the size of the fibre and its oxidative capacity (van der Laarse et al., 1998; van Wessel et al., 2010). According to this inverse relationship it is to be expected that an increase in FCSA during hypertrophy would be accompanied with a reduction in oxidative capacity. Yet, during compensatory hypertrophy the oxidative capacity has been reported to increase (Degens et al., 1993c), while the capillary density is reduced (Degens et al., 1992; Degens et al., 1993c). It may well be that a limit is reached here, and when one considers that the capillary density in

old muscles is already reduced, such an increase in both FCSA and oxidative capacity may compromise the muscle. It maybe that this looming compromised muscle structure and function underlies the blunted hypertrophic response in old age. The relationship between capillarisation, oxidative capacity and fibre size in the normal muscle and the degree of hypertrophy that can be developed is hitherto not been investigated. An additional possibility is that in old age the hypertrophy of the fibre is accompanied with decreased rather than increased oxidative capacity. Hence, we investigated whether the hypertrophic response was impaired at old age and how the oxidative capacity, FCSA and fibre capillarisation were related in both normal and hypertrophied old mouse *m. plantaris*.

6 Effects of resveratrol on muscle

During the production of ATP by the electron transport chain also reactive oxygen species (ROS) are formed. ROS are very reactive molecules and when reacting with proteins, DNA and other structures cause damage to these molecules, which is called oxidative stress (Fig. 1.1). The oxidative modifications impair the functionality of the affected structure and could lead to long-lasting reductions in muscle homeostasis and function (Marzetti et al., 2013). Fortunately the body has different systems to counteract (the effects of) ROS. One of these is the neutralisation of ROS by anti-oxidants, like vitamin C and E (see for review (Powers and Lennon, 1999)). Supplementation of different kinds of anti-oxidants could thus potentially reduce oxidative stress.

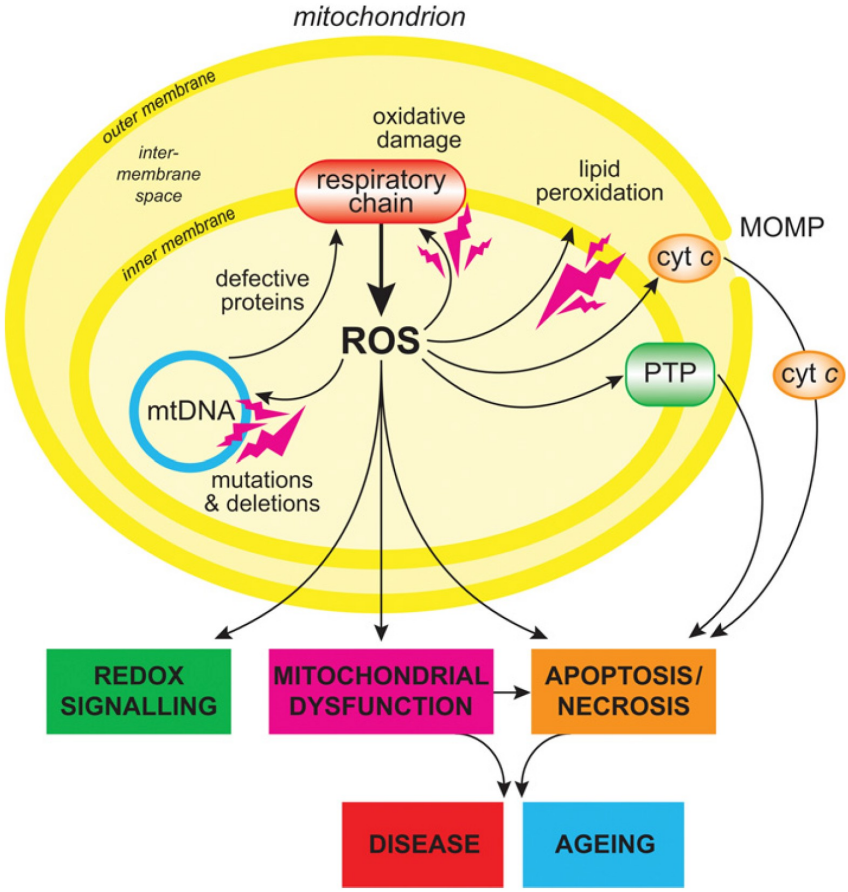


Figure 1.2 Formation of ROS by the electron transport chain and its consequences leading to oxidative stress. From Murphy 2009, Biochem J.

It has been suggested that impaired organelle function, such as enhanced mitochondrial uncoupling, may via positive feedback loops cause further impaired organelle function and hence reduce muscle function during ageing (Degens, 2010). If oxidative stress is one of those factors it may prove beneficial to administer anti-oxidants, like resveratrol, to improve muscle function, and enhance the hypertrophic response via attenuation of oxidative stress-induced activation of proteolytic pathways (Tisdale, 2005). Resveratrol (3,5,4'–trihydroxystilbene) is an anti-inflammatory anti-oxidant polyphenol (Timmers et al., 2012) present in several plants and in the skin of red grapes. Howitz et al. were the first to find a potential connection of

resveratrol with Sirt-1 (Howitz et al., 2003), a factor linked to, among other things, longevity. Although the latter has not been conclusively proven (Marchal et al., 2013), resveratrol has been linked to beneficial effects on metabolism and healthy ageing in humans, by decreasing chronic diseases (Timmers et al., 2012).

6.1 Effect of resveratrol on age-related muscle weakness

The effects of resveratrol on muscle and more specifically sarcopenia are less clear. There is an ongoing debate, whether resveratrol directly activates AMPK, which would then upregulate Sirt-1 (Dasgupta and Milbrandt, 2007; Park et al., 2012; Um et al., 2010), or whether resveratrol directly stimulates Sirt-1 (Price et al., 2012). Either way, both Sirt-1 and AMPK are up-regulated in the presence of resveratrol, potentially resulting in the stimulation of many pathways conferring health benefits. For instance, AMPK stimulates PGC-1α, which is a factor responsible for mitochondrial biosynthesis. Indeed, beneficial effects of resveratrol on mitochondrial function and endurance performance have been reported (Lagouge et al., 2006; Murase et al., 2009). However, not everybody observed effects of resveratrol on maximal or sustained muscle force (Jackson et al., 2011; Ryan et al., 2010) in aged mice. In addition, age-related muscle wasting does not seem to be attenuated by resveratrol (Jackson et al., 2011). Therefore, the efficacy of resveratrol on age-related muscle weakness is still not clear. On the other hand, resveratrol has been reported to improve markers of oxidative stress (Jackson et al., 2011; Olesen et al., 2013; Ryan et al., 2010) and mitochondrial biogenesis (Murase et al., 2009) in aged muscle. It remains to be investigated whether the reduction in oxidative stress induced by resveratrol supplementation is enough to improve SC function and augment age-related attenuated hypertrophy in aged muscle.

6.2 Appropriate doses of resveratrol

The conflicting results on the effect of resveratrol on the aged muscle may be due to differences in supplemented resveratrol doses. Even though resveratrol down-regulated mitochondrial-mediated apoptotic signalling pathways, it did not attenuate sarcopenia (Jackson et al., 2011). In that study, the 0.05% resveratrol in the diet of the male C57BL6 mice for 10 months may have been too little to prevent muscle wasting, as the effects of resveratrol are concentration dependent (Dolinsky et al., 2012; Lagouge et al., 2006; Murase et al., 2009). Together, this suggests that a higher resveratrol concentration may attenuate sarcopenia (Marzetti et al., 2011).

7 The outline of the thesis

This thesis describes a series of studies on the effects of ageing on muscle force generating and endurance capacity in a mouse model. The aim of this thesis was to obtain a better insight in the understanding of the factors contributing to muscle dysfunction in old age. In addition, it was studied whether, and if so, why the hypertrophic response was attenuated in old compared to adult muscle. In addition, studies were performed to elucidate the effect of resveratrol on age-related muscle weakness and whether it could rescue the age-related reduction in muscle force generating capacity. Finally, it was investigated how muscle capillarisation and oxidative capacity are affected by ageing and how they change in overloaded young and old mouse muscle.

To this end, the effects of age, overload and resveratrol administration on the maximal isometric force and fatigue resistance of the *in situ m. plantaris* of 9- and 25-month-old mice were studied. The morphology of the muscles was investigated to gain insight in the factors underlying the age-related, overload- and resveratrol-induced changes in muscle function. Further details of the conducted studies are described below.

Chapter 2 describes a literature study comparing determinants of force generating capacity in human and rodent models of muscle ageing.

Chapter 3 investigated the relative contribution of changes in key determinants of maximal muscle force – i.e. number of muscle fibres, muscle cross-sectional area (CSA) and specific muscle force to the loss of muscle force between 9 and 25 months in mice.

The impact of overload and resveratrol on muscle force during ageing was studied in **chapter 4** in the same mouse model. The first objective of chapter 3 was to assess whether the hypertrophic response was attenuated in old muscle, and if so, whether this was due to a reduced number of muscle fibres in old muscle or due to an attenuated muscle fibre hypertrophy. The second objective was to determine whether the attenuated response was associated with a lower SC content in the older muscles. The third objective was to establish the possible beneficial effects of resveratrol on muscle (specific) force and the hypertrophic response of the old muscle.

Muscle capillarisation plays an important role in fatigue resistance**.** To study muscle capillarisation the method of capillary domains is often used. **Chapter 5** describes the validation of a new semi-automated method to establish capillary domains. Since the method of capillary domains is a time consuming task, a Matlab ® based application was developed to improve this. The new method was used to study the effects of ageing, overload and resveratrol on muscle capillarisation in **chapter 6**. In addition, muscle capillarisation was linked to mitochondrial function (SDH) and fatigue resistance. The first objective was to establish whether the fatigue resistance in old muscle was impaired compared to adult muscle and if so, to what extent this was related to age-related changes in muscle oxidative capacity and capillarisation. The second objective was to obtain more knowledge on the impact of overload and resveratrol on indices of muscle oxygenation in both adult and old muscle.

In **chapter 7** the results of this thesis are summarized and discussed. Furthermore, future perspectives are given and final conclusions are drawn.

Chapter 2

Aging related changes in determinants of muscle force generating capacity: A comparison of muscle aging in men and male rodents

This chapter is published as:
Sam B. Ballak, Hans Degens, Arnold de Haan, Richard T. Jaspers. Aging related changes in determinants of muscle force generating capacity: A comparison of muscle aging in men and male rodents. *Aging research reviews (2014) 14: 43-55*

Abstract
Human aging is associated with a progressive decline in skeletal muscle mass and force generating capacity, however the exact mechanisms underlying these changes are not fully understood. Rodent models have often been used to enhance our understanding of mechanisms of age-related changes in human skeletal muscle. However, to what extent age-related alterations in determinants of muscle force generating capacity observed in rodents resemble those in humans has not been considered thoroughly. This review compares the effect of aging on muscle force generating determinants (muscle mass, fiber size, fiber number, fiber type distribution and muscle specific tension), in men and male rodents at similar relative age. It appears that muscle aging in male F344*BN rat resembles that in men most; 32-35-month-old rats exhibit similar signs of muscle weakness to those of 70-80-yr-old men, and the decline in 36-38-month-old rats is similar to that in men aged over 80 yrs. For male C57BL/6 mice, age-related decline in muscle force generating capacity seems to occur only at higher relative age than in men. We conclude that the effects on determinants of muscle force differ between species as well as within species, but qualitatively show the same pattern as that observed in men.

Comparing human and rodent models of sarcopenia and their underlying mechanisms

1. Introduction

In humans, aging is accompanied by a progressive decline in muscle mass and force generating capacity, referred to as sarcopenia (Fielding et al., 2011), which starts at the age of 40-60 (Faulkner et al., 2007b; Porter et al., 1995). At the age of 80, the muscle force generating capacity is on average approximately 60% of that at the age of 20-30 (Doherty, 2003). This progressive decline in muscle function and muscle mass is presumed to be a significant contributor to the increased incidence of falls, transition to a dependent life-style and reduced quality of life in old age (Kamel, 2003; Roubenoff and Hughes, 2000; Visser and Schaap, 2011). Furthermore, a decrease in muscle mass is associated with an increase in morbidity and mortality (Morley, 2003; Morley et al., 2006; Newman et al., 2001). This and the impact of increased frailty, incidence of falls and associated fractures on the associated cost of care pose an enormous burden on healthcare systems. Moreover, demographic prognoses of economically developed countries worldwide predict that the proportion of older people increases at the fastest pace ever (United Nations, 2011), which makes muscle aging investigations even more urgent.

Clearly, maintenance of muscle mass up to old age or even reversal of the age-related muscle wasting and weakness has important beneficial implications for the quality of life of the older person. To develop adequate strategies to maintain muscle function, a profound understanding of the mechanisms and causes of age-related muscle wasting are a prerequisite. Daily life physical activities require skeletal muscle power, which is the product of muscle force and velocity of contraction. While the force generating capacity is largely determined by the number of sarcomeres arranged in parallel, the maximal shortening velocity is determined by the number of sarcomeres arranged in series and myosin heavy chain (MHC) composition (Degens and Larsson, 2007; Larsson and Moss, 1993). The latter has been reported to shift to

a slower profile during aging (Degens and Alway, 2006; Larsson and Ansved, 1995). Thus, not only the age-related decrease in muscle mass, but also slowing of the muscle as a result of a shift to a slower myosin heavy chain profile would negatively impact the ability to produce power (Brooks and Faulkner, 1994c).

The age-related muscle wasting (Degens, 2010; Degens and Alway, 2003; Deschenes, 2004; Doherty, 2003; Janssen et al., 2000) is attributable to a loss of muscle fibers and an atrophy of the remaining muscle fibers (Faulkner et al., 2007b; Larsson and Ansved, 1995; Lexell et al., 1988). While the loss of muscle mass contributes significantly to muscle weakness in old age, this is aggravated by a reduction in the specific tension, the force generating capacity per cross-sectional area of the remaining muscle tissue (Degens et al., 2009a; Frontera et al., 2000b; Gonzalez et al., 2000; Larsson et al., 1997b; Morse et al., 2005). It is, however, difficult to measure force, and particularly the physiological cross-sectional area of a human muscle *in vivo*, accurately. This stresses the importance of models investigating sarcopenia. Animal models of sarcopenia may be helpful in overcoming some of these problems. In particular rodents have been used to study the impact of aging on skeletal muscle structure and function. An important question is to what extent the information gained from aging animal models can be translated to humans.

In this review we will address this question by evaluating the similarities in muscle aging in rodents and men. We will give particular attention to the widely used male Fischer344, Fischer344 x Brown Norway (F344*BN) and Wistar rats, and C57BL/6 mice. In addition, the vast majority of the studies on muscle aging in rodents used male animals (~70% vs ~20% female, ~10% are undefined). Therefore, only studies using male rodents were included, because this decreases the variability and complexity of the comparisons. Furthermore, factors influencing the ‘rate of muscle aging’ (Degens, 2012), such as maximal life span, environment, nutrition and activity levels are discussed. We conclude with a

summary of the strengths and weaknesses of these models and discuss to what extent aging rodents can be used as models of muscle aging in men.

2. Advantages of rodent models of muscle aging

Aging rodents have often been used as models to enhance our understanding of processes and biological mechanisms of age-related changes in human skeletal muscle (Alway et al., 2005; Cartee, 1995). The use of rodents to study muscle aging has some obvious advantages. First of all, longitudinal studies in humans require a very long follow-up period, which makes these studies more difficult, in contrast to the relatively short life span of rodents (only 2-3 yrs), which makes it much easier to study the aging process. Secondly, it is ethically less problematical to perform invasive procedures in rodents than in humans (Cartee, 1995). Thirdly, environmental conditions and nutrition can be tightly controlled, and activity can be accurately monitored in laboratory animals, which again is virtually impossible in humans (Alway et al., 2005). Fourthly, terminal experiments can be performed in rodents, allowing one to dissect whole muscles, which increases the opportunities to study whole muscle morphology and cellular and molecular mechanisms, compared to human studies where at best a small muscle biopsy can be analyzed. Finally, laboratory animals have a much more homogeneous gene pool than humans (Alway et al., 2005). Although it may be argued that the experimental control of animal models compromises the validity and relevance of these models to humans (Alway et al., 2005), it is this possibility to control many factors that will help us gain a better understanding of the extent at which various environmental factors contribute to the observed variation in the rate of muscle aging between people (Degens, 2012).

3. Age-related changes in force generating capacity

During aging there is a progressive reduction in the force generating capacity approximately amounting up to 40% when comparing healthy young (20-30 yrs) and old (70-80 yrs) humans (Doherty, 2003; Porter et al., 1995), continuing in the ninth decade and beyond (Murray et al., 1985; Murray et al., 1980; Vandervoort and McComas, 1986).

An important factor to consider when comparing human and rodent aging studies is how a given age of a rodent compares with that of humans. In order to do so we introduce the 'relative age' in this review. 'The relative age' is expressed as a percentage of the mean life span (MLS) of men and rodent species or rodent strain. Table 2.1 shows values of MLS reported for men, different male rat strains and C57BL/6 mice. Since our interest is aging, relative differences in the duration of the nurture stage and time to reach sexual maturity between humans and rodents (Flurkey, 2007; Quinn, 2005) are of less interest in the context of this review.

Table 2.: *Mean and maximal reported life spans of men in developed countries, male C57BL/6 mice and male F344*BN, F344 and Wistar rats.*

	Mean Life Span (MLS)	**Maximal life span (men) / 10% life survival (rodents)**	**References**
Men	80yrs	120yrs	(Smith, 1993; WHO, 2013)
Male C57BL/6	26.7mths	31.5mths	(Blackwell et al., 1995; Sheard and Anderson, 2012; Storer, 1966; Turturro et al., 2002; Wolf et al., 2000; Yuan et al., 2009; Zurcher and Van Zweiten, 1982)
Male F344*BN	32.7mths	38mths	(NIA, 2012; Thompson and Brown, 1999; Turturro et al., 1999)
Male F344	24.6mths	27.3mths	(Masoro, 1980; NIA, 2012; Shimokawa et al., 1993; Thurman et al., 1994; Wolf et al., 2000; Yu et al., 1982)
Male Wistar	23.5mths	36mths	(Edstrom and Larsson, 1987; Masoro, 1980; van der Meer et al., 2011b)

For each species and strain, the average reported life expectancy was taken at 100% MLS. This allows to calculate relative ages from reported absolute ages in months and years.

Note that most human studies have investigated the age-related changes in force generating capacity of the *quadriceps femoris* muscles, while in rodents mainly muscles of the *triceps surae* complex have been studied. This may complicate direct comparisons between rodents and humans as thigh and calf muscles may show different age-related changes. The two human studies (Davies et al., 1986; Vandervoort and McComas, 1986) we are aware of that investigated age-related changes in the force generating capacity of *m. triceps surae* showed a similar decline (approximately 40% between 20-30 yrs and 70 yrs and older) as that observed in the *m. quadriceps femoris* (Frontera et al., 1991; Frontera et al., 2000b; Murray et al., 1980; Young et al., 1984; Young et al., 1985). In addition, in both muscle groups the decline in force generating capacity was shown to be negligible before the sixth decade of life (Doherty et al., 1993; Vandervoort and McComas, 1986). Effects of aging on the masses of

both *soleus* and *m. vastus lateralis* muscles within the same animal showed considerable reductions in *m. vastus lateralis* (58-60%) and *soleus* (36-50%) mass (Bua et al., 2002; McKiernan et al., 2004). These observations indicate that in both human and rodents the *quadriceps femoris* and *triceps surae* show comparable age-related reductions in force generating capacity and muscle mass. This suggests that the use of *triceps surae* complex in rodent models does not preclude a fair comparison with the age-related changes in the human quadriceps complex.

When comparing studies on men and rodent maximal muscle force generating capacity, it appears that both human and rodent muscles exhibit their peak force at similar relative MLS (men 25-44%; rodents 24-51% MLS) (Brooks and Faulkner, 1988; Brooks and Faulkner, 1991; Brown and Hasser, 1996; Degens and Alway, 2003). Figure 2.1 shows that after reaching this peak in maximal force (set at 100%), it decreases after ~80% MLS. At MLS, force generating capacity in muscles of men and male rats and mice was reduced by ~40%, ~30% and ~20%, respectively, compared to peak value. For mice and rats, the reduction in muscle force generating capacity seems to occur at a relatively later stage of life, compared to that for men. In addition, a large variation was observed between rat strains. Male F344*BN rats show the largest age-related decline in maximal muscle force (45%) at 100% MLS, whereas male Wistar rats lose only 10% of maximal muscle force at 100% MLS.

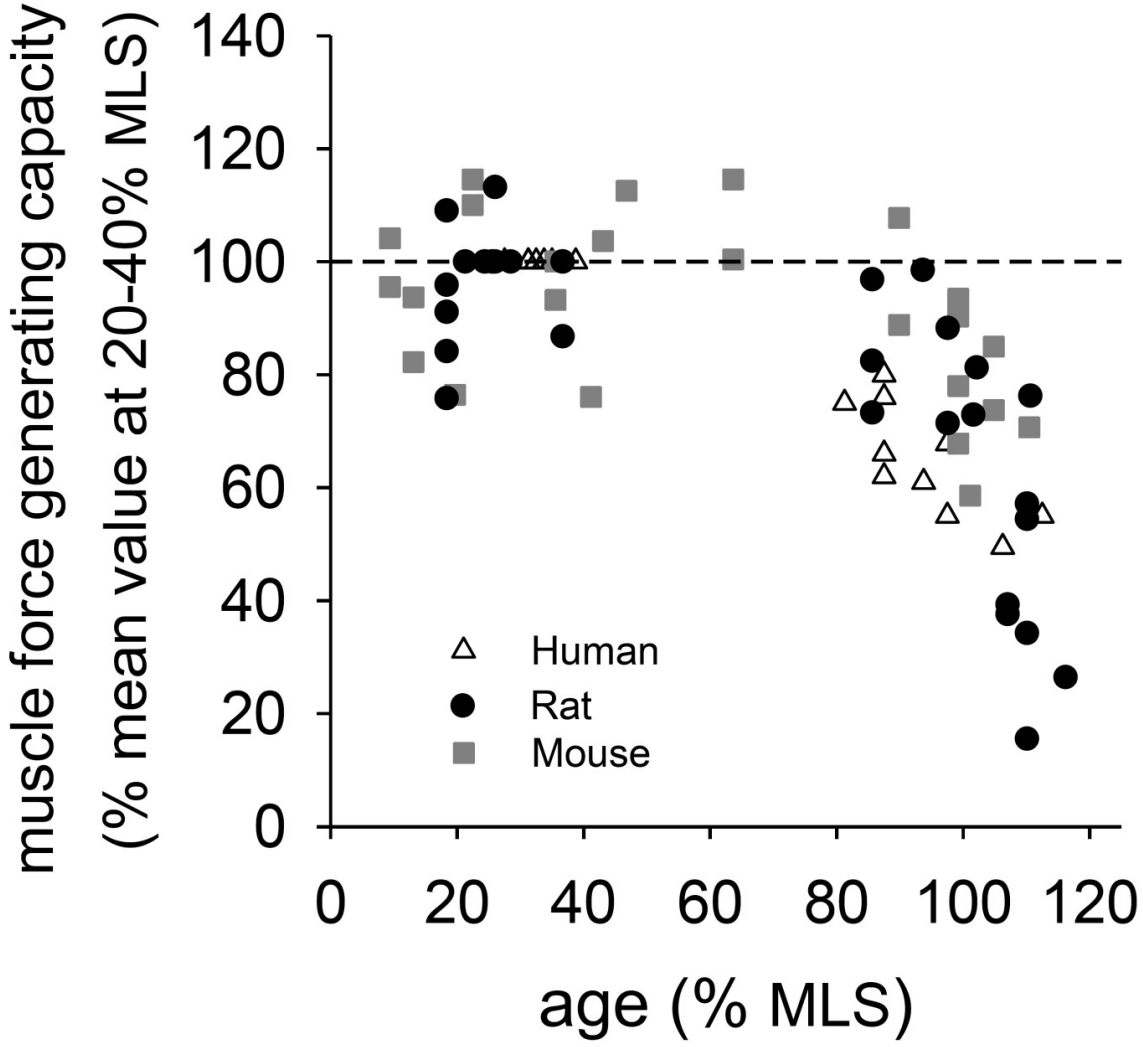


Figure 2.: Effects of aging on men and male rodent muscle force generating capacity for men male rats and mice. Literature-based values of muscle force generating capacity are expressed as function of age. The force generating capacity represents maximal in situ isometric muscle force or maximal net joint moments. These values are normalized for the mean force generating capacity calculated from reported values in literature. Mean life span (MLS) was calculated for each species and strain from reported life expectancies (Table 6.1). For men, peak force was taken as the maximal force or net moment reported between 25-44% MLS and for rodents between 24-51%. Average values were calculated when multiple studies reported for the same muscles, and sampling techniques were similar. The dashed line

represents 100% of maximal force/net moment as estimated for the respective periods of life. Note that for rodents only hind limb muscles were tested, whereas for humans mostly knee flexor and extensor muscles were tested (Aniansson et al., 1986; Blough and Linderman, 2000a; Brooks and Faulkner, 1988; Brooks and Faulkner, 1991; Brown and Hasser, 1996; Carter et al., 2010; Davies et al., 1986; Gutmann and Carlson, 1976; Ivey et al., 2000; Kadhiresan et al., 1996; Kayani et al., 2010a; Larsson and Edstrom, 1986; Larsson et al., 1979; Linderman and Blough, 2002; Lynch et al., 2001; Murray et al., 1980; Overend et al., 1992; Pettigrew and Gardiner, 1987; Phillips et al., 1991; Rice et al., 2005; Vandervoort and McComas, 1986; Young et al., 1985; Zerba et al., 1990b).

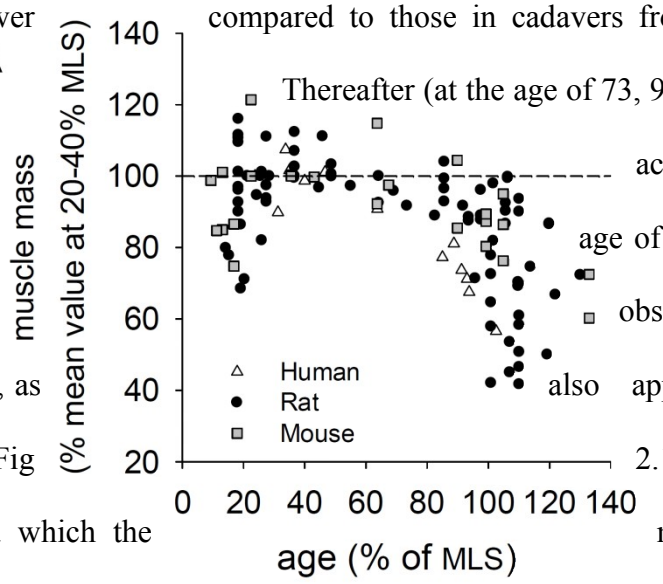
Although there is a difference in magnitude, all models show a drop in muscle force generating capacity with increasing age. To answer whether in the different models the major determinants of maximal muscle force are equally affected during aging, we listed the reported effects of aging on muscle mass, muscle fiber CSA, muscle fiber number, motoneurons, muscle fiber type and specific tension, which will be discussed in the next section.

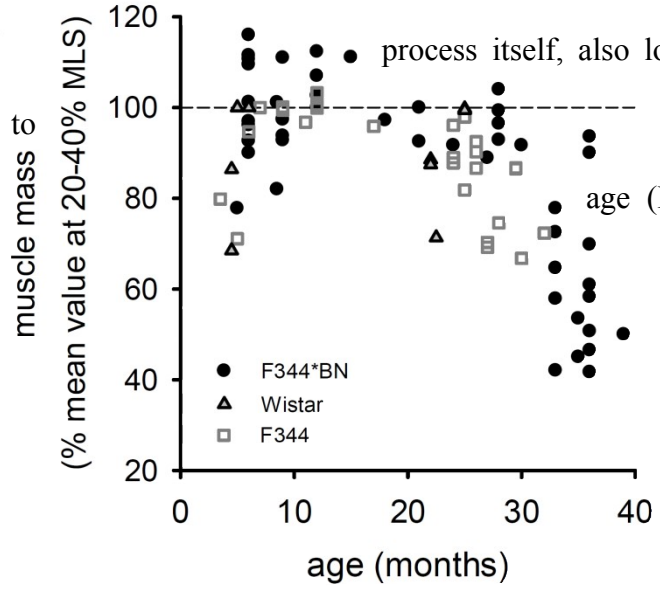
4. Determinants of muscle function

4.1 Age-related changes in muscle mass

As mentioned above, a large proportion of the loss in muscle force generating capacity with age is attributable to a reduction in muscle mass. Most of the studies examining anatomical cross-sectional area (ACSA) of muscles in humans, used computed tomography or MRI (Klitgaard et al., 1990; Overend et al., 1992; Rice et al., 1989; Young et al., 1984; Young et al., 1985), because a direct measurement of muscle mass in humans is not possible. These studies reported an average decrease for the human *quadriceps femoris*, elbow flexors and

extensors in ACSA of ~35%, from young (24-31 yrs, 30-39% MLS) to old (68-75 yrs, 85-94% MLS) (Klitgaard et al., 1990; Rice et al., 1989; Young et al., 1984; Young et al., 1985). The ACSA of the *m. vastus lateralis* from human cadavers at the age of 51 (64% MLS) was shown to be ~10% lower compared to those in cadavers from 19-yrs-old people (24% MLS). Thereafter (at the age of 73, 91% MLS), the decrease of ACSA accelerated (Lexell et al., 1988). At an age of 80 yrs (100% MLS) a 40% decline was observed (Lexell et al., 1988). This indicates, as also applies to the force generating capacity (Fig 2.1), that there is a threshold age beyond which the muscle wasting accelerates. It is important to take into account that besides the aging process itself, also lower levels of physical activity contribute to a decreased muscle mass at advanced age (Morley et al., 2001).





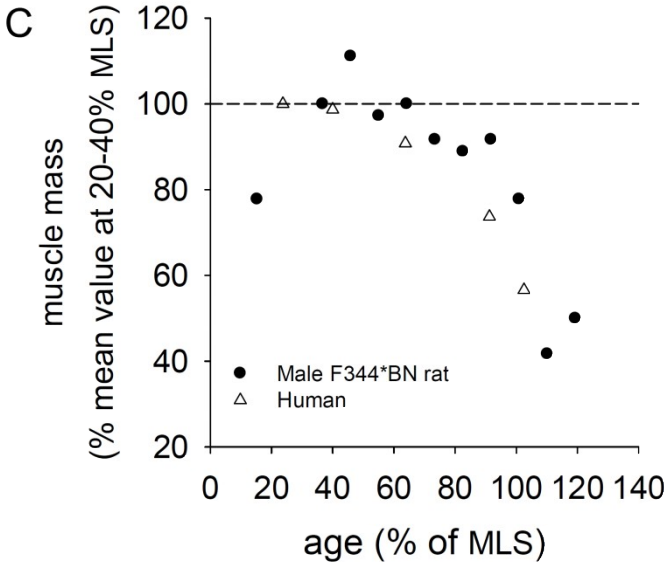


*Figure 2.2: Comparison of age-related changes in muscle mass between men and male rodents. A) Muscle mass normalized for the mean mass calculated from reported values for an age range varying between 24-51% MLS (Ansved and Larsson, 1989; Blough and Linderman, 2000a; Brooks and Faulkner, 1988; Brown and Hasser, 1996; Carter et al., 2010; Daw et al., 1988; Degens and Alway, 2003; Eddinger et al., 1985; Edstrom and Larsson, 1987; Frontera et al., 2000b; Jackson et al., 2011; Kadhiresan et al., 1996; Kanda et al., 1996; Klitgaard et al., 1990; Larkin et al., 2003; Larsson and Edstrom, 1986; Lexell et al., 1988; Linderman and Blough, 2002; Lushaj et al., 2008; Lynch et al., 2001; McKiernan et al., 2004; Overend et al., 1992; Pagala et al., 1998; Pettigrew and Gardiner, 1987; Rowan et al., 2012; Schuenke et al., 2011; Sheard and Anderson, 2012; van der Meer et al., 2011b; Young et al., 1985; Zerba et al., 1990b). B) Comparison of age-related changes on muscle mass between male F344*BN, F344 and Wistar rats as function of absolute age. C) Comparison of age-related mass of normalized m. vastus lateralis muscle (%) between male F344*BN rat (Lushaj et al., 2008) and humans (Lexell et al., 1988) expressed as a function of relative age. For rodents, mean maximal muscle mass was calculated for the age ranging from 24 to 51% MLS, when multiple studies reported for the same muscles and sampling techniques were similar. The dashed line represents 100% muscle mass as estimated for the respective periods of life. Note that for rodents only hind limb muscles were tested, whereas for humans mostly knee flexor and extensor muscles were tested.*

The question remains whether age-related decreases in men and rodent muscle mass are comparable. Figure 2.2A shows age-related reductions in muscle mass reported for men and male rats and mice as a function of the relative age. At 100% MLS, the average loss of muscle mass in adult rat and mouse was ~20% (mean of all rat species) and ~15%, respectively. These changes are somewhat smaller than the changes in maximal muscle force

generating capacity reported for these species (Fig. 2.1). However, those relative reductions in muscle mass for rodents are strikingly lower and occur later in life than those reported for muscles of men.

A more detailed analysis of the studies regarding effects of aging on rat muscle reveals that there is a huge variety in age-related losses in muscle mass. For some rat species (Wistar) the decline in muscle mass is rather small (~5%), whereas for the F344*BN rats this was ~25%, which is much closer to that reported for muscles in men (Frontera et al., 2000b; Klitgaard et al., 1990; Lexell et al., 1988; Young et al., 1985). This variance could be the result of a different prevalence of co-morbidities (which is discussed more extensively below), affecting potentially identical life spans in all rat strains. Figure 2.2B indeed shows that a part of the variation between rat strains may be explained by absolute age.

It is interesting to compare the decline in muscle mass and the relative threshold age (losing <10% muscle mass) of the *m. vastus lateralis*, because of its common use in human studies. Although the magnitude of the decline seems to be equivalent, the threshold age for male F344*BN rats (30 months, ~90% MLS (Lushaj et al., 2008)) seems to be later in life than for men (60 yrs, ~75% MLS) (Fig. 6.2C) (Lexell et al., 1988).

For mice, an age-related decrease of only ~15% was observed in muscle mass at 100% MLS. This age-related loss of muscle mass was much lower than that in men. Furthermore, the relative age at which mice start losing muscle mass (~90% MLS) was higher than that for men (~75% MLS).

In summary, age-related reductions in muscle strength and mass seem to take place in both rodents and men. In both rodents and men there appears to be a threshold age beyond which muscle wasting and weakness develops. However, particularly in mice the reduction in muscle mass seems to occur relatively later in life than in men. To better understand the initial

stages of age-related muscle deterioration, more data on middle-aged men and rodents is needed.

4.2 Age-related changes in muscle fiber number

Grimby and Saltin were among the firsts to recognize that muscle fiber atrophy alone could not explain all of the age-related loss of muscle mass (Grimby and Saltin, 1983). Later on, Lexell et al. in a cadaver study showed that the reduction in *m. vastus lateralis* mass of men with increasing age was mainly due to a loss in the number of muscle fibers (Lexell and Downham, 1992). These observations were confirmed by other studies, reporting substantial fewer fibers at 70-80 years (88-100% MLS), compared to those in young adults (Lexell et al., 1986; Lexell et al., 1988). This is, however equivocal, as other studies report no change in fiber number in the *m. vastus* lateralis (29% MLS vs. 89% MLS) (Nilwik et al., 2013), or biceps brachii (21 yrs; 26% MLS vs. 82 yrs; 103% MLS) (Klein et al., 2003). However, these latter studies calculated the fiber number indirectly, opposed to Lexell et al. (Lexell et al., 1988). Why such differences exist between indirect and direct measurements of muscle fiber number still remains to be elucidated. It is also possible that difference in activity level may cause differences in the rate of fiber loss.

Also in rats, the effect of aging on muscle fiber number is equivocal and differs between strains (Fig. 2.3). For Wistar and F344 rats, reported reductions in muscle fiber number of gastrocnemius medialis, soleus, extensor digitorum longus and tibialis anterior muscles range from 5-15% at 100% MLS (Ansved and Larsson, 1989; Brown, 1987; Daw et al., 1988; Eddinger et al., 1985; Edstrom and Larsson, 1987; Kadhiresan et al., 1996; Larkin et al., 2003; Larsson and Ansved, 1995; Larsson and Edstrom, 1986). For both strains this loss explains ~50% of the reported reduction in muscle mass. In contrast, at MLS for F344*BN rats, the reported muscle fiber number is reduced by ~20%. Note that most of these studies

investigated quadriceps muscles (*m. vastus lateralis* and *medialis*, and *m. rectus femoris*) (Bua et al., 2002; Lushaj et al., 2008; McKiernan et al., 2004; Wanagat et al., 2001). The *soleus* muscle, the only other muscle of which muscle fiber number was investigated in this rat strain, showed a decrease of ~15% at MLS (Bua et al., 2002; McKiernan et al., 2004).

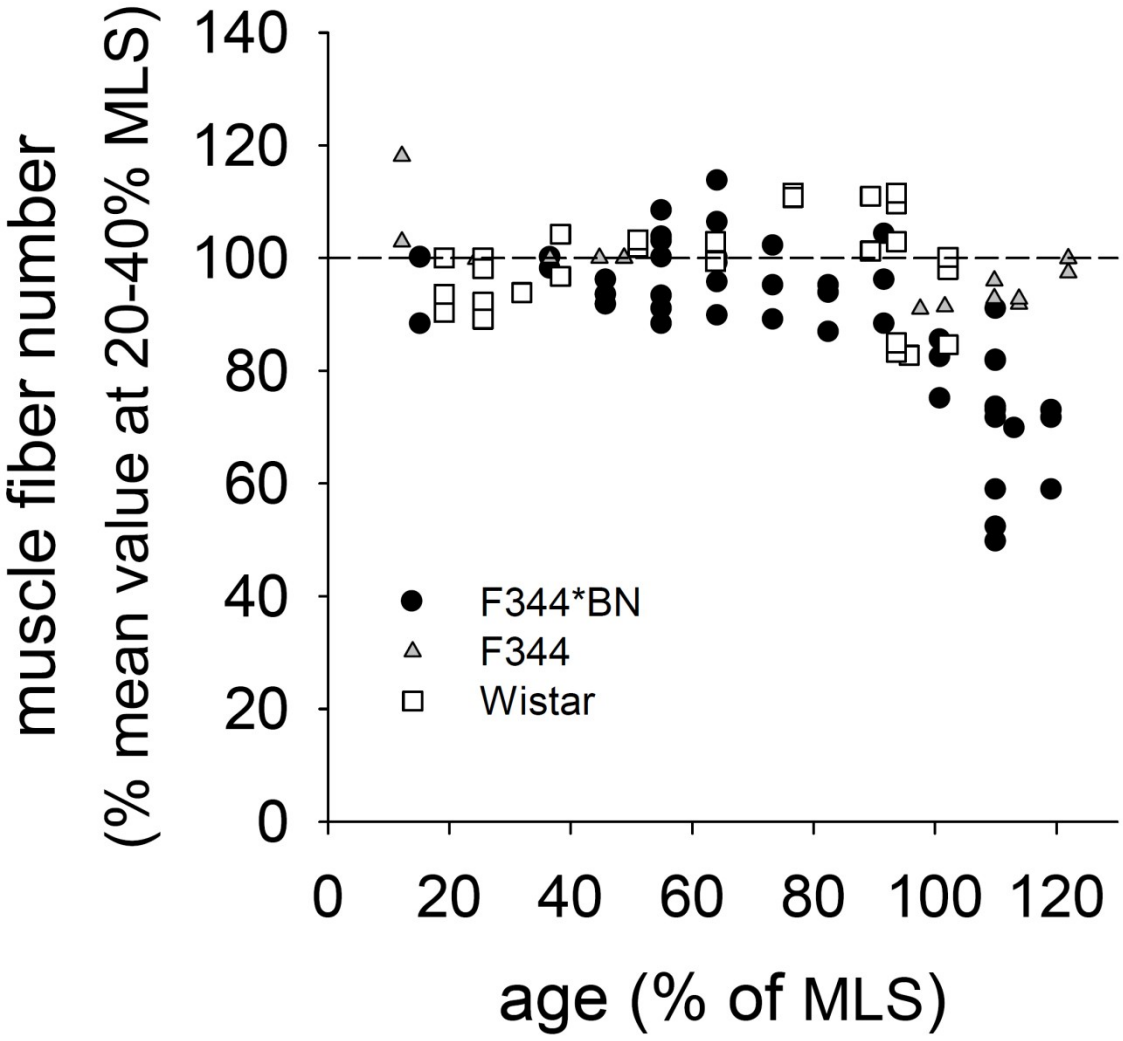


*Figure 2.3: Comparison between rat strains of literature-based age related changes in muscle fiber number. Peak fiber number for male F344*BN, F344 and Wistar rats was taken as the maximal fiber number reported between 24-51% (Ansved and L., 1990; Brown, 1987; Bua et al., 2002; Daw et al., 1988; Eddinger et al., 1985; Edstrom and Larsson, 1987; Kadhiresan et al., 1996; Larkin et al., 2003; Larsson and Ansved, 1995; Larsson and Edstrom, 1986; Lushaj*

et al., 2008; McKiernan et al., 2004; Wanagat et al., 2001). Mean maximal fiber number was calculated for the 24-51% interval, when multiple studies reported for the same muscles and sampling techniques were similar. The dashed line represents 100% maximal fiber number as estimated for the respective periods of life.

Very little is known about the effects of aging on muscle fiber number in male C57BL/6 mice (Sheard and Anderson, 2012; Zerba et al., 1990b). The muscle fiber number in *extensor digitorum longus* of old (26-27 months, 97-101% MLS) mice was 15% lower compared to that in young mice (2-3 months, 7-11% MLS) (Zerba et al., 1990b). Furthermore, muscle fiber numbers within *extensor digitorum longus* and *soleus* muscles of 24-months-old mice (90% MLS) were 12% and 10% lower compared to that in mice at the age of 4-6 months (15-22% MLS) (Sheard and Anderson, 2012). These results suggest that for mice the effects of aging on muscle fiber number become substantial only after an age of 90% MLS (> 24 months).

Taken together, at 100% MLS, human muscles (Lexell et al., 1986; Lexell et al., 1988) seem to lose relatively more muscle fibers, when counting all muscle fibers, than those of most rodents, even those of F344*BN rats. It is likely that similar mechanisms of neuronal death and loss of motor units cause this loss of fibers in both human and rodent muscles. Male F344*BN is the only rodent model which displays muscle fiber loss similar to that reported in men, but this occurs only later in life (~110%) (Bua et al., 2002; Lushaj et al., 2008; McKiernan et al., 2004; Wanagat et al., 2001). Therefore, studies investigating the effects of aging on muscle fiber number in rodent models should use rodents at >100% MLS.

4.3 Age-related change in muscle fiber cross-sectional area

In humans, numerous studies have investigated the effects of aging on muscle fiber size in *m. vastus lateralis* (see for review) (Lexell, 1995; Porter et al., 1995; Vandervoort, 2002). Comparison of muscle fiber cross-sectional area (FCSA) of subjects at ~25% MLS with those of ~90%, and ~75% with ~115% MLS consistently shows that aging is accompanied by a 20-50% atrophy of type II muscle fibers, while type I muscle fiber size remains largely unaffected (Essen-Gustavsson and Borges, 1986; Larsson, 1978; Lexell et al., 1988; Nilwik et al., 2013; Scelsi et al., 1980; Singh et al., 1999; Tomonaga, 1977).

To our knowledge no studies have investigated this issue in male Wistar rats, therefore only male F344*BN and F344 rats were included in this section. Both strains showed a larger decrease in FCSA for type IIB than type I and IIA muscle fibers, when comparing muscles from rats at the age of 20-30% MLS with those of rats at the age of 90-100% MLS (Brown and Hasser, 1996; Carter et al., 2010; Kadhiresan et al., 1996; Larkin et al., 2003; Rowan et al., 2012; Schuenke et al., 2011; Wineinger et al., 1995). Of both rat models, only male F344*BN rats show age-related reductions in FCSA similar to those of men. For this rat strain, reductions in FCSA of type I and IIA fibers were less evident (~10%), whereas reductions for type IIB fibers were shown to be 30% at 100% MLS (Brown and Hasser, 1996; Carter et al., 2010; Rowan et al., 2012; Wineinger et al., 1995). Furthermore, it seems as if the age-related drop in FCSA for type I muscle fibers is more distinct for slow muscles that have no or only small numbers of type IIB fibers, while the age-related drop in FCSA for type IIB muscle fibers is more pronounced for fast muscles, at least for the F344*BN strain (Brown and Hasser, 1996; Carter et al., 2010; Rowan et al., 2011). For F344 rats the effects of aging on FCSA are less pronounced. Plantaris and gastrocnemius medialis muscles of male F344 showed similar decreases in all FCSAs of less than 25% at 100% MLS (Kadhiresan et al., 1996; Larkin et al., 2003; Schuenke et al., 2011).

Only one study investigated the effect of aging on FCSA in C57BL6 mice. In both *extensor digitorum longus* and *soleus* muscles no age-related decrease in FCSA was observed (Sheard and Anderson, 2012), most likely because the 6-months-old mice did not reach their peak muscle mass yet. Therefore, the effect of aging on the FCSA in C57BL6 mice could not be established.

Taken together, the data on aging related effects on FCSA suggest that over the same relative age range, muscle fibers of male F344*BN rats seem to undergo age-related atrophy similar to that observed in men. The difference between F344*BN and F344 rats may exist because F344*BN rats survive much longer than other rat strains. Therefore, they can be tested at higher absolute ages (up to 39 months), which clearly results in a larger cumulative effects of the aging induced fiber atrophy and a larger muscle atrophy at a given MLS.

4.4 Age-related changes in motoneurons

Age-related degenerative changes in the nervous system, particularly in the motoneurons have been suggested to be an important cause of reductions in muscle performance (Aagaard et al., 2010; Doherty et al., 1993), muscle mass (Aagaard et al., 2010; Doherty, 2003; Doherty et al., 1993; Faulkner et al., 2007b; Lexell, 1995), muscle fiber muscle CSA (Aagaard et al., 2010; Lexell, 1995), muscle fiber number (Aagaard et al., 2010; Doherty et al., 1993; Faulkner et al., 2007b; Lexell, 1995), and in particular preferential type II muscle fiber loss (Doherty et al., 1993; Lexell, 1995). As a result of an on-going denervation-reinnervation process, other consequences of age-related motoneuron loss are fiber type grouping (Scelsi et al., 1980), larger variation in fiber sizes (Degens et al., 2009b) and irregular muscle fiber shapes (Kirkeby and Garbarsch, 2000).

Tomlinson and Irving provided the first evidence of age-related motoneuron loss in the human lumbosacral cord (Tomlinson and Irving, 1977), in which up to the age of 60 yrs the

loss of motoneurons was negligible. Thereafter, at the age of 80 (100% MLS), a gradual loss of motoneurons of ~25% was observed, with even greater losses beyond that age (Tomlinson and Irving, 1977). Another study observed a motoneuron loss of approximately 10% between the 2nd and 8th decade at lumbar vertebrae 3 to 5 (Kawamura et al., 1977). In addition, for humans age-related reductions in both numbers and diameters of motor axons in the ventral roots have been reported to be 5% per decade (Kawamura et al., 1977). In accordance, a drop of 25% was observed between the age of 25 yrs (31% MLS) and 80 yrs (100% MLS) (Mittal and Logmani, 1987).

Like humans, also different rat strains (male F344*BN, F344, F344/Crj rats and female Wistars) showed a 20% motoneuron loss (Kanda et al., 1996; Rowan et al., 2012; Sugiura and Kanda, 2004) at the age of 30 months or older. Note that here male F344Crj and female Wistar rats were included in the comparison between species and strains, since a considerable part of the studies reported in the literature on this topic used these two particular rat strains. However, no MLS for these strains was determined and therefore these strains were excluded in the discussion about the other determinants.

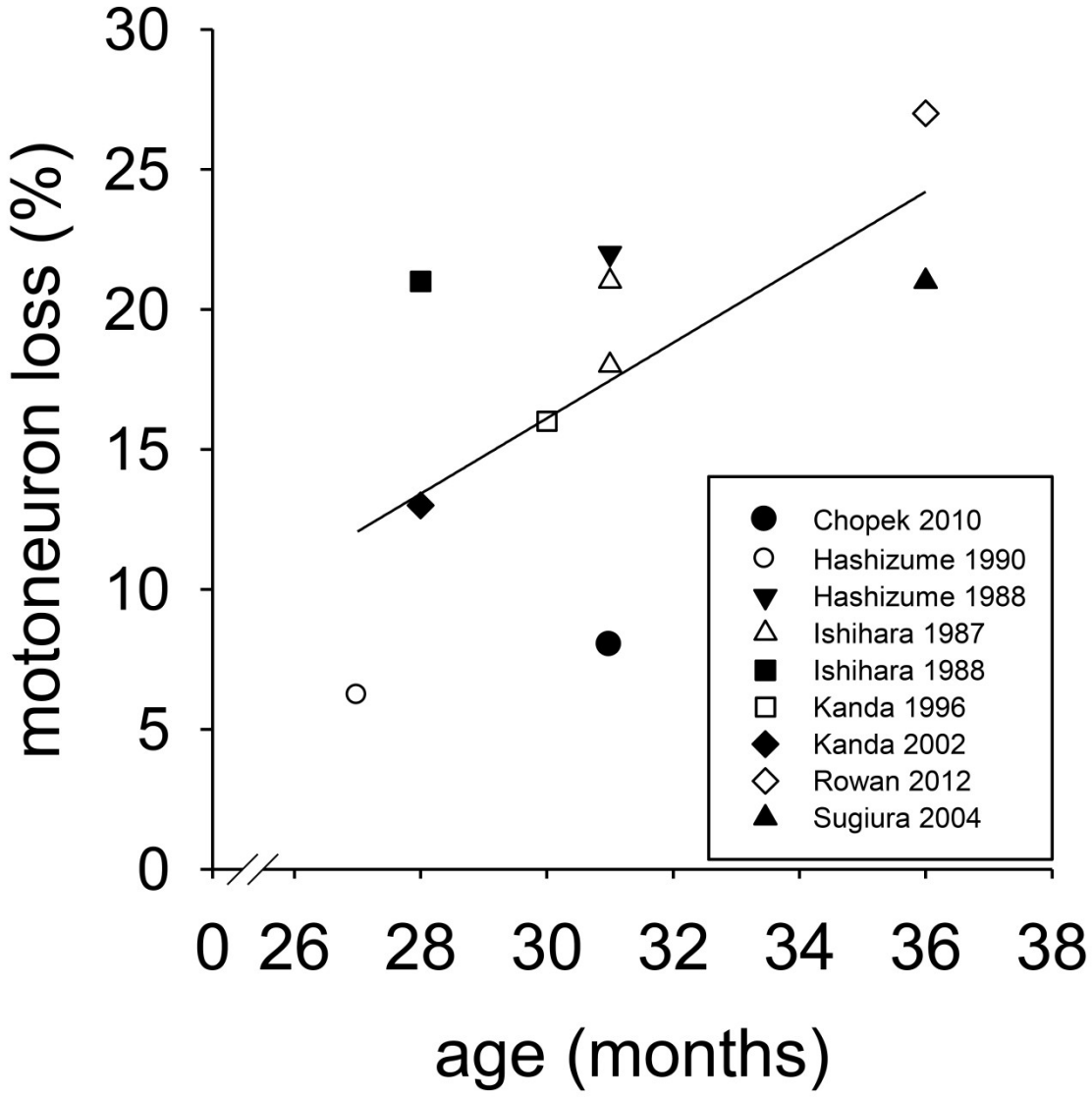


Figure 2.4: Literature-based values for motoneuron loss in different rat strains expressed as a function of age. An inventory of the loss of motoneurons in rat over the age range from 27 to 36 months shows a significant loss of 1.4% of the population motoneurons per month (Chopek and Gardiner, 2010; Hashizume and Kanda, 1990; Hashizume et al., 1988b; Ishihara and Araki, 1988; Ishihara et al., 1987; Kanda, 2002; Kanda et al., 1996; Rowan et al., 2012; Sugiura and Kanda, 2004) ($R^2 = 0.40$, $P = 0.05$).

Age rather than strain seems an important determinant of the percentage of motoneuron loss (Fig. 2.4). The same has been shown for humans (Kawamura et al., 1977; Tomlinson and Irving, 1977) and this indicates that the absolute age of rats is important when studying factors determining muscle function during aging. Female Wistar rats of 28-31 months showed a decrease in motoneurons, particularly α-motoneurons of approximately 20% for the *tibialis anterior* and *extensor digiorum longus* muscles, compared to 2.5-month-old rats (Ishihara and Araki, 1988; Ishihara et al., 1987). This distinct α-motoneuron loss (30% compared to 22% loss of total number of motoneurons) was also found in *gastrocnemius medialis* of male F344/DuCrJ rats (comparing 31 months with 11.5-months-olds) (Hashizume et al., 1988b). However, of all rat species, 36-month-old (110% MLS) F344*BN rats showed the largest decrease (27%) in motoneuron count vis-à-vis to that of adult rats at 28% MLS (Rowan et al., 2012). Therefore, the male F344*BN model seems the model that resembles aging changes in human motoneuron number and size most, because rats are tested at more advanced ages. To our knowledge, age-related motoneuron loss in male C57BL/6 mice has not yet been investigated.

Although aging related motoneuron loss seems to be involved in sarcopenia and critically determines the decline in muscle force generating capacity, little is known about the mechanisms underlying age-related motoneuron loss. Possibly, reactive oxygen species (ROS) compromise metabolism in motoneurons, which are very vulnerable to oxidative stress due to their long life span (post-mitotic cells) (Sohal et al., 1994). Indeed, a pronounced 4-to-5 fold higher accumulation of age-related (oxidative) damaged proteins was found within myelinated peripheral nerves in 38 months (116% of MLS) compared to 8-months-old (24% MLS) male F344*BN rats (Opalach et al., 2010). Moreover, aging related increases in expression of tumor necrosis factor-α (TNF-α), a cytokine playing an important role in chronic low-grade inflammation (Degens, 2010), were found in peripheral nerves (Opalach et

al., 2010). Such increased cytokine expression may contribute to the progression of degeneration of motoneurons, slowing axonal regeneration and may contribute to neuromuscular junction remodeling, adding up to the decline in muscle function during aging (Manini et al., 2013).

In summary, the literature indicates that there is a 20% loss of motoneurons in both humans and rats at 100-120% MLS (Mittal and Logmani, 1987; Rowan et al., 2012; Tomlinson and Irving, 1977). In addition, in both rats (Hashizume et al., 1988b; Ishihara and Araki, 1988; Ishihara et al., 1987) and in humans (Brown, 1972; Campbell et al., 1973; Doherty and Brown, 1993; Doherty and Brown, 1997), evidence suggests that the age-related loss of muscle mass is in part a consequence of loss of α-motoneurons.

4.5 Age-related changes in muscle fiber type proportion

Since some (Bottinelli et al., 1996; Bottinelli et al., 1999; Frontera et al., 2000b; Widrick et al., 1996a), but not all studies (Degens and Larsson, 2007; Larsson and Moss, 1993) report that slow fibers have a lower specific tension than fast fibers, it is possible that part of the age-related reduction in specific tension is due to a fast-to-slow transition. Preferential loss of large α-motoneurons during aging (discussed in the previous section) has been proposed to cause this shift, via denervation of IIB fibers and subsequent innervation by slower motoneurons. Several studies investigating human *m. vastus lateralis* found this age-related shift towards slow muscle fibers (Hakkinen et al., 1998; Larsson, 1978; Scelsi et al., 1980). However, there are also studies, including longitudinal assessments and a post-mortem study, which did not observe such a shift (Aniansson et al., 1986; Frontera et al., 2000a; Grimby et al., 1984; Kirkeby and Garbarsch, 2000; Lexell et al., 1988; Verdijk et al., 2012). In a 12-yrs longitudinal study, in which a group of old men was investigated, the proportion of type II muscle fibers increased from 40% at the age of 65 (82% MLS) to 60% at the age of 78

(97% MLS) (Frontera et al., 2000a), suggesting an increased rather than a decreased proportion of type II fibers! More evidence that no shift towards a slower muscle fiber type occurs with aging was found by another 7-yrs longitudinal study, in which 70-year-old men (88% MLS) did not show a significant change in fiber type composition (Aniansson et al., 1986). Furthermore, a study which investigated fiber type distributions within the quadriceps femoris by counting fibers in every 48th square (1 x 1 mm) throughout the whole muscle, found no age-related difference in fiber type proportion, although a very pronounced effect of aging on muscle mass and the total number of fibers was found (Lexell et al., 1988). The latter study investigated the whole muscle, instead of taking a single biopsy. It therefore was not prone to sampling bias when analyzing muscle biopsies, because of the large variability in muscle fiber type proportions within a whole muscle (Lexell et al., 1985). Part of this variation is related to differences in fiber type composition along the length and the depth of the muscle (Andersen, 2003). For a biopsy consisting of ~600 muscle fibers with 50% type I muscle fibers, the 95% confidence interval has been shown to be as large as 40-60% (Lexell et al., 1985). Given that the differences in specific tension between fiber types, if existent, are rather small, the relatively small fast-to-slow transition, if at all occurring, is likely to have only a minor impact on the specific tension of the muscle as whole.

Even in rodents, in which usually whole muscles are analyzed, there is considerable variation in the reported age-related changes in fiber type composition. Several studies have investigated age-related muscle fiber type distribution changes in male F344*BN rats at an age of at least 107% MLS (Brown and Hasser, 1996; Carter et al., 2010; Lushaj et al., 2008). In fast twitch muscles (*m. vastus lateralis, m. rectus femoris* and *m. vastus medialis*) at 110% MLS, an increase in proportion of type I muscle fibers was reported (Lushaj et al., 2008). In agreement with those results, an increase in proportion of slow type muscle fibers was shown in *gastrocnemius medialis* of male F344*BN rats, when comparing rats at the age of ~25%

and 107% MLS (Carter et al., 2010). In contrast, for high-oxidative *soleus* muscle, a shift towards fast MHC was observed (Carter et al., 2010). The authors argued that the aging related direction of the fiber type shift was determined by the prevailing muscle fiber type, i.e. the shift is away from the dominant adult MHC isoform (Carter et al., 2010). This is however contradicted by another report, in which the shift was reported to be towards the dominant MHC isoform, when comparing rats of 18% and 110% MLS (Brown and Hasser, 1996). Also, studies in other rat strains (male Wistar and F344) reported ambiguous results (Eddinger et al., 1985; Kadhiresan et al., 1996; Larsson and Edstrom, 1986).

To our knowledge, in C57BL mice this issue has been investigated using immunohistochemistry in only one study, which reported an age-related muscle fiber type shift towards a slower muscle fiber type in soleus and towards a faster muscle fiber type in *extensor digitorum* longus muscles in male C57BL/6J mice (Sheard and Anderson, 2012). These results contradict a shift away from the dominant muscle fiber type as shown in several rat studies. However, the lack of changes in force-velocity curves in *extensor digitorum longus* muscle of 26-27 compared to that in 9-12-months-old male C57BL/6 mice suggests that an age-related shift in myosin composition may not occur (Brooks and Faulkner, 1988; Brooks and Faulkner, 1994a), although a shift between type II isoforms could not be excluded (Brooks and Faulkner, 1994a). More information on fiber type distribution in male C57BL mice is therefore needed.

Abovementioned findings are difficult to interpret with the preferential loss of fast (α-) motoneurons as established in rats (Ishihara and Araki, 1988; Ishihara et al., 1987). Surely the method of analysis, type of muscle, rodent strain and (relative) age also affect the results. It is a possibility that the preferential loss of fast (α-) motoneurons is not as widespread and common in humans and rodents as generally presumed. Therefore, for humans and rodents the age-related shift towards a slower phenotype, but also the possible differences in muscle fiber

specific tension, should not be regarded as established. More investigation on this subject, for instance whole muscle studies in rodents, is therefore necessary.

4.6 Age-related changes in specific muscle tension

In addition to changes in muscle physiological cross-sectional area (PCSA), the maximal muscle force is also determined by the specific tension of the muscle. As discussed above, aging is accompanied with a decrease in muscle mass, but the decrease in force generating capacity is more than proportional to the decrease in muscle mass (Degens et al., 2009a; Delmonico et al., 2009; Jubrias et al., 1997). One explanation for the reduced force generating capacity is the reduction in specific tension. In aged men (76 yrs, ~95% MLS), specific muscle tension is reported to be approximately 20% lower than in (young) adults (25 yrs, ~31% MLS) (Davies et al., 1986; Klein et al., 2001; Klitgaard et al., 1990; Morse et al., 2005; Vandervoort and McComas, 1986; Young et al., 1985). However, some studies did not find an age-related reduction in specific tension (Frontera et al., 1991; Frontera et al., 2000b; Young et al., 1984). A possible explanation for this discrepancy may be an inappropriate age window (50 vs. 69 yrs) to pick up changes in specific tension (Frontera et al., 1991). Two longitudinal studies, investigating men beyond the age of 60 yrs (>75% MLS), showed a reduction in specific tension (Delmonico et al., 2009; Frontera et al., 2008a). These results suggest that in men a reduction (~20%) in specific muscle tension above 60 yrs does exist and contributes significantly to the age-related muscle weakness.

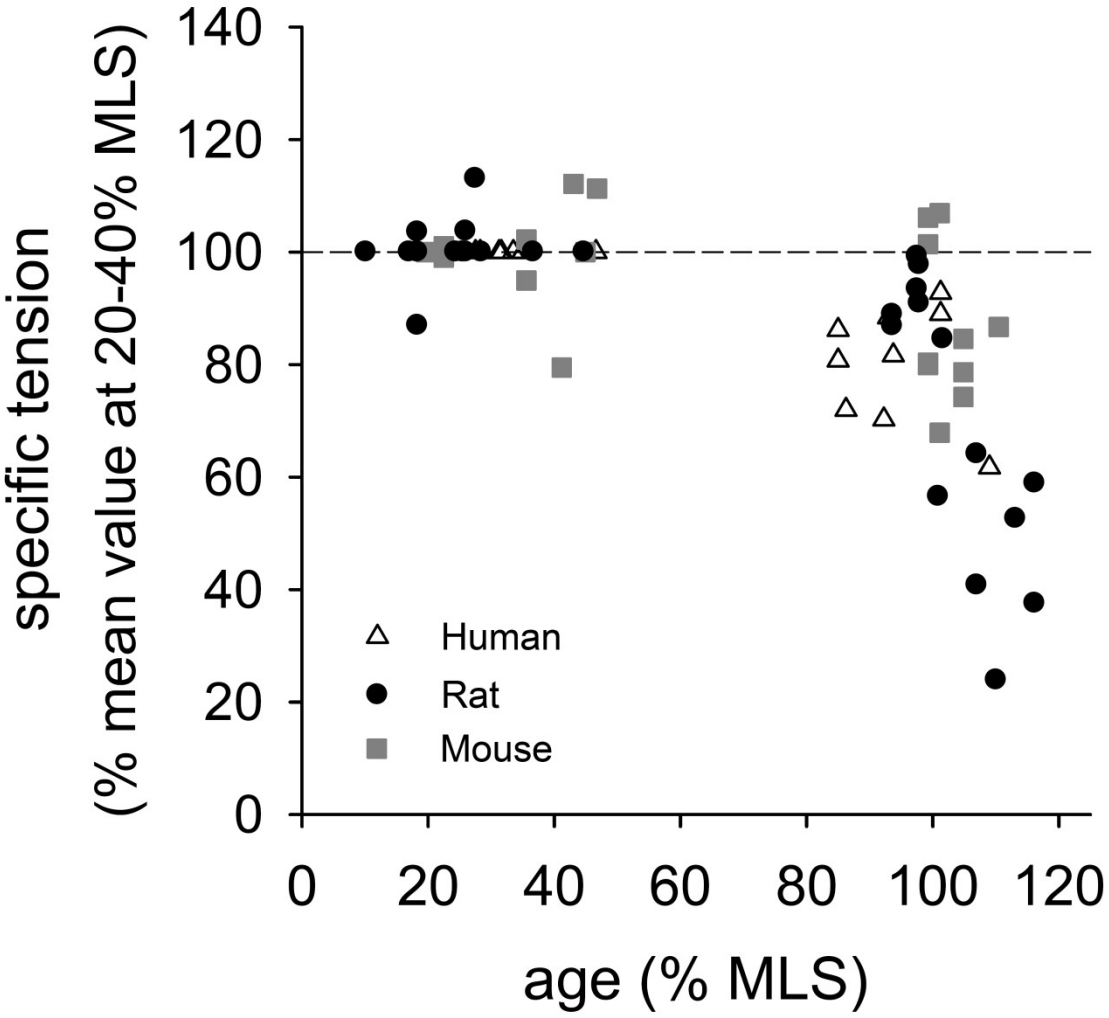


Figure 2.5: Literature-based values for age-related specific muscle tension in men and male rat and mice. Specific muscle tension is expressed as a percentage of the mean values of those reported in literature for the age range from 24 to 51% MLS. Mean maximal specific tension was taken as the maximal tension reported between 25-44% MLS for men and 24-51% MLS for rodents. For the calculation of the estimate of the maximal specific tension multiple studies were included reporting for the same muscles and using similar sampling techniques. The dashed line represents 100% specific tension. Note that for rodents only hind limb muscles were tested, whereas for humans mostly knee flexors and extensors were tested (Blough and Linderman, 2000a; Brooks and Faulkner, 1988; Brooks and Faulkner, 1991;

Carter et al., 2010; Davies et al., 1986; Degens and Alway, 2003; Frontera et al., 2000b; Kadhiresan et al., 1996; Kayani et al., 2010a; Klein et al., 2001; Klitgaard et al., 1990; Larsson and Edstrom, 1986; Linderman and Blough, 2002; Lodder et al., 1993; Lynch et al., 2001; Morse et al., 2005; Phillips et al., 1991; Phillips et al., 1993; Rice et al., 2005; Thompson and Brown, 1999; Vandervoort and McComas, 1986; Wineinger et al., 1995; Young et al., 1985; Zerba et al., 1990b).

In contrast to men, in rodents the precision and reliability of estimations of PCSA is higher (Brooks and Faulkner, 1994c). Overall in rats, similar to men, a decrease in specific muscle tension with increasing age has been shown (Fig. 2.5). All studies using male F344*BN rats comparing adult rats of 8 months (24% MLS) with aged rats of 35 months or older (>107% MLS) showed a significant reduction in specific tension (varying from 36 to 72%) (Blough and Linderman, 2000a; Carter et al., 2010; Degens and Alway, 2003; Rice et al., 2005; Thompson and Brown, 1999). For male F344 and Wistar rats age-related reductions were also reported, but these were only ~15% and 10%, respectively (Kadhiresan et al., 1996; Larsson and Edstrom, 1986; Linderman and Blough, 2002; Lodder et al., 1993).

Also in male C57BL/6 mice, decreases in specific muscle tension have been reported of ~15% (Brooks and Faulkner, 1988; Brooks and Faulkner, 1991; Brooks and Faulkner, 1994a; Lynch et al., 2001; Phillips et al., 1993; Zerba et al., 1990b). These values are in the same order of magnitude as those reported for Wistar and F344 rat strains.

The F344*BN rats where studied at a relatively higher age (107% MLS) than men and other rodents (strains). Therefore, it is a possibility that the decrease in specific muscle tension accelerates when the mean life span is reached.

Several explanations have been proposed for the observed reduction in specific muscle tension in older men. The age-related decrease in specific muscle tension may be due to a loss

of specific muscle fiber tension (D'Antona et al., 2003; Gonzalez et al., 2000; Larsson et al., 1997a; Larsson et al., 1997b; Lowe et al., 2001; Thompson and Brown, 1999). In biopsies of *m. vastus lateralis* from older persons at 90% MLS, the myosin concentrations of both type I and IIA muscle fibers were lower than in those from adult controls at 38% MLS, suggesting that a decrease in the potential maximum number of cross-bridges may in part explain the reduced muscle fiber specific tension (D'Antona et al., 2003). Furthermore, a reduction in peak intracellular Ca^{2+} in muscles from aging mice would lead to a larger number of ryanodine receptors (Ryrs1) being uncoupled to dihydropyridine receptors (DHPR) in skeletal muscle fibers (Wang et al., 2000), causing an impaired excitation-contraction coupling and therewith a decline in specific muscle force (Delbono, 2002; Gonzalez et al., 2000).

Note that in addition to these explanations other factors such as the degree of selective type II muscle fiber atrophy, an increase in the co-activation of antagonist muscles and/or a decrease in agonist muscle activation may all further contribute to the reduction of maximal muscle force generating capacity (Macaluso et al., 2002). Likewise, the inclusion of denervated muscle fibers in the assessment of PCSA has been shown to contribute to a reduced muscle force to PCSA ratio in old rats (Urbanchek et al., 2001). Although the amount of connective tissue content increases in both men (Overend et al., 1992; Rice et al., 1989) and rodents (Lushaj et al., 2008), this does not seem to be a factor that reduces the specific tension significantly (Brooks and Faulkner, 1994c; Degens et al., 1995).

5. Discussion

5.1 Are age-related reductions in muscle mass and force during aging comparable in men and male rodent models?

This review provides an overview of the factors that contribute to the loss in muscle mass and maximal muscle force. For this purpose we compared data of men with those of male rats and mice.

The rat strains included in this review were male Fischer344 x Brown Norway, Fischer344 and Wistar. Mouse studies were only included when male C57BL/6 mice were used. These rodent strains were chosen for practical and scientific reasons. Most of the determinants of aging related muscle weakness have been studied in the four abovementioned strains.

Rodents and humans differ considerably with respect to body size, life span and metabolic rate. It might therefore be difficult to directly compare humans with rodents. However, for the proper translation of knowledge gained in rodents to humans, this comparison has to be made. To do this, it is important to compare them at similar relative ages. Therefore, mean and maximal life span were used to determine relative ages per species. For men, we used a mean life span of 80 yrs and a maximal life span of 120 yrs. The mean and maximal life spans of the rodent models discussed in this review are shown in table 6.1.

The time to reach sexual maturity is relatively shorter in rodents (~8% MLS) compared to humans (~15% MLS) (Flurkey, 2007; Quinn, 2005). Since the purpose of this review was to provide an overview of the effects of aging on skeletal muscle function and its determinants, the difference in growth rates up to adulthood did likely not affect the validity of the comparisons made in this review. After sexual maturation both rodent and human muscles continue to develop. We found that the peak values for muscle force and all determinants were reached between 25-51% MLS.

One obvious difference between male F344*BN rats and other rat strains is the difference in life expectancy (NIA, 2012; Turturro et al., 1999). The MLS of F344*BN rats is 32.7 months and the 10% survival is 38 months (NIA, 2012; Turturro et al., 1999). In

comparison, for both male F344 and Wistar rats, MLS is only around 24 months (Deerberg et al., 1980; Masoro, 1980; Takeuchi et al., 2009; Thurman et al., 1994; Wolf et al., 2000). The difference in effects of aging on muscle force generating capacity between the F344*BN and the other inbred strains may exist because inbreeding can lead to homozygosity of detrimental recessive genes which is minimized by crossing inbred strains (Sprott and Ramirez, 1997). Therefore, a lower abundance of homozygous detrimental genes in hybrid models is likely to be responsible for the delayed onset of co-morbidities in the F344*BN strain. This increases the period in which age-related changes in the body can be observed (Lipman et al., 1996; Sprott, 1991). In addition, this strain does not develop renal pathology like F344 rats, and is less susceptible to develop specific tumors than other common rat models (Sprott, 1991). If co-morbidities do not kick in at the early stage in life, it is likely that the life span and the muscle aging processes of the other rat strains are similar (Degens and Alway, 2003). This raises the question whether the F344 and Wistar model can show signs of advanced muscle aging at all? Therefore, the choice of rodent strains as models for sarcopenia or muscle atrophy during cachexia may have implications for testing interventions to maintain muscle function.

5.2 Is the age-related reduction in muscle force explained by the sum of individual determinants and is this the same for men and male rodents?

For all determinants contributing to the age-related loss in muscle force generating capacity the effects of aging were estimated at 100% MLS by linear interpolation, compared to adult controls (24-51% MLS) (Table 2.2). This table shows that men have lost ~40% of maximal muscle force when they reach the age of 80, while the interpolated reductions in muscle mass and specific tension are ~35% and ~20%, respectively. Based on the multiplication of the remaining fractions of muscle mass and specific muscle tension, the

change is calculated to be 48%. This seems to be more than the reported decline in maximal muscle force estimated at MLS. For F344*BN rats, no difference between the interpolated and combined reduction in muscle force was observed. The estimated reduction for maximal muscle force was found to be 45%. The multiplication of the remaining fractions of muscle mass and specific tension, the age-related decreases were 25% and 30%, respectively, would account for a 47% reduction in maximal muscle force. Interpolation of the mice data established a reduction in maximal muscle force of 20%, while a loss of 23% was calculated when the remaining fractions in mass and specific muscle tension at MLS were multiplied. What may explain any discrepancies between the sum of determinants and the reported age-related decline in force at MLS, mostly observed in men?

One reason may be that different studies investigated determinants individually and not in combination. Alternatively, when studies did investigate multiple determinants, but reported opposite outcomes, this can affect the current calculations enormously. For example, one study reported that fiber loss explained most of the loss in muscle ACSA (Lexell and Downham, 1992). In contrast, a recent study explained the age-related decrease in ACSA of the *m. vastus lateralis* solely by atrophy of type II fibers (Nilwik et al., 2013). Finally, if muscle mass and specific tension do not change independently, but these changes are interrelated, this could explain part of the overestimation of the calculated muscle force generating capacity.

Several studies investigated effects of age on reductions in muscle fiber CSA and specific tension as well as those on maximal muscle force of the quadriceps of men of 30 yrs (38% MLS) and 75 yrs (94% MLS) (Frontera et al., 2000b; Young et al., 1985). The average age-related loss of maximal muscle force was 37%, of which 75% was explained by the loss of muscle mass and 25% by the loss of specific tension (Frontera et al., 2000b; Young et al., 1984; Young et al., 1985). Comparable studies in the F344*BN model found ~60% loss of

muscle force in lower leg muscles at 110% MLS, of which 50% was explained by the loss of muscle mass and the other 50% by reduction in specific tension (Blough and Linderman, 2000b; Brown and Hasser, 1996; Carter et al., 2010; Degens and Alway, 2003; Thompson and Brown, 1999). In both F344 and Wistar strain the decline in maximal muscle force was much less. Nevertheless, the decline was explained by an equal reduction in muscle mass and specific tension. Likewise, in mice the decline in muscle force was shown to be 23% of which ~50% was explained by both the age-related loss of muscle mass as well as specific tension, in *extensor digitorum longus* and *soleus* muscles at an average of 101% MLS (Brooks and Faulkner, 1988; Brooks and Faulkner, 1991; Lynch et al., 2001; Zerba et al., 1990b). Therefore, these studies indicate that in men the age-related loss of muscle mass is the most important factor contributing to the reduced maximal muscle force, while in rodents the contribution of the loss of muscle mass and specific tension seems to be equal.

*Table 2.2: Effects of aging on determinants of maximal muscle force or net joint moment in men and male C57BL mice and male F344*BN, F344 and Wistar rats.*

Male Wistar	Male F344	Male F344*BN	Male C57BL	Men	
10%	20%	45%	20%	40%	**ΔForce**
10%	15%	25%	15%	35% (CSA)	**ΔMass**
5%	10%	20%	20%	25%	**ΔMyofiber number**
5-10% (I) **15-20% (IIA)** **15-20% (IIB)**	0-10% (I) 0-15% (IIA) 10-25% (IIB)	10% (I) 10% (IIA) 30% (IIB)	-	5% (I) 35% (II)	**ΔMyofiber CSA**
0%	10%	30%	10%	20%	**ΔSpecific tension**
-	10%	20%	-	10-25%	**ΔMoto-neuron**

(Ansved and L., 1990; Brown, 1987; Edstrom and Larsson, 1987; Gutmann and Carlson, 1976; Ishihara et al., 1987; Larsson and Ansved, 1995; Larsson and Edstrom, 1986; Lodder et al., 1993; van der Meer et al., 2011b)	(Daw et al., 1988; Degens and Alway, 2003; Eddinger et al., 1985; Kadhiresan et al., 1996; Kanda et al., 1996; Larkin et al., 2003; Linderman and Blough, 2002; Pettigrew and Gardiner, 1987; Schuenke et al., 2011)	(Blough and Linderman, 2000a; Brown and Hasser, 1996; Bua et al., 2002; Carter et al., 2010; Degens and Alway, 2003; Lushaj et al., 2008; McKiernan et al., 2004; Rice et al., 2005; Rowan et al., 2012; Thompson and Brown, 1999; Wanagat et al., 2001; Wineinger et al., 1995)	(Brooks and Faulkner, 1988; Brooks and Faulkner, 1991; Jackson et al., 2011; Kayani et al., 2010a; Lynch et al., 2001; Pagala et al., 1998; Phillips et al., 1991; Phillips et al., 1993; Sheard and Anderson, 2012; Zerba et al., 1990b)	(Andersen, 2003; Aniansson et al., 1986; Davies et al., 1986; Essen-Gustavsson and Borges, 1986; Frontera et al., 2000b; Hakkinen et al., 1998; Ivey et al., 2000; Kawamura et al., 1977; Klein et al., 2001; Klitgaard et al., 1990; Larsson, 1978; Larsson et al., 1979; Lexell et al., 1988; Morse et al., 2005; Murray et al., 1980; Overend et al., 1992; Scelsi et al., 1980; Tomlinson and Irving, 1977; Tomonaga, 1977; Vandervoort and McComas, 1986; Young et al., 1985)	**References**

The determinants shown are: force generating capacity (force), muscle mass (mass), total myofiber number, myofiber CSA, specific muscle tension and motoneuron number. The percentages show per determinant age-related reductions (Δ) at 100% MLS, compared to adult controls (24-51% MLS). The age-related reductions were interpolated by linear regression with all datapoints between 60-120% MLS. As data on muscle mass in men are not available, this value has been replaced with muscle CSA.

Commencing from the collected data, male F344*BN rats at an age of 32-35 months may be more appropriate to study aging effects corresponding to those of 70-80 year old men (Lushaj et al., 2008), whereas these effects in rats of 36-38-months-old seem to be similar to those of men aged over 80 yrs (Vandervoort and McComas, 1986).

In mice, the reported aging related reductions in maximal muscle force and mass, are much lower than those reported for both men and F344*BN rats, while specific tension seems to be affected almost equally. This does not necessarily mean that sarcopenia in mice is a

result of other mechanisms. This could possibly be explained by the fact that in mice the aging related muscle deteriorating effects occur at a higher relative age.

In summary, in both men and male rodents the loss of force generating capacity seems affected by a loss in muscle mass and specific tension. However, the contribution of these factors may differ.

5.3 Comparison of age-related changes in factors determining muscle force generating capacity in men and male rodents

The observed effects of aging on muscle function reported for the different rat strains (F344*BN, F344 and Wistar) are most severe in F344*BN rats, and lowest in Wistar rats. Old F344*BN rats were tested at a high absolute (33 to 39 months) and relative (101% to 119% MLS) ages. The F344*BN model shows a similar reduction in maximal muscle force, a slightly lower loss of muscle mass, but a higher reduction in specific tension with age compared with men is shown, when the effects are interpolated to 100% MLS using linear regression. Furthermore, at MLS male F344*BN rats exhibit a lower loss in muscle fiber number, but show a similar reduction in muscle fiber CSA. This suggests that male F344*BN rats show comparable age-related losses as men on most determinants of maximal muscle force.

Male Wistar rats show very low signs of age-related muscle deterioration. A major reason for this seems to be the low relative and absolute ages at which these animals are tested. In most studies, old male Wistar rats were between 20 to 24 months (85% to 102% MLS). With a MLS of ~24 months, testing at an older age seems not feasible, because of the high disease rate from this age. Because of this, the Wistar rat is not an appropriate model to study progression of sarcopenia at advanced age. But it may well be suitable to study early signs of sarcopenia, such as the occurrence of a reduction in specific tension (Degens et al.,

1995; Degens et al., 1993a) and increased variability in muscle fiber sizes (Degens et al., 2009b), where the latter may be an early sign of the on-going denervation-reinnervation process. For male F344 rats, the determinants of maximal muscle force are more affected by age than for Wistars. One factor could be the age range at which F344 rats were tested, which was from 24 months up to 32 months (i.e. 95% to 130% MLS), which was absolutely and relatively higher than that at which Wistar rats were tested. Note that for male Wistar rats it is not exactly known how the determinants of force generating capacity develop between the age of ~6 and ~12 months, because most studies investigating muscle aging have been done with control rats at the age of only ~6 months. Furthermore, studies investigating male Wistar rats at an age of ~12 months are rare. Studies with female Wistar rats of 5 and 13 months of age, indicate a small increase in muscle mass (Degens et al., 1995; Degens et al., 1993a). It is possible that male Wistar rats undergo the same process, which may cause a small underestimation of the effect of aging on muscle wasting in Wistar rats in this review.

The relative age at which male C57BL/6 mice were tested was close to MLS (90% to 105%). Furthermore, compared to men it seems that age-related effects of sarcopenia in mice appear only later in life. A reason for this could be that the MLS of C57BL/6 mice has been underestimated, as it has been shown to increase over the years (Fig. 2.6). Another explanation may be that 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 human beings, who otherwise would have died. Thereby, even with the best intensions, the number of aged individuals with an impaired quality of life and physical fitness grows, which exposes them to (additional) age-related muscle weakness, compared to rodents.

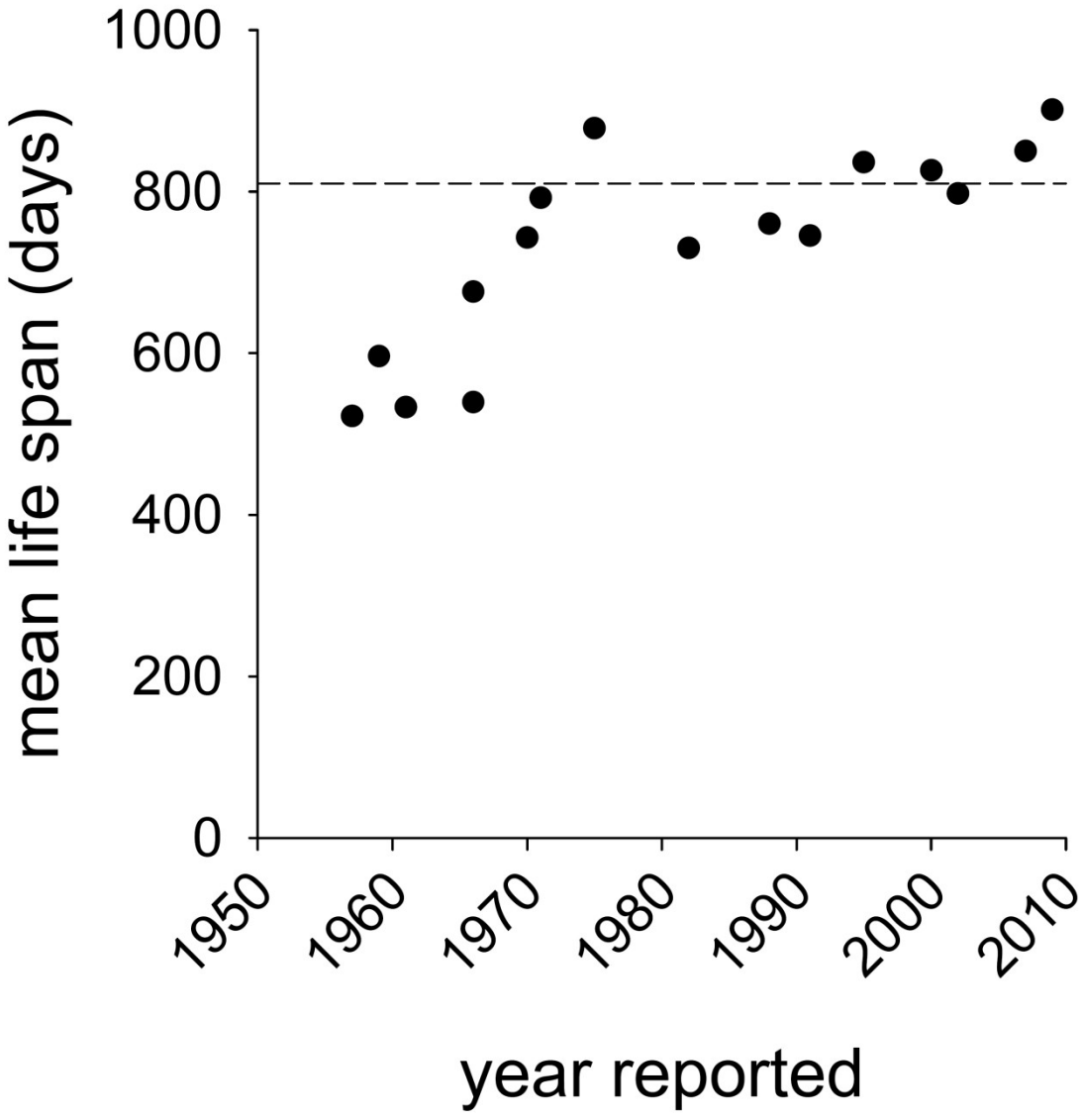


Figure 2.6: Increase in reported mean life span in male C57BL/6 mice with time, from 1957 until 2009 (Blackwell et al., 1995; Flurkey, 2007; Kunstyr and Leuenberger, 1975; Turturro et al., 2002; Wolf et al., 2000; Yuan et al., 2009). The dashed line indicates 810 days (i.e. 26.7 months), the mean reported life span for these mice since 1970.

So there are some differences between rodents which in part can be explained by differences in the rate or degree of senescence. The number of co-morbidities occurring in old age seems to be closely related to the latter. Furthermore, in all models, all determinants are

affected, meaning that for all the investigated organisms the mechanism underlying sarcopenia is comparable. This implies that when comparisons are made in a correct way, results are transferable between male rodents and men. In addition, for most determinants of muscle force, the degree of muscle aging in male F344*BN rats between 32 and 35 months seems to be similar to that of a 80 year-old man, when compared to adult controls (20-40% MLS).

In this review we focused on male rodents and men, because limited data is available for female rodents. There are several reasons why the rate and onset of sarcopenia may differ between men and women and male and female rodents. For instance, the hormonal differences between men and women may affect sarcopenia in a qualitative and/or quantitative different way. In addition, there may be differences in life expectancy as well as the age at which adulthood is reached, which both would affect the MLS comparison between male and female rodents. In order to focus the manuscript on the processes occurring during aging in different species, we decided to avoid extensive comparisons between genders. More detailed investigations at similar relative ages are warranted to establish gender-dependent effects of aging on muscle force generating capacity.

Normalizing age to MLS allows one to compare maximal muscle force and its determinants between models at similar relative ages. Obviously, variations in the calculated MLS may have consequences for the outcomes of this review. Per species and strain, we estimated MLS based on data from the literature, realizing that even after extensive searches the calculated values would be approximations. Furthermore, MLS is prone to change over time, due to improving living conditions, environmental factors, and changing genetic backgrounds of strains. Some of these factors and their effects on muscle aging and MLS are discussed below.

5.4 Factors affecting life span of humans and rodents

Life expectancy is determined by a multitude of factors, among which genetic and environmental factors such as nutrition, exercise and stress, which may all differ between humans and rodents. Housing conditions, protein intake, physical activity and co-morbidities are factors that are discussed below.

Housing facilities have improved over time. For instance the introduction of specified pathogen free (SPF) barrier facilities has resulted in an increased life expectancy of laboratory rodents, especially C57BL mice, throughout the last decades (Fig. 2.6). The introduction of SPF organisms also had a dramatic impact on the quality and validity of research (Sprott, 1991). The consequence 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 animals are generally fed standard industrial food. These diets often contain 20% or more protein (Blackwell et al., 1995; Masoro, 1980; Turturro et al., 2002; Turturro et al., 1999; Wineinger et al., 1995; Wolf et al., 2000), which is higher than the average protein intake (15%) of humans throughout life (Fulgoni, 2008; National institute for public health and the environment, 2011). The effect of a chronic high protein diet on muscle mass, especially in aging rodents, is largely unknown. However, for *gastrocnemius* muscle of male F344 rats at ~100% MLS it was shown that one month of amino acids administration normalized total muscle protein content (Chen Scarabelli et al., 2008). In old Wistar rats (85% MLS) long-term oral supplementation (8 weeks) of amino acids increased muscle mass by 10% (Pansarasa et al., 2008). More specifically, for type I muscle fibers, age-related atrophy at 85% MLS was attenuated by 20%, compared to a young control group (Pansarasa et al., 2008). Another study showed that in male C57BL/6 mice administration of branched-chain amino acids from 67% to 79% MLS preserved muscle fiber size, and resulted in an 70 day increase in the mean life span (D'Antona et al., 2010). These studies suggest that in rodents a

high protein diet may slow down sarcopenia. Theoretically, extra supplementation of anti-oxidants, decreases oxidative stress, and may therefore have beneficial health effects. However, whether the life-time intake of anti-oxidants differs between humans and rodents and whether this actually has a different effect on life span remains to be elucidated.

The intensity and duration of daily physical activity may also affect the lifespan and the process of sarcopenia (Degens, 2012). During aging, physical activity of humans is substantially reduced. The amount of energy spend during daily life is drastically decreased in old (90 yrs, ~115% MLS) compared to young people (~25 yrs, ~30% MLS) (Westerterp, 2000). For laboratory rodents discussed in this review, an age-related decline in physical activity has also been reported (Ingram, 2000). In a study on male F344 and F344*BN rats it was shown that both species' total activity count decreased by ~30% and ~50%, from 7 to 24 months (98% MLS) (F344) and 31 months (F344*BN) (95% MLS), respectively (Spangler et al., 1994). Also for 13-month-old female 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 seems 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.

In summary, it is important to be aware of the fact that some factors influencing life expectancy and the rate of muscle aging may differ between men and male rodents.

6. Conclusions

In this review we present an overview of the magnitude of age-related muscle force deterioration and underlying causes for different aging models. The main questions of this

review were if knowledge about age-related reductions in maximal muscle force generating capacity acquired in rodents could be translated to humans, and if so, how do they relate to each other?

Using relative age allows one to compare effects of human aging on muscle force generating capacity with those of three different rat strains and one mouse strain. The male F344*BN model resembles muscle aging in men most. At MLS male F344*BN rats show a similar age-related reduction in maximal muscle force, a slightly lower loss of muscle mass, but a higher reduction in specific tension than men. The most important reason for this is that F344*BN reach an older age than other rat strains and C57BL/6 mice, likely because of a delayed onset of co-morbidities.

In designing future rodent studies to investigate mechanisms underlying sarcopenia and muscle weakness, attention should be paid to the age and determinants that are of interest. Male F344*BN rats at an age of 32-35 months seem to exhibit age-related signs of muscle weakness comparable with those of 70-80-yr-old men, whereas rats of 36-38-months-old seem to show similar declines as men aged over 80 yrs. Wistar and F344 rats seem to be less appropriate for studying advanced effects of age-related muscle weakness. Instead, these models may be more useful to study the initial stages of sarcopenia.

Most aging studies on mice have investigated muscle force and its determinants at around 90-100% MLS. At this age, the effects are moderate compared to those reported for men and F344*BN rats. At present, C57BL/6 mice seem to live longer, also without co-morbidities, compared to 20-30 yrs ago, most probably caused by better environmental conditions like housing facilities. These improvements make the current model much more suitable for studying the initial stages of muscle aging. Furthermore, choosing older mice (≥25 months) and for example changing the diet, may make them appropriate for studying advanced aging processes in skeletal muscle. Moreover, mouse models are advantageous

because of the large availability and of genetically engineered and mutant mice strains, which allows one to investigate the molecular mechanisms behind aging related muscle wasting.

Although the rate of age-related changes in muscle force generating capacity and its determinants differs between species, in each of them, all determinants of muscle force are affected by age, suggesting that the mechanism behind sarcopenia in male rodents and men is comparable. This implies that when comparisons are made in a correct way, results can be translated from male rodents to men. However, the exact effects of aging on some parameters involved in sarcopenia are still not elucidated. Therefore, more research is warranted, in particular studies that integrate changes of all determinants of muscle aging within the same model.

Acknowledgements

This study was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate program (2011-2015). The authors have no conflict of interest to declare.

Chapter 3

Plantaris muscle weakness in old mice: Relative contributions of changes in specific force, muscle mass, myofiber cross-sectional area and number

This chapter is based on:
Sam B. Ballak, Hans Degens, Tinelies Busé-Pot, Arnold de Haan and Richard T. Jaspers.

Plantaris muscle weakness in old mice: Relative contributions of changes in specific force, muscle mass, myofiber cross-sectional area and number. *Age* (2014) 36:9726

Abstract

Background: The age-related decline in muscle function contributes to the movement limitations in daily life in old age. The age-related loss in muscle force is attributable to loss of myofibers, myofiber atrophy and a reduction in specific force. The contribution of each of these determinants to muscle weakness in old age is, however, largely unknown.

Objective: To determine whether a loss in myofiber number, myofiber atrophy and a reduction in specific muscle force contribute to the age-related loss of muscle force in 25-month-old mouse.

Methods: Maximal isometric force of *in situ m. plantaris* of C57BL/6j male adult (9-months) and old (25-months) mice was determined and related to myofiber number, myofiber size, intramuscular connective tissue content and proportion of denervated myofibers.

Results: Isometric maximal plantaris muscle force was 13% lower in old than adult mice (0.97±0.05N vs. 0.84±0.03N; $P<0.05$). *M. plantaris* mass of old mice was not significantly smaller than that of adult mice. There was also no significant myofiber atrophy or myofiber loss. Specific muscle force of old mice was 25% lower than that of adult mice (0.55±0.05 vs. 0.41±0.03N·mm^{-2}, $P<0.01$). In addition, with age the proportion of type IIB myofibers decreased (43.6% vs. 38.4%, respectively), while the connective tissue content increased (11.6% vs. 16.4%, respectively).

Conclusion: The age-related reduction in maximal isometric plantaris muscle force in 25-month-old male C57BL/6j mice is mainly attributable to a reduction in specific force, which is for 5% explicable by an age-related increase in connective tissue, rather than myofiber atrophy and myofiber loss.

1. Introduction

Aging is associated with a progressive decline in muscle power generating capacity (Runge et al., 2004) that contributes to an increased incidence of falls, a decreased independence and reduced quality of life (Kamel, 2003; Roubenoff and Hughes, 2000). Improvement of muscle function in the older person may alleviate many of these problems. To develop strategies to preserve, slow or even reverse muscle weakness in advanced age, a profound understanding of the etiology of age-related muscle weakness is needed.

An important determinant of muscle power is the force generating capacity of the muscle. Muscle force generating capacity in humans can decrease as much as 60% between the age of 30 and 80 years (Faulkner et al., 2007a). Also in rodents a 25-65% age-related deterioration in the muscle force generating capacity has been reported (Ballak et al., 2014b; Brooks and Faulkner, 1988; Carter et al., 2010; Degens and Alway, 2003), which is not only due to a loss of muscle mass but also a reduction in the force generating capacity per muscle cross-sectional area, specific force (Degens et al., 2009a). It could be that during the initial stages of sarcopenia a reduction in specific force precedes the loss in muscle mass.

Concomitant with the loss of muscle force generating capacity there is a loss of muscle mass. In the human *m. vastus lateralis* this amounted to approximately 30% between 40 and 80 years (Janssen et al., 2000; Klitgaard et al., 1990; Maden-Wilkinson et al., 2014b; Overend et al., 1992; Rice et al., 1989; Young et al., 1984; Young et al., 1985). In rodents, at ages representative of these ages in humans, a similar age-related reduction in muscle mass is observed (Ballak et al., 2014b). Typically, the age-related decrease in muscle mass is due to both a reduction in myofiber number (Lexell et al., 1988; Lushaj et al., 2008; McKiernan et al., 2004; Sato et al., 1984; Zerba et al., 1990a) and (preferential type II) myofiber atrophy (Brown and Hasser, 1996; Carter et al., 2010; Klitgaard et al., 1990; Larsson, 1978; Tomonaga, 1977; Verdijk et al., 2007). However, the loss in maximal muscle force with age is

more than proportional to the decrease in muscle mass (Degens et al., 2009a). This implies age-related intrinsic changes of the muscle affecting the quality of the remaining muscle tissue (Frontera et al., 2008b). In fact, several studies in both humans (Frontera et al., 2008b; Klein et al., 2001; Morse et al., 2005) and rodents (Degens and Alway, 2003; Degens et al., 1995; Kadhiresan et al., 1996; Rice et al., 2005) have reported a reduced force per unit cross-sectional area (referred to as specific force) during aging. An increased connective tissue content (Brooks and Faulkner, 1994b; Lushaj et al., 2008; Overend et al., 1992), inclusion of denervated, and thus non-force producing myofibers (Urbanchek et al., 2001), and a selective atrophy of type II myofibers that reportedly have a higher specific force than type I fibers (Yu et al., 2007), may also contribute to the age-related reduction of specific muscle force.

It thus appears that the three prime factors that contribute to the age-related muscle weakness are a reduction in myofiber number, myofiber atrophy and a reduction in specific force. Yet, these determinants of the age-related muscle dysfunction are hitherto not evaluated collectively, especially in early stages of sarcopenia. Since the contribution of each of these determinants to the age-related changes in muscle force generating capacity cannot be investigated in humans, we used *m. plantaris* in adult (9-months) and old (25-months) mice. We hypothesized that skeletal muscle weakness in old age is in order of importance due to a) a loss in muscle quantity, b) a reduction in muscle quality.

To investigate this we determined the maximal isometric force of mouse *m. plantaris in situ*. To determine the relative contribution of the three abovementioned factors, tetanic force was related to *m. plantaris* mass, myofiber CSA, myofiber number and specific force. Histological analysis was also performed to determine connective tissue content and proportion of denervated, and hence non-force producing, myofibers.

2. Methods

2.1 Animals

Experiments were performed on eleven 9-month-old (adult) and ten 25-month-old (old) male C57BL/6J mice (Janvier, France). Mice were housed individually and kept under specific-pathogen-free conditions at 20-22 ^{0}C at a 12-h light/dark cycle. Animals were given free access to water and chow (Ssniff® S8189-S095). For the experiments described below we analyzed histological and contractile force characteristics of the right leg. At the age of 7.5 (adult) or 23.5 (old) months, in the left leg of the mice the *m. gastrocnemius* and *m. soleus* were denervated to overload the *m. plantaris* muscle for six weeks. The effects of overload are beyond the scope of the present study.

All experiments were approved by the local animal use and care committee of the VU University Amsterdam and conformed to the guide of the Dutch Research Council for care and use of laboratory animals.

2.2 Surgery and preparation

Fifteen minutes prior to the start of the experiment, mice received a subcutaneous injection of 0.06 mL 1% Temgesic (Reckitt Benckiser, UK) as an analgesic and were anaesthetized with 4% isoflurane, 0.1 L·min^{-1} O_2 and 0.2 L·min^{-1} air. Subsequently, the level of anesthesia was maintained with 1.5-2.5% isoflurane. A humidifier moistened the inhaled air to prevent dehydration due to respiration. The mice were placed on a heated pad to maintain body temperature at ~36.5 ^{0}C.

The experiments were performed as explained previously (Degens and Alway, 2003). The *m. plantaris* was dissected free from surrounding tissue while maintaining its innervation and blood supply. The sciatic nerve was severed and the proximal end was placed over an electrode for stimulation of the muscle. The distal tendon of the *m. plantaris* was dissected free and tightened with a Kevlar thread via a small steel link to a force transducer, which was

mounted on the lever arm of an isovelocity measuring system. The femur was fixed by a clamp on the condyle of the femur. During the experiment, the muscle and its surrounding were kept moist at physiological temperature (34-36 ^{o}C) with a water-saturated airflow.

Figure 3.1 Experimental set-up as used during all measurements. This is a modification of the set-up used by de Haan et al. (de Haan et al., 1989). The anaesthetized mouse was placed on a heated pad, while the m. plantaris was attached to the force transducer, which allowed to measure muscle force in situ.

1

2.3 Experimental setup and force measurements

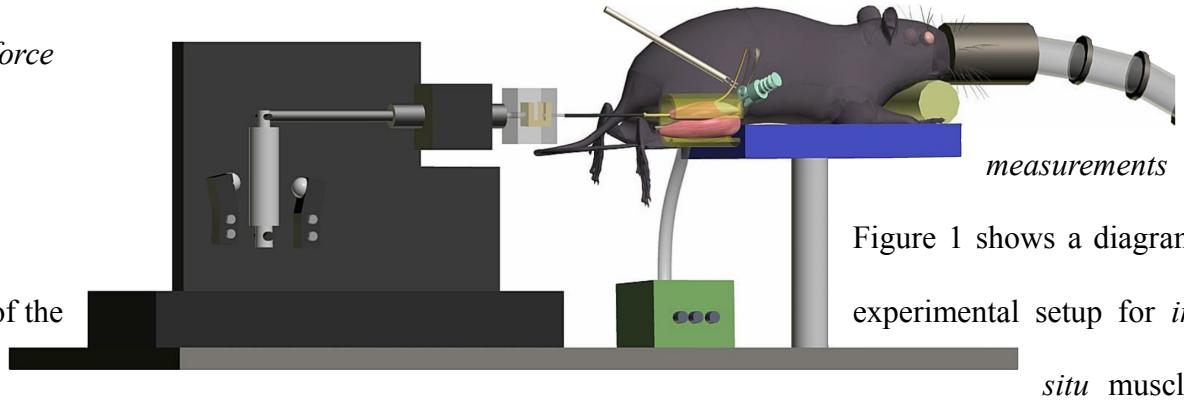


Figure 1 shows a diagram of the experimental setup for *in situ* muscle force measurements which is a modification of that described (de Haan et al., 1989). Contractions were induced by supramaximal electrical stimulation of the sciatic nerve at a constant current (2 mA; 200-µs pulse width). Optimal muscle length ($\square_o$) was defined as the muscle length at which maximal tetanic isometric force was generated. To determine $\square_o$, first the length at which the muscle produced maximal twitch force was assessed. To fine adjust $\square_o$ tetani (150 Hz, 150 ms) were applied once every 2 minutes. Two minutes after the last tetanus the muscle was stimulated by applying a 400-Hz, 80-ms pulse train to determine the maximal rate of force development (MRTD). Finally, the muscle was stimulated by applying a 150-Hz, 150-ms pulse trains once every second for 5 min to deplete the glycogen in fibers expressing type IIB myosin. Force and length signals (1–10 kHz) were stored on disk. At the end of the

experiment, the *m. plantaris* was excised and weighed and the mouse was killed by cervical dislocation.-

2.4 Analyses

The *m. plantaris* was embedded at □$_o$ in a gelatin-tyrode (NaCl, 128.3 mM; KCl, 4.7 mM; MCl_2·$_2O$, 1.05 mM; NaH_2PO_4·H_2O, 0.42 mM; $NaHCO_3$, 20.2 mM; EGTA, 15.0 mM; Gelatine 15% (w/v), pH 7.2) solution and frozen in liquid nitrogen. Subsequently, *m. soleus* and *m. gastrocnemius medialis* were excised and also frozen in liquid nitrogen. All chemicals were obtained from Sigma Aldrich (The Netherlands) unless stated otherwise.

2.4.1 Cutting of histological sections

Within a month after the contraction protocol, serial cross-sections (10 µm) were cut from the midbelly of the *m. plantaris* using a cryostat at -20 ºC. The in gelatin-tyrode embedded sections were mounted with Tissue-Tek (Jung, Leica Microsystems, Germany) onto a specimen holder, avoiding direct contact between Tissue-Tek and the part of the muscle that would be sectioned to prevent freeze-thaw artefacts. Sections were mounted on glass slides (Menzel-Gläser, superfrost® plus, Germany), air-dried and stored at -80 ºC until further use.

2.4.2 Myosin heavy chain composition

Serial sections of the *m. plantaris* were immunohistochemically stained against type I, IIA, IIX and IIB MHC using monoclonal antibodies BAD5 (1µg/mL), SC-71 (1µg/mL), 6H1 (10µg/mL) and BF-F3 (1-10µg/mL) (Developmental Studies Hybridoma Bank, USA), respectively. In short, sections were fixed with acetone for 10 minutes at 4 ºC and washed in phosphate buffered saline with 0.05% tween (PBST) three times for 5 minutes. After blocking

with 10% normal swine serum for 30 minutes, the sections were incubated with the primary antibody for 90 minutes. Subsequently, sections were washed in PBST three times for 3 minutes and incubated in the dark with secondary antibody (Alexa 488 anti-mouse, 1:500, Life Technologies The Netherlands) for 30 minutes. After washing with PBST, incubating with wheat germ agglutinin (WGA, 1:50, Life Technologies, The Netherlands) for 20 minutes, washing with PBST and afterwards washing once more with PBS all in the dark, the sections were enclosed with Vectashield®-hardset mounting medium with DAPI (1.5µg/mL; Vector Laboratories, USA). The public domain software ImageJ 1.45s (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to count myofiber number, type and myofiber cross-sectional area.

2.4.3 Determination of denervated myofibers by PAS-staining

The glycogen content of individual myofibers was determined in 10-µm thick sections using the periodic acid-Schiff (PAS) staining reaction (van der Laarse et al., 1992). The sections were air-dried and fixed for 5 min in 4% formaldehyde in 20 mL 0.2 M imidazole and 180 mL acetone. Subsequently, sections were incubated in 44 mM periodic acid solution for 30 minutes at room temperature. After incubation, sections were briefly washed in 0.1 M HCl and stained with Schiff's reagents for 25 min at room temperature.

2.4.4 Intramuscular connective tissue content

Intramuscular connective tissue was determined using Sirius Red. The sections were air-dried and fixed for 30 min in Bouin solution. Next, sections were washed for 10 min, before 30 min incubation in Sirius red saturated with picric acid. Sections were dehydrated by rapidly dipping in absolute ethanol. For a better image quality sections were cleared with

xylene and mounted with DPX (Sigma Aldrich, UK). For each muscle the connective tissue was assessed by averaging the connective tissue of one picture in the glycolytic and another picture in the oxidative region of the muscle. A Matlab-script (version R2012a) was used to quantify the connective tissue content per image. The RBG threshold was set at R>140, B<110, G<110, to create a binary image allowing to filter all red pixels. The same threshold was applied to all images that were stained at the same time.

2.5 Statistics

To determine significant differences between age-groups Student's *t*-tests were used. To test for differences in fiber CSA a two-way ANOVA was used with factors age and fiber type. Effects were considered significant at $P<0.05$. Data are expressed as mean±SEM. All calculations were performed using IBM SPSS version 20.

3. Results

3.1 Mice characteristics

Table 3.1 shows that body mass, muscle optimum length (l_o), myofiber number and muscle mass / body mass ratio did not differ significantly between adult and old mice. The masses of the plantaris, gastrocnemius medialis and soleus muscles (PGMS) combined were ~8% less in old than adult animals ($P=0.03$; Table 3.1). Mass of *m. plantaris* alone was, however, not significantly reduced in the old compared to the adult group ($P>0.05$; Table 3.1).

Table 3.1 Mice characteristics

	Adult mice (n=11)	**Old mice (n=10)**
Body mass (g)	**36.1±3.0**	**35.6±0.9**
PGMS muscle mass (mg)	**102.6±2.5**	**94.7±2.3[a]**
Plantaris muscle mass (mg)	**23.6±0.6**	**22.4±0.7**
Muscle ACSA (mm^2)	**1.83±0.12**	**2.08±0.10**
Pooled myofiber CSA (μm^2)	**1498±65**	**1546±110**
l_o(mm)	**12.7±0.37**	**12.88±0.38**
Myofiber number (#)	**1027±58**	**1104±55**

Ratio muscle/body mass (mg·g^{-1})	**0.67±0.04**	**0.63±0.03**
Denervated myofibers (%)		
%total	**1.15±0.37**	**1.33±0.38**
%IIB	**2.33±0.68**	**3.48±1.09**
Specific force (N·mm^{-2})	**0.55±0.05**	**0.41±0.03[a]**
Specific force (N*g^{-1})	**40.94±1.4**	**37.58±1.4**
MRTD (mN·ms^{-1})	**32.3±2.1**	**29.1±2.1**
MRTD/Fmax (mN·ms^{-1}·N^{-1})	**36.4±2.2**	**37.9±1.5**
RT50 (ms)	**28.1±0.9**	**32.3±2.8[a]**

Values are mean±SEM. PGMS: Plantaris, gastrocnemius medialis and soleus. □$_o$: plantaris muscle length at which tetanic force was maximal. MRTD: Maximal rate of force development. RT50: half relaxation time. [a] Significantly different from adult mice at P<0.05.

2

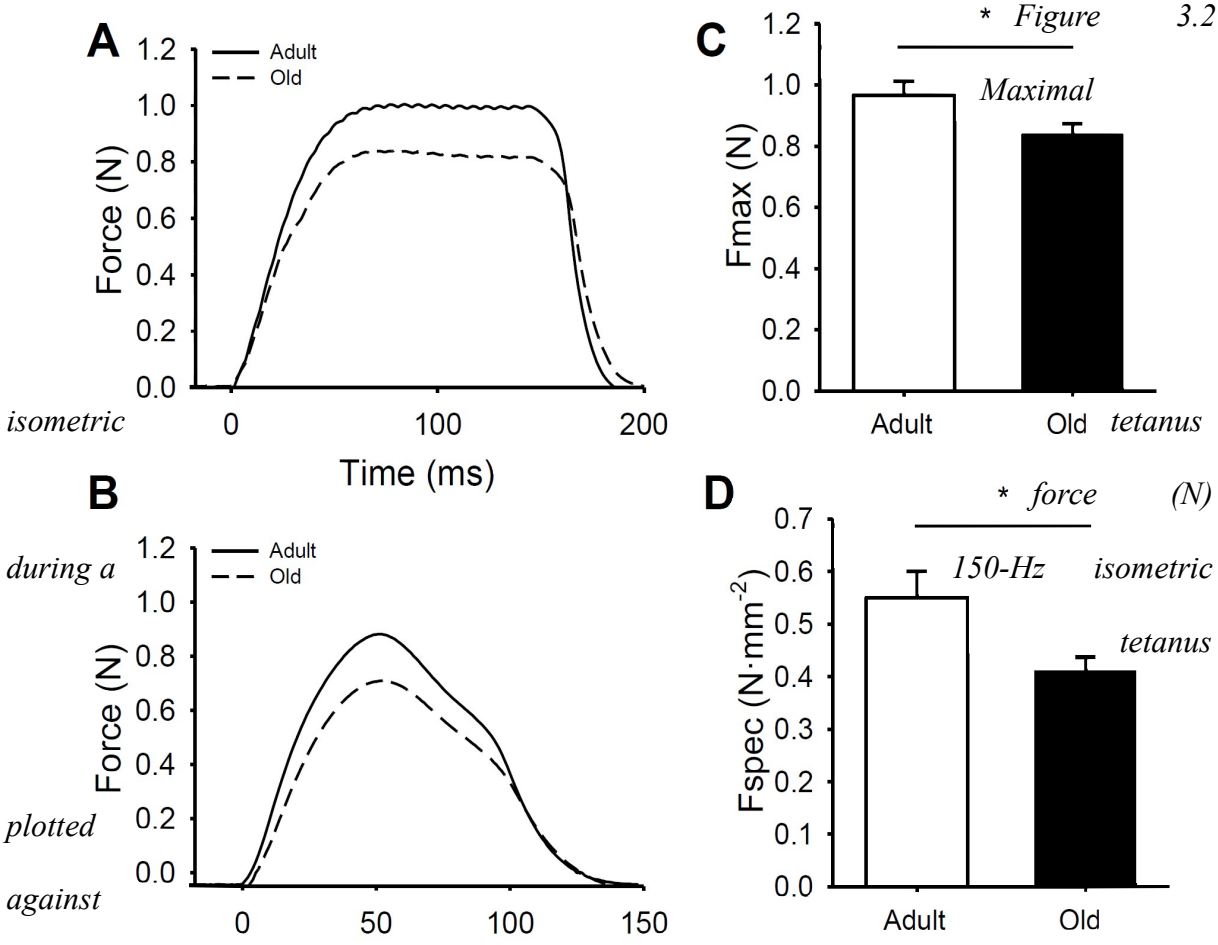


Figure 3.2 Maximal isometric tetanus force (N) during a 150-Hz isometric tetanus plotted against time (ms) for adult and old m. plantaris of mice (A). Force (N) during a 400-Hz isometric tetanus plotted against time for adult and old m. plantaris of mice (B).

Maximal force (mN) in adult mice was higher than that in old mice (: P<0.05) (C). Specific force (N·mm^{-2}) was less in old than adult m. plantaris (*: P<0.05). Values are mean±SEM.*

3.2 Age-related changes in morphology and in situ force characteristics of m. plantaris

The ACSA of the *m. plantaris* muscle did not differ significantly between adult and old mice (Table 3.1). Maximal isometric force of *m. plantaris* of old mice was 13% lower compared to that of adults (Figs. 3.2A and C; P<0.05). Maximal tetanic force divided by muscles ACSA (specific tetanic force) of old muscles was 25% lower than that of adult muscle (P<0.01; Fig. 3.2D). Five percent of this lower specific force was explicable by the higher relative content of connective tissue in the old (16.4% vs. 11.6% in adult) muscles (specific muscle force corrected for collagen content: adult: 0.63±0.06N·mm^{-2}; vs old 0.50±0.03N·mm^{-2}; P>0.05). However, specific tetanic force expressed as maximal tetanic force per muscle mass did not differ significantly between adult and old muscles (Table 3.1).

3

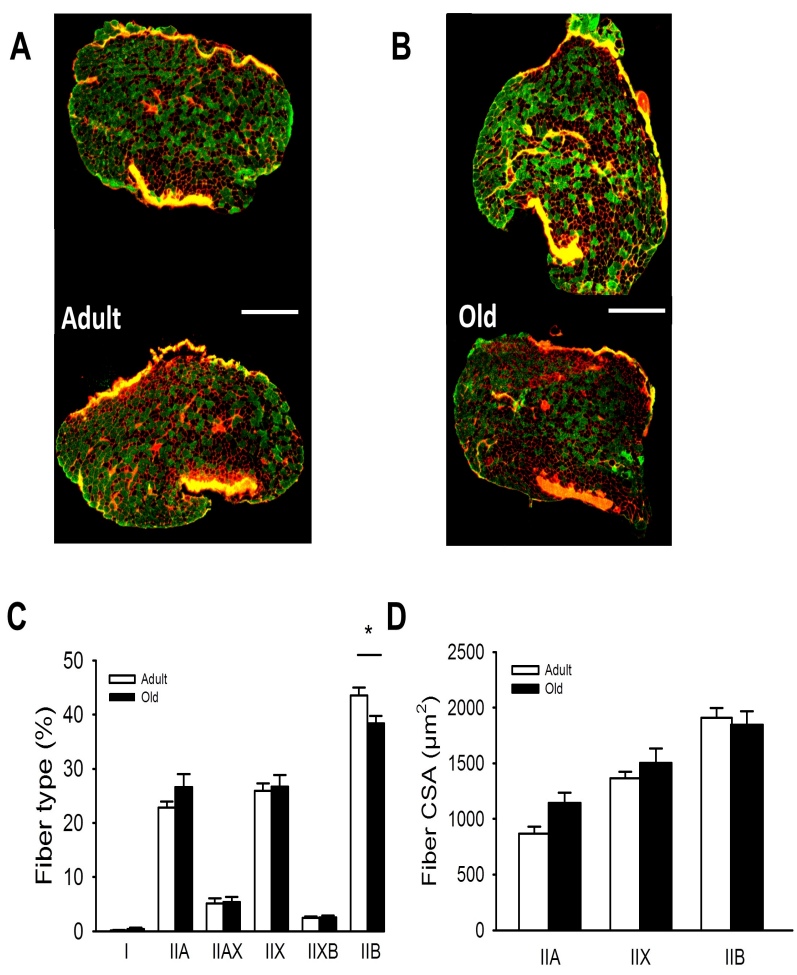


Figure 3.3 Examples of immunofluorescent staining for type IIB myosin in an adult (A) and old (B) m. plantaris. Bar represents 500µm. Type IIB myofiber proportion (%) in adult and old mice was significantly different (P<0.05). Type I, IIA, IIAX, IIX and IIXB proportions did not change significantly (C). Myofiber CSA (µm²) did not significantly decline for any fiber type (P>0.05) (D). Note that type I myofibers were not included in this analysis, because of the low presence of type I myofibers within the m. plantaris of C57Bl/6 mice. Values are mean±SEM.

Figure 3.3 shows examples of *m. plantaris* cross-sections stained specifically for type IIB myosin heavy chain. The percentage of type IIB myofibers decreased with age (43.6% vs. 38.4% in adult and old, respectively; P<0.05; Fig. 3C). The CSAs of type IIA, IIX and IIB

myofibers did not differ significantly between adult and old mice (Fig. 3.3D). Likewise pooled CSA did not change significantly with age (Table 3.1).

4

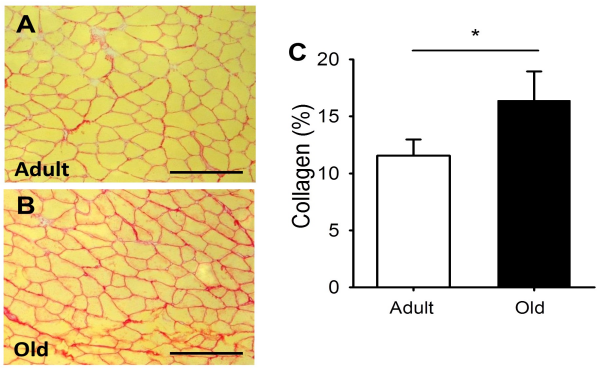


Figure 3.4 Collagen staining of m. plantaris of adult (A) and old mice (B). Bar represents 100µm. Threshold analysis (C) revealed a significant age-related increase (: P<0.05). Values are mean±SEM.*

Figures 3.4A and 3.4B show examples of sirius red stained muscle cross-sections. The percentage of collagen in the *m. plantaris* was higher in the old group compared to that in the adult group (P=0.03; Fig. 3.4C). Figure 3.5 shows examples of serial muscle sections stained for type IIB myosin and PAS to quantify the number of denervated myofibers that express IIB myosin. The percentage of all fibers and the percentage of IIB myosin expressing myofibers that were denervated did not differ significantly between adult and old mice (Fig. 3.5E).

Figures 3.4A and 3.4B show examples of sirius red stained muscle cross-sections. The percentage of collagen in the *m. plantaris* was higher in the old group compared to that in the adult group (16.4% vs. 11.6%, respectively; P=0.03; Fig. 3.4C). Figure 3.5 shows examples of serial muscle sections stained for type IIB myosin and PAS to quantify the number of

denervated myofibers that express IIB myosin. The percentage of all fibers and the percentage of IIB myosin expressing myofibers that were denervated did not differ significantly between adult and old mice (Fig. 5E).

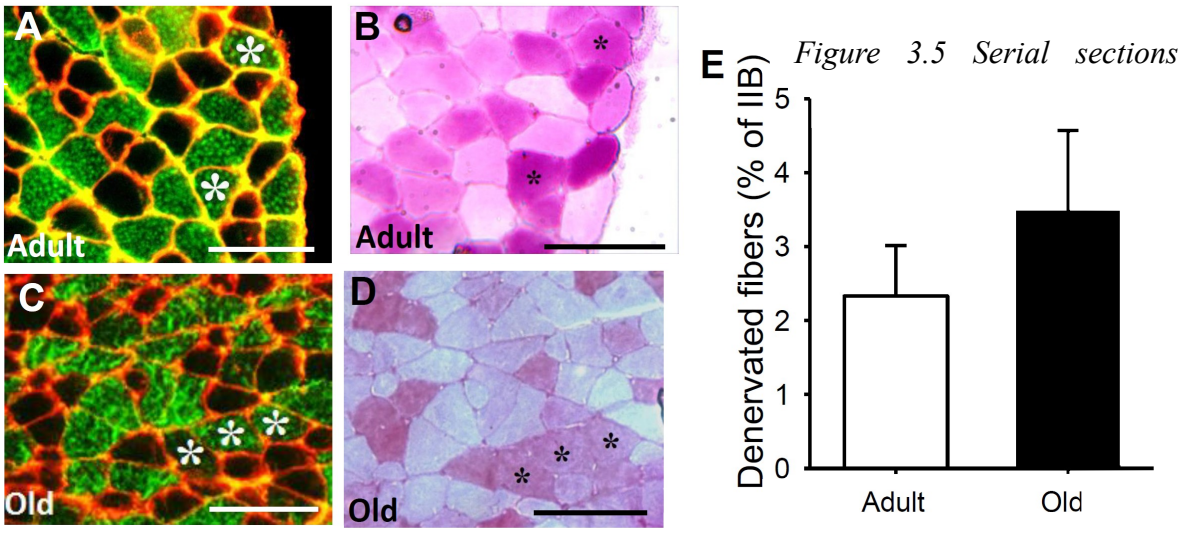


Figure 3.5 Serial sections stained for type IIB myosin and PAS of adult (A and B) and old (C and D) muscles. Bar represents 100µm. Corresponding denervated myofibers are specified with an asterisk. Analysis revealed no significant difference between adult and old denervated myofiber count expressed as percentage of all IIB myofibers (E). Values are mean±SEM.

Maximal rate of force development (MRTD) did not significantly differ between adult and old plantaris muscles (Fig. 3.2B; Table 3.1). Also normalized MRTD (MRTD/Fmax) did not decrease with age. However, half relaxation time of the 400-Hz contraction was significantly longer in the old than adult muscles ($P<0.01$) (Table 3.1), indicating that the old muscles were slower.

4. Discussion

The main finding of the present study is that the age-related loss in maximal force of plantaris muscle in 25-month-old male C57BL/6 mice is mainly attributable to a reduction in

specific force. In contrast to our hypothesis, plantaris muscles of old mice did not show a reduction in total and functional myofiber number, nor myofiber CSA.

4.1 Age-related decline in force generating capacity is explained by loss of muscle mass and reductions in specific force

Here we observed a 13% lower force generating capacity in 25- compared to male 9-month-old mice. Our aim was to determine to what extent reductions in muscle mass (quantity) and specific muscle force (quality) contribute to this age-related decline in muscle force generating capacity.

Other studies in male mice have reported that the ~25% age-related decline in muscle force in both slow and fast hindlimb muscles is explained for 55% by the age-related loss of muscle mass, and for ~45% by a reduction in specific force in 26-28-month-old mice (Brooks and Faulkner, 1988; Brooks and Faulkner, 1991; Lynch et al., 2001; Zerba et al., 1990a). We did not observe a decrease in *m. plantaris* mass which is different from what has been reported in other studies in which a 15-26% lower muscle mass was reported in 26-28-month-old mice (Brooks and Faulkner, 1988; Jackson et al., 2011; Lynch et al., 2001; McArdle et al., 2004). The discrepancy between the effects of aging on muscle mass and maximal force shown in our study and those previously reported is unlikely related to the muscles used, as other studies observed the effects of aging in both fast and slow muscles. It is more likely that the use of older animals (26-28-months) in other studies than in our study (25-months) contributes to the discrepancy, as larger effects of aging on maximal muscle force are likely to be observed in the older the animal (Ballak et al., 2014b; Brooks and Faulkner, 1988; Lynch et al., 2001; McArdle et al., 2004). Indeed (Chan and Head, 2010) observed that even in 20-22-month-old mice specific force was already reduced without significant muscle atrophy. Combination of the data of these studies with slightly older and younger mice than the ones

used in the present study suggests that a reduction in muscle quality precedes a reduction in muscle mass. If so, it is likely that the contribution of a loss of muscle mass to the age-related muscle weakness increases with increasing age.

4.2 Effects of aging on atrophy and loss in myofiber number

An age-related reduction in muscle mass can be the result of both a loss of myofibers and myofiber atrophy (Lexell et al. 1988; Lusjah et al. 2008). Here, no myofiber atrophy nor myofiber loss was observed. Our observations are in line with other reports in which age-related decline in myofiber CSA could not be observed in *m. extensor digitorum longus* and *m. soleus* of 24- compared to 5-month-old mice (Sheard and Anderson, 2012; Zerba et al., 1990a). It is possible that the age-related myofiber atrophy and loss occur only at a later age as a small decrease in myofiber number of 10-15% has been observed in *m. extensor digitorum longus* and *m. soleus* of 24-27-month-old C57Bl/6 mice (Sheard and Anderson, 2012; Zerba et al., 1990a). Also human myofiber loss has not unequivocally been reported. Recently, in *m. vastus lateralis* of healthy 70 year old subjects a decrease in muscle mass was solely explained by a decrease in type II fiber CSA, and not by a loss of myofibers (Nilwik et al., 2013). Overall, it appears that in 25-month-old male C57BL/6j mice myofiber number and size are well maintained. Therefore, if these factors are involved in aging related reduction in maximal muscle force, the impact on the decline in maximal isometric muscle force will be minor and may only become significant at later stages of the aging process.

4.3 Effects of aging on fibrosis, denervation and myofiber type changes

The specific force (force per anatomical cross-sectional area) of 25-month-old *m. plantaris* was 25% lower compared to that of 9-month-old mice. The magnitude of the decrease in specific force was similar to that reported by others (Brooks and Faulkner, 1988;

Lynch et al., 2001; McArdle et al., 2004). Therefore, the age-related decrease in specific force appears to be an important factor contributing to an age-related decline in muscle force.

Theoretically, the loss of specific muscle force may be the result of an increase in connective tissue content, a larger proportion of denervated myofibers, a decrease in the proportion of fast type IIB myofibers, and/or a reduced force generating capacity of individual myofibers (reduced specific force). The fraction of intramuscular connective tissue within the *m. plantaris* was higher in muscles from old than adult animals. Others have also observed an increase in connective tissue content with age (Brooks and Faulkner, 1994c; Lushaj et al., 2008; Overend et al., 1992). When we corrected the specific force for the connective tissue content the difference between adult and old muscles was not significant anymore. Thus, part of the reduction in specific force in muscles from old mice is attributable to an increased content of connective tissue.

Another factor that could potentially reduce specific muscle force is an age-related increase in the number of denervated myofibers as a result of motor neuron death (Hashizume et al., 1988a; Ishihara et al., 1987; Larsson and Ansved, 1995). Using glycogen depletion, we were able to assess the proportion of non-recruited, presumable denervated fibers. However, no significant difference in the proportion of non-recruited myofibers was observed between the age groups, and therefore the lower specific force in the older animals is not attributable to loss of functional myofibers.

The proportion of myofibers expressing type IIB myosin decreased with age. The longer half relaxation time of the 400-Hz tetanus in aged than in adult muscle fits this observed reduction in the proportion of fibers expressing type IIB myosin. It is debatable, however, how much this actually contributes to the lower specific force. It is controversial whether the specific force of type IIB fibers is higher than that of other fiber types (Bottinelli et al., 1996; Widrick et al., 1996b) or not (Degens and Larsson, 2007; Larsson and Moss,

1993). Even if specific force of type IIB fibres was 1.41 times as high of that of other fibers (Larsson et al. 1993; Bottinelli et al., 1996 IIB vs. I) the decrease from 44% IIB fibres in adult to 38% in old muscle would only cause a 2% reduction in specific force. This is even an overestimation as the plantaris muscle of the mouse barely contains type I fibres and the differences in specific force between fast fibres, if existent, are much smaller than those reported in (Bottinelli et al., 1996) between type I and type IIB fibers. It is thus unlikely that the fiber type shift contributes to the observed age-related decline in specific force.

Finally, the observed age-related decrease in specific muscle force may be due to a loss of specific myofiber force with age (D'Antona et al., 2003; Gonzalez et al., 2000; Larsson et al., 1997a; Larsson et al., 1997b; Lowe et al., 2001; Thompson and Brown, 1999). This reduced skeletal muscle force is possibly related to a decreased number of cross-bridges (D'Antona et al., 2003), oxidative modifications (Degens and Larsson, 2007; Gilliver et al., 2010b; Lowe et al., 2001), protein glycation (Snow et al., 2007) and/or an impaired excitation-contraction uncoupling (Delbono, 2002; Gonzalez et al., 2000). While an age-related Ca-release may cause reductions in specific force during a given submaximal stimulation intensity and frequency, this will not cause a reduction in the maximal force generated during a tetanic contraction of the duration we applied (Andersson et al., 2011; Delbono et al., 1997; Lamboley et al., 2015). Together, with the aforementioned factors, this may explain the decrease in specific muscle force observed in this study.

4.4 Muscle aging in 25-month-old mice mimics the initial stages of sarcopenia in humans

The decreases in maximal muscle force observed in this and other mice studies are rather small, compared to the effects reported at a similar relative mean life expectancy (see for review (Ballak et al., 2014b). Nevertheless, the 13% decrease in muscle strength between

9- and 25-months of age in our C57BL/6J mice is comparable to that between the age of 25-30 year old and 60-70year-old humans (Ballak et al., 2014b).

4.5 Implications

Future studies could investigate the time course of the relative contribution of decrements in specific force and muscle mass to the loss of muscle strength. To do so, both somewhat younger and also older C57BL/6j mice (e.g. 26-30-months) should be studied, while making sure that the impact of disease is minimized. Furthermore, nutritional intake may be changed (e.g. lower protein intake) to observe whether a poor diet causes not only a lower specific muscle force and a larger loss of muscle mass at a given age, but also an accelerated rate of sarcopenia.

To counteract sarcopenia it is essential to know the mechanisms and causes of muscle aging, especially in the initial stages of sarcopenia. The present data suggest that interventions should, at least during the early stages of sarcopenia, aim primarily to stop or reverse the age-related decline in specific muscle force. This could be achieved by resistance training that has been shown to increase specific force (Erskine et al., 2011). The impact of training may decrease with increasing age (Degens, 2012; Slivka et al., 2008) and it thus appears essential to start interventions before sarcopenia takes its toll.

5 Conclusion

In conclusion, the age-related reductions in maximal isometric muscle force in male C57BL/6j mice were mainly attributable to a reduced specific force. Part of the reduction in specific force was due to an age-related increase in connective tissue content, but not to an increase in the percentage of denervated IIB myofibers. It is conceivable that the age-related reduction in specific force is the result of loss of specific force of individual myofibers. We

did not observe an age-related decline of myofiber number and myofiber CSA. Thus, our model seems to mimic force reductions similar to those reported in the early stages of sarcopenia in humans, and it is suggested that the decline in muscle quality precedes the decline in muscle quantity with age. Therefore, interventions to constrain early sarcopenia should aim particularly to improve specific muscle force.

Acknowledgements

This research was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate program (2011-2015). The authors would like to thank Guus Baan for excellent technical assistance. Furthermore, we would like to acknowledge Frank van't Hoff and Joshua Dunnink for their support.

Chapter 4

Blunted hypertrophic response in old mouse muscle is associated with a lower satellite cell content and is not alleviated by resveratrol.

This chapter has been published as:
Sam B. Ballak, Richard T. Jaspers, Louise Deldicque, Sreeda Chalil, Eva L. Peters, Arnold de Haan, Hans Degens. Blunted hypertrophic response in old mouse muscle is associated with a lower satellite cell content and is not alleviated by resveratrol. *Experimental gerontology.62 (2015) 23-31*

Abstract

Background: Sarcopenia contributes to the decreased quality of life in the older person. While resistance exercise is an effective measure to increase muscle mass and strength, the hypertrophic response may be blunted in old age.

Objectives: To determine 1) whether hypertrophy in the *m. plantaris* of old mice was blunted compared to adult and 2) whether this was related to a reduced satellite cell (SC) density and 3) how resveratrol affects hypertrophy in old mice.

Methods: In adult (7.5 months, n = 11), old (23.5 months, n = 10) and old-resveratrol-treated (n = 10) male C57BL/6J mice, hypertrophy of the left *m. plantaris* was induced by denervation of its synergists. The contralateral leg served as control.

Results: After six weeks, overload-induced myofiber hypertrophy and IIB–IIA shift in myofiber type composition were less pronounced in old than adult mice (P=0.03), irrespective of resveratrol treatment. Muscles from old mice had a lower SC density than adult muscles (P=0.002). Overload-induced SC proliferation ($P < 0.05$) resulted in an increased SC density in old, but not adult muscles (P=0.02), while a decrease occurred after resveratrol supplementation (P=0.044). Id2 and myogenin protein expression levels were higher in old than adult muscles ($P < 0.05$). Caspase-3 was expressed more in hypertrophied than control muscles and was reduced with resveratrol ($P < 0.05$).

Conclusion: The blunted hypertrophic response in old mice was associated with a lower SC density, but there was no evidence for a lower capacity for proliferation. Resveratrol did not rescue the hypertrophic response and even reduced, rather than increased, the number of SCs in hypertrophied muscles.

1. Introduction

During aging there is a progressive loss of skeletal muscle mass and function (Ballak et al., 2014; Degens and Korhonen, 2012). While resistance exercise is a widely used strategy to counteract the age-related loss in muscle mass and strength (Brown et al., 1990; Frontera et al., 1988; Leenders et al., 2013), the hypertrophic response to resistance training or overload may be blunted in old humans (Grimby et al., 1992; Kosek et al., 2006; Martel et al., 2006; Raue et al., 2009; Slivka et al., 2008) and rodents (Alway et al., 2002a; Blough and Linderman, 2000; Degens and Alway, 2003). It has been postulated that the age-related blunted hypertrophy is largely due to a decrease in the number of functional myofibers (Degens, 2012), but a reduced ability of old myofibers to hypertrophy may also contribute. The latter may be a consequence of a reduced mechanosensitivity of myofibers, a reduced number of satellite cells (SCs) and/or the SC's ability to proliferate and/or differentiate.

Since the activation of the AKT/mTOR pathway during overload was not diminished in muscles from old rodents, it is unlikely that a blunted rate of protein translation is a major cause of the attenuated hypertrophic response in old age (Mayhew et al., 2009). Since mechanical growth factor (MGF) and IL-4 are involved in SC activation (Aline and Sotiropoulos, 2012; Della Gatta et al., 2014), an attenuated loading-induced induction of MGF (Owino et al., 2001) and serum response factor, that controls the expression of IL-4, in old age (Lahoute et al., 2008) may result in impaired activation of SCs. This in turn could attenuate the accretion of myonuclei within the myofiber in aged muscle. SCs are thought to play an important role in the development of hypertrophy, where proliferation and differentiation of SCs prevent an excessive increase in the size of the myonuclear domain, the amount of cytoplasm controlled by a myonucleus (Van der Meer et al., 2011a). The potential importance of the addition of new myonuclei to the hypertrophying myofiber is reflected by the observation that individuals that developed the largest degree of hypertrophy in response

to a training program also had the largest accretion of new myonuclei (Petrella et al., 2006) and the highest number of SCs per myofiber before training (Petrella et al., 2008). This suggests that not only an age-related decline in SC number (Chakkalakal et al., 2012), but also impaired proliferation and/or differentiation may underlie the blunted hypertrophic response in old age. That this may indeed occur is reflected by the blunted increase in myogenin and MyoD protein expression in overloaded *m. plantaris* in old rat (Alway et al., 2002a), indicative for an impaired SC proliferation and differentiation, respectively.

The blunted hypertrophic response in old rats was associated with an increased expression of inhibitors of differentiation (Id) proteins and apoptosis (Alway et al., 2002a). An elevated expression of Id proteins is significant, as overexpression of these proteins leads to myofiber atrophy (Gundersen and Merlie, 1994), possibly by stimulating proliferation and apoptosis (Florio et al., 1998) and inhibition of differentiation of SCs via their inhibitory action on the myogenic regulatory factors, such as MyoD and myogenin (Alway et al., 2003). Furthermore, increased oxidative stress and low-grade inflammation, often observed in aged muscle (Degens, 2010), have been linked to increased expression of Id proteins (Mueller et al., 2002). In addition, TNFα can impair the transcription of MyoD and myogenin (Guttridge et al., 2000) and stimulate their breakdown in the proteasome (Langen et al., 2004) particularly when they are dimerized with Id proteins (Abu Hatoum et al., 1998). This then would hamper the transcription of muscle specific genes, as in fact observed in old rat plantaris muscle (Alway et al., 2002b).

Treatment with an anti-inflammatory anti-oxidant may restore the SC proliferation and differentiation and thereby the hypertrophic response in old age. Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenol with anti-oxidant properties that has been associated with a number of health benefits. Its effect on myoblasts in particular seems to be anti-proliferative and pro-differentiation (Kaminski et al., 2012; Montesano et al., 2013; Saini et al., 2012). This

suggests that resveratrol is a potent supplement to counteract sarcopenia (Marzetti et al., 2011), enhance SC differentiation and restore the hypertrophic response to mechanical overload in old age.

Therefore, the aim of this study was threefold: 1) to test whether the hypertrophic response to a 6-week period of overload by synergist denervation was blunted in old mice compared to that in adult mice; 2) to establish whether the blunted hypertrophy was related to a reduced number of SCs and 3) to assess whether resveratrol supplementation increases the number of SCs and restores the hypertrophic response in old muscles.

2. Methods

2.1. Animals

For this study we used the following groups of male C57BL/6J mice (Janvier, France): adult (9-month-old; n = 11), old (25-month-old; n = 10) and old-resveratrol-treated (old-res; 25-months-old; n =10). In the left leg of the mice, the *m. plantaris* was overloaded to induce hypertrophy, while the right *m. plantaris* served as an internal control. Mice were housed individually under specific-pathogen-free conditions at 20–22 °C and a 12-h light/dark cycle. Animals were given free access to water and chow (Ssniff® S8189-S095, the same as provided at the supplier).

The mice in the old-res group received 0.4% resveratrol (98.57% pure, Polygonum cuspidatum extract; 21st Century Alternative, UK) in the chow. The food intake for these mice is about 3 g per day and given a body mass of ~35 g, the daily intake of resveratrol was approximately 0.4 mg per gram body mass per day. Since the body mass did not change throughout the last six weeks prior to the terminal experiment and no difference was observed between the old and old-res group (P = 0.70), it is likely that the food intake was similar between the old and old-res group. At the age of 7.5 or 23.5 months, the *m. gastrocnemius* and

m. soleus of the left leg were denervated to impose an overload onto the *m. plantaris* for six weeks as described previously (Degens and Alway, 2003), while the non-operated right leg served as internal control.

All experiments were approved by the local animal use and care committee of the VU University Amsterdam and conformed to the Dutch Research Council's guide for care and use of laboratory animals.

2.2. Preparation for in situ muscle function

Six weeks after induction of overload, the terminal experiment was conducted. Fifteen minutes prior to surgery, mice received a subcutaneous injection of 0.06 mL 1% Temgesic (Reckitt Benckiser, UK) as an analgesic and were anesthetized with 4% isoflurane, 0.1 L·min−1 O2 and 0.2 L·min−1 air. After nociceptive responses had ceased, the level of anesthesia was maintained with 1.5–2.5% isoflurane. A humidifier moistened the inhaled air to prevent dehydration due to respiration. The mice were placed on a heated pad to maintain body temperature at ~36.5 °C. In situ force measurements were performed as described previously (Degens and Alway, 2003). The *m. plantaris* was dissected free from surrounding Tissue while maintaining its innervation and blood supply. The sciatic nerve was severed and the proximal end was placed over an electrode to stimulate the muscle. The distal tendon of the *m. plantaris* was dissected free and tightened with a Kevlar thread via a small steel link to a force transducer, which was mounted on the lever arm of an isovelocity measuring system (de Haan et al., 1989). The femur was fixed by a clamp on the condyle of the femur. During the experiment, the muscle and its surrounding were kept at 34–36 °C with a water saturated airflow.

2.3. Experimental setup and force measurements

The order of the experiments was randomized. Contractions were elicited by supramaximal electrical stimulation of the sciatic nerve at a constant current (2 mA; 200 μs pulse width). Optimal length (ℓo) was defined as the muscle length where maximal tetanic isometric force was generated. To set ℓo, first the length at which the muscle produced maximal twitch force was determined. To fine adjust ℓo, tetani (150Hz, 150ms) were applied once every 2 min. To deplete glycogen in the fast type IIB myofibers, the muscle was subsequently stimulated for 5 min with 150-ms trains of 150 Hz once every second. Type IIB myofibers that were not glycogen depleted after this protocol were considered to be denervated myofibers. Force and length signals were digitized (1–10 kHz) and stored on disk. At the end of the experiment, the *m. plantaris* was excised, blotted dry and weighed. Then the mice were killed by cervical dislocation.

2.4. Cryosectioning

The *m. plantaris* was embedded at ℓo in gelatin-tyrode (NaCl, 128.3 mM; KCl, 4.7 mM; MCl2∗6H2O, 1.05 mM; NaH2PO4∗H2O, 0.42 mM; NaHCO3, 20.2 mM; EGTA, 15.0 mM; gelatin 15% (w/v), pH 7.2) solution and frozen in liquid nitrogen. Subsequently, the *m. soleus* and *m. gastrocnemius medialis* were excised from the hind limbs and weighed. Within a month after the contraction protocol, serial cross-sections (10 μm) were cut from the mid-belly of *m. plantaris* in a cryostat at −20 °C. Sections were mounted on glass slides (Menzel-Gläser, Superfrost® plus, GER), air-dried and stored at −80 °C until further use. All chemicals were obtained from Sigma Aldrich (The Netherlands) unless stated otherwise.

2.4.1. Myofiber type composition

Serial sections of the *m. plantaris* were immunohistochemically stained against type I, IIA, IIX and IIB myosin heavy chains to distinguish myofiber types. Thereto, monoclonal antibodies BAD5, SC-71, 6H1 and BF-F3 (Developmental Studies Hybridoma Bank, USA) against types I, IIA, IIX, and IIB, respectively were used. In short, sections were fixed with acetone for 10 min at 4 °C and washed three times for 5 min in phosphate-buffered saline (PBS) plus tween (PBST). After blocking with 10% normal swine serum for 30 min, sections were incubated with primary antibody. Subsequently, sections were washed three times in PBST for 3 min and incubated in the dark with secondary antibody (Alexa 488 anti-mouse, Molecular Probes) for 30 min. After washing with PBST, incubating with wheat germ agglutinin (WGA) (Life Technologies) for 20 min, washing with PBST and subsequently washing once more with PBS all in the dark, sections were enclosed with Vectashield®-hardset mounting medium with DAPI (Vector Laboratories, USA). The public domain software ImageJ 1.45s (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to determine the number of myofibers in a cross-section, the myofiber type proportions and myofiber cross-sectional area. Myofiber outlines were manually traced.

2.4.2. Determination of denervated myofibers by PAS-staining

The glycogen content of individual myofibers was determined in 10-µm thick sections using the periodic acid-Schiff (PAS) staining (van der Laarse et al., 1992). Sections were air-dried and fixed for 5 min in 4% formaldehyde in 20 mL 0.2 M imidazole and 18 mL acetone. Subsequently, sections were oxidized in 44 mM periodic acid solution for 30 min at room temperature. After incubation, sections were briefly washed in 0.1 M HCl and stained with Schiff's reagents for 25 min at room temperature.

2.4.3. Intramuscular connective tissue content

Intramuscular connective tissue was determined using Sirius Red. Briefly, sections were air-dried and subsequently fixed for 30 min in Bouin fixative. Next, the sections were washed for 10 min with water and then incubated for 30 min in Sirius Red solution saturated with picric acid. The sections were dehydrated rapidly in absolute ethanol. For a better image quality, the sections were finally cleared with xylene and mounted with Entellan. A Matlab-script (version R2012a) was used to quantify % connective tissue per picture. The RBG threshold was set at R>140, B<110, and G <110, to create a binary image allowing to filter all red pixels. The same threshold was applied to all images.

Table 4.1 Mice' characteristics.

	Adult mice (n=11)		Old mice (n=10)		Old-res mice (n=10)	
	Con	vs. experimental	Con	vs. experimental	Con	vs. experimental
Body mass (gr)	**36.1±3.0**		**35.6±0.9**		**35.9±1.2**	
Muscle mass (mg)						
M. plantaris	**24.2±0.7**	**32.1±1.2***	**22.4±0.7[a]**	**28.1±1.1***	**23.8±0.8**	**29.8±0.9***
M. soleus	**13.3±0.3**	**8.9±0.4***	**12.2 ±0.4[a]**	**8.8±0.4***	**13.2±1.5**	**9.3±0.7***
M. gastrocnemius	**65.2±1.8**	**26.5±0.6***	**60.1 ±1.7[a]**	**28.1±0.8***	**58.7±2.8**	**28.5±2.0***
Muscle length (mm)	**12.8±0.4**	**12.8±0.4**	**12.9±0.4**	**13.0±0.3**	**12.3±0.3**	**12.7±0.3**
Muscle CSA (mm^2)	**1.83±0.12**	**2.61±0.16***	**2.08±0.10**	**2.48±0.12***	**2.07±0.10**	**2.53±0.17***
Myofiber number (#)	**1027±58**	**1098±67**	**1104±55**	**1299±73**	**1131±66**	**1235±47**
Ratio muscle/body mass ($mg \cdot g^{-1}$)	**0.67±0.04**	**0.89±0.03***	**0.63±0.03[a]**	**0.77±0.05***	**0.67±0.02**	**0.84±0.03***
Connective tissue (%)	**11.6±1.1**	**12.0±1.3**	**16.4±1.8**	**14.1±1.4**	**12.8±1.3**	**11.6±1.7**
Hybrid myofibers (%)	**11.2±2.4**	**27.2±4.7***	**6.4±3.1**	**20.2±3.6***	**8.7±1.9**	**22.8±5.2***
Denervated myofibers (%total)	**1.2±0.4**	**0.8±0.1**	**1.3±0.4**	**1.1±0.3**	**1.3±0.3**	**0.9±0.2**
Denervated myofibers (%IIB)	**2.3±0.7**	**3.8±1.0**	**3.5±1.1**	**2.7±0.6**	**2.7±0.8**	**2.5±0.6**

Values are mean ± SEM. Significantly different compared to control muscle ($P < 0.05$). [a] and with adult muscle $P < 0.05$). [c] For the muscles in the experimental group, the m. plantaris was overloaded. [b] while the m. soleus and m. gastrocnemius were denervated. Note that in the second, but not the first study, the age-related decrease in muscle mass was significant. The apparent discrepancy is related to the fact that in study 2 the overloaded muscles of the old mice were also smaller than those in the adult mice, increasing in the ANOVA the power of the test.*

2.4.4. Quantification of myonuclei and SC number

Sections were co-stained with DAPI and anti-Pax7 antibodies according to van der Meer et al. (2011b). Briefly, sections were fixed in 4% formaldehyde PBS for 10 min, washed with PBST and blocked in 10% normal donkey serum (NDS) in PBS for 30 min. Subsequently, sections were incubated in a 0.1% bovine serum albumin (BSA)–PBS solution containing 4 μg·mL−1 Pax7 antibody (Developmental Studies Hybridoma Bank, USA) in the dark for 60 min, washed in PBST, incubated with Alexa Fluor 488 (1:500, Molecular Probes, Life Technologies) donkey anti-mouse secondary antibody (Invitrogen, Breda, The Netherlands) and washed with PBST. Then the sections were incubated in the dark for 20 min with Texas red-WGA (1:50) conjugate (Invitrogen, Breda, The Netherlands) and washed with PBS. Finally, the sections were enclosed with Vectashield®-hardset mounting medium with DAPI (Vector Laboratories, USA). The images were captured at 10× magnification using a CCD camera (PCOl Sensicam, Kelheim, Germany) connected to a fluorescence microscope (Axiovert 200M; Zeiss, Göttingen, Germany) with image processing software Slidebook 4.1 (Intelligent Image Innovations, Denver, Colorado). The number of myonuclear fragments per myofiber cross-section was counted manually in an average of ~70 myofibers per muscle, while SC fragments per myofiber cross-section were counted throughout the whole muscle.

2.4.5. Western blots

Frozen muscle tissue (~5–10 mg)was homogenized with a Polytron mixer in ice-cold buffer (1:40, w/v) [50 mM Tris–HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 0.1% Triton-X 100 and a complete protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected and immediately stored at −80 °C. The protein concentration was measured using the DC protein assay kid (Bio-Rad laboratories, Nazareth, Belgium). Forty micrograms of proteins was separated by SDS-PAGE (10–12% gels) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5%

non-fat milk for 1 h and afterwards incubated overnight (4 °C) with the following antibodies (1:1000): MyoD, myogenin, Id2 (BD Biosciences, Oxford, UK), caspase-3, caspase-9 and eEF2 (Cell Signaling, Leiden, The Netherlands). Horseradish peroxidase-conjugated anti-mouse (1:10,000) or anti-rabbit (1:5000) secondary antibodies (Sigma-Aldrich, Bornem, Belgium)were used for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genetools and Genesnap software (Syngene, Cambridge, UK), respectively. The results are presented as the ratio protein of interest/eEF2. eEF2 was used as a loading control as preliminary experiments showed that eEF2 expression was stable across the different treatments and conditions.

2.5. Statistics

To test for differences between age and treatment groups we used an univariate ANOVA with factors age and overload, or resveratrol and overload. Effects were considered significant at $P < 0.05$. Data are expressed as means ± standard error of the mean (SEM). All calculations were performed using IBM SPSS version 20.

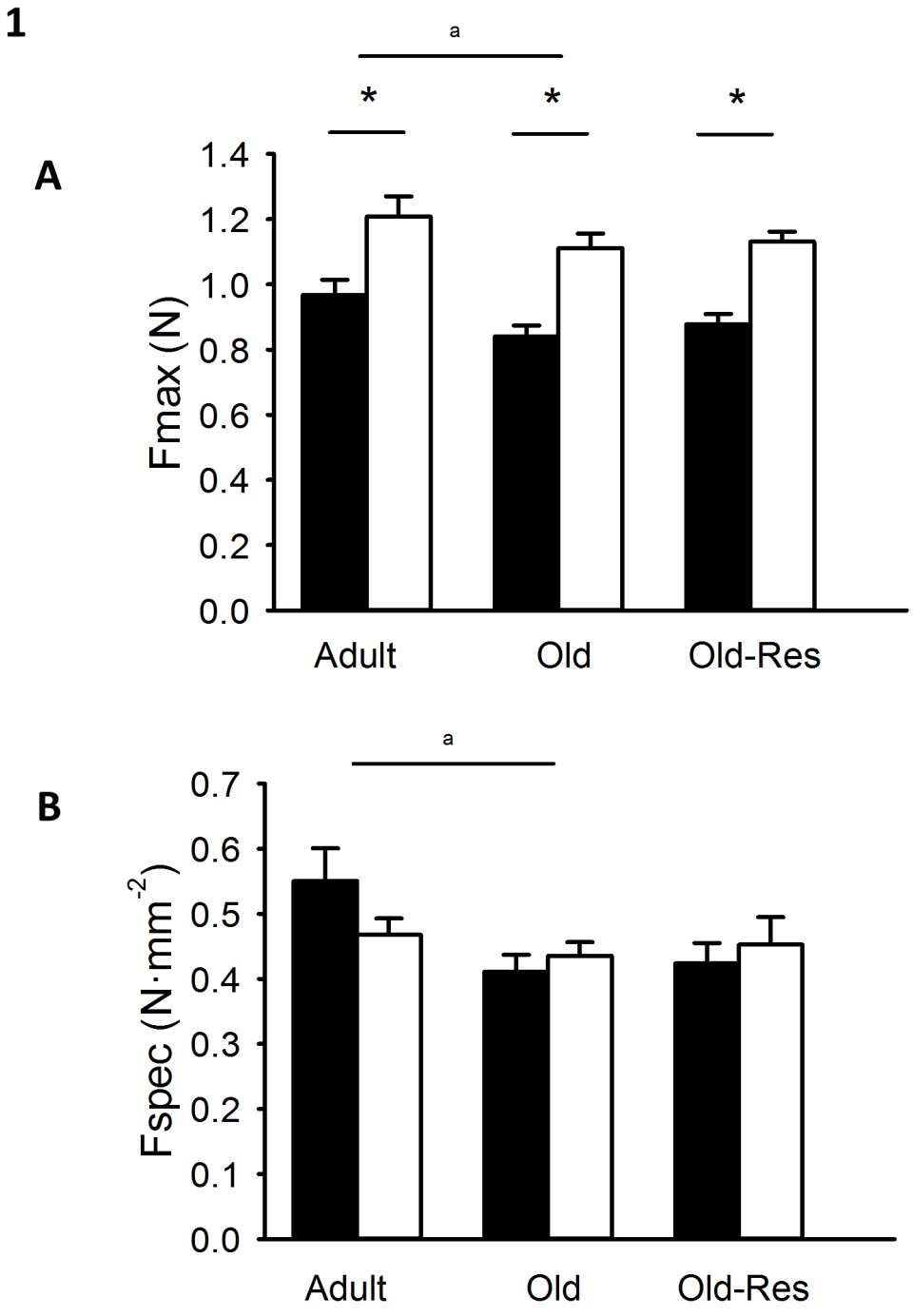


Fig. 4.1. Effects of aging and resveratrol on maximal isometric force. (A) Maximal isometric force (N) of rat m. plantaris muscle during a 150-Hz isometric tetanus and (B) specific tension (N·mm−2) of the m. plantaris of male adult (9-months-old), old (25-months-old) and old-res C57Bl/6 mice. (A) A main age effect was observed for force ($P < 0.05$),while resveratrol had no effect ($P > 0.05$). Force was increased by overload in all groups ($P < 0.001$). (B) Muscle specific force was higher for adult than for old ($P < 0.01$), while resveratrol had no effect on muscle specific force ($P > 0.05$). An Age ∗ Overload interaction ($P < 0.05$) was observed for specific tension, which indicates that a reduction in specific

tension after overload in adult but not in old m. plantaris had occurred. [*]: different from control. [a]: different from adult. Values are mean ± SEM.

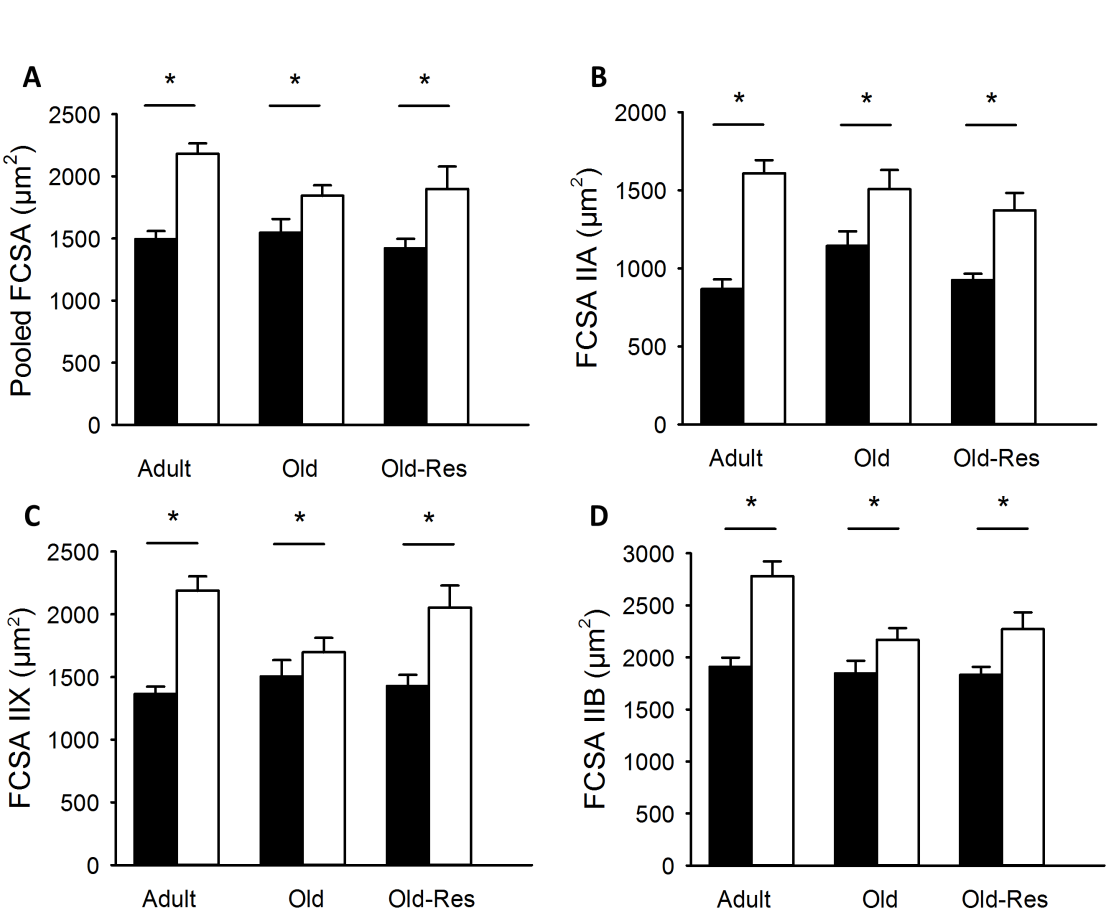


Fig. 4.2. Pooled and individual myofiber CSA for adult, old and old-res m. plantaris of C57Bl/6 mice. (A) Overload increased myofiber CSA (µm2) in all groups ($P < 0.001$), however this increase was higher in adult than in old muscle (interaction effect Age ∗ Overload $P=0.032$). (B) Type IIA, (C) X and (D) B myofibers hypertrophied after overload for all groups, however the magnitude of this increase was higher for adult muscle than old (interaction effect Age ∗ Overload for all three myofiber types $P < 0.05$). [*]: different from control. Values are mean±SEM.

3. Results

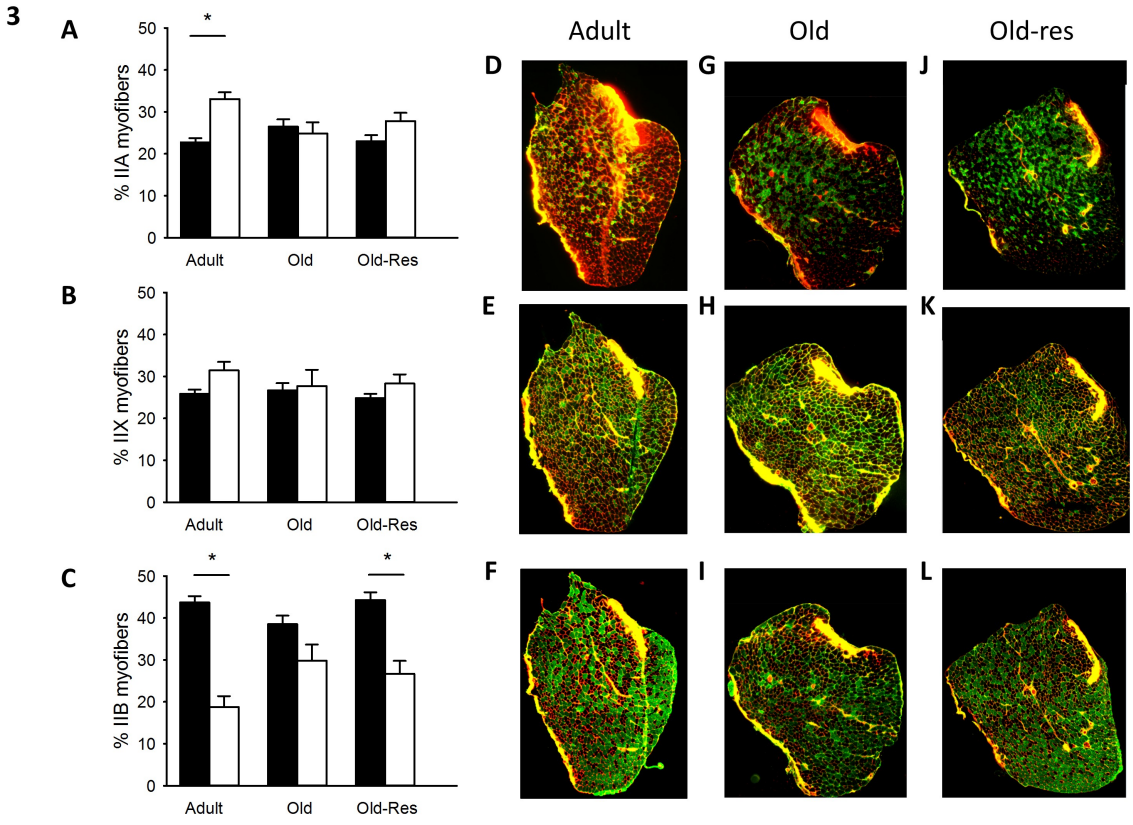
3.1. Mice and muscle characteristics

The data of the control muscles have been published previously (Ballak et al., 2014a), but are included here for comparison with the overloaded muscles and resveratrol-treated mice. Body mass, muscle length, myofiber number, number of denervated myofibers and connective tissue were not significantly affected by age, overload or resveratrol treatment (Table 4.1). In all groups, masses of the denervated gastrocnemius medialis and soleus muscles were significantly smaller than those of the contralateral leg ($P < 0.001$). *M. plantaris* mass was lower in aged mice compared to that in adult mice ($P < 0.01$). The overloaded *m. plantaris* were ~30% heavier than control muscles ($P < 0.001$), irrespective of age or resveratrol treatment. In addition, overload induced an increase in muscle cross-sectional area ($P < 0.001$; Table 4.1).

3.2. Muscle function

Maximal isometric muscle force was 13% lower in muscles from old than adult mice ($P = 0.02$) and was 27% higher in overloaded than in control muscles ($P < 0.001$; Fig. 4.1A). The absence of an interaction between age and overload indicates that the increase in force was similar in adult and old overloaded muscles. Resveratrol had no effect on maximal isometric muscle force. Specific tension was lower in the *m. plantaris* from old than adult mice ($P < 0.01$). The significant interaction between age and overload is reflected by a lower specific tension in overloaded adult muscle ($P= 0.047$), but not old muscles. In both adult and old muscles, the pooled myofiber CSA (FCSA) increased during overload ($P=0.01$),while resveratrol had no significant effect. The increase in pooled FCSA was, however, more in adult than in old *m. plantaris* (Age ∗ Overload interaction; $P = 0.03$; Fig. 4.2A). This Age ∗ Overload interaction was also shown for each individual myofiber type (Age ∗ Overload $P < 0.05$; Figs. 4.2B–D). In addition, in all groups the hypertrophic response was larger for IIA myofibers (56%), than IIX (40%) and IIB (21%) myofibers. Note that the proportion of type I myofibers was so small (<1%) that it was left out of all analyses. The proportion of type IIB myofibers decreased with overload, but less so in old than adult mice (Fig. 4.3; Age ∗ Overload $P=0.013$). This decrease in the proportion of type IIB myofibers was accompanied

by an increase in the proportion of hybrid (type IIA/X and IIX/B) (Table 4.1; $P < 0.001$) and in adult also type IIA myofibers (Fig. 4.3; Age ∗ Overload P=0.002). Resveratrol had no significant impact on myofiber type proportions (Fig. 4.3). Examples of whole muscle myofiber type staining for adult (Figs. 4.3D–F), old (Figs. 4.3G–I) and old-res (Figs. 4.3J–L) are provided.



4.3. *The proportion of type IIA, IIX and IIB myofibers inside the m. plantaris of adult, old and old-res C57Bl/6 mice. (A) No effect of age or resveratrol was observed in the proportion of type IIA myofibers. (B) No effect of age, resveratrol or overload was observed for the proportion of type IIX myofibers. (C) In both adult and old-res mice the proportion of type IIB myofibers decreased after overload ($P < 0.01$), but not for old. Overload increased the proportion of IIA myofibers and decreased the proportion of IIB myofibers more in adult than old muscle (interaction effect Age ∗ Overload $P < 0.01$ and $P < 0.05$, respectively). Examples of immunohistochemical staining for type IIA, IIX and IIB myofibers in adult (Fig. 3.3D–F), old (Fig. 3.3G–I) and old-res (Fig. 3.3J–L) muscles are provided.* : different from control. Values are mean ± SEM.*

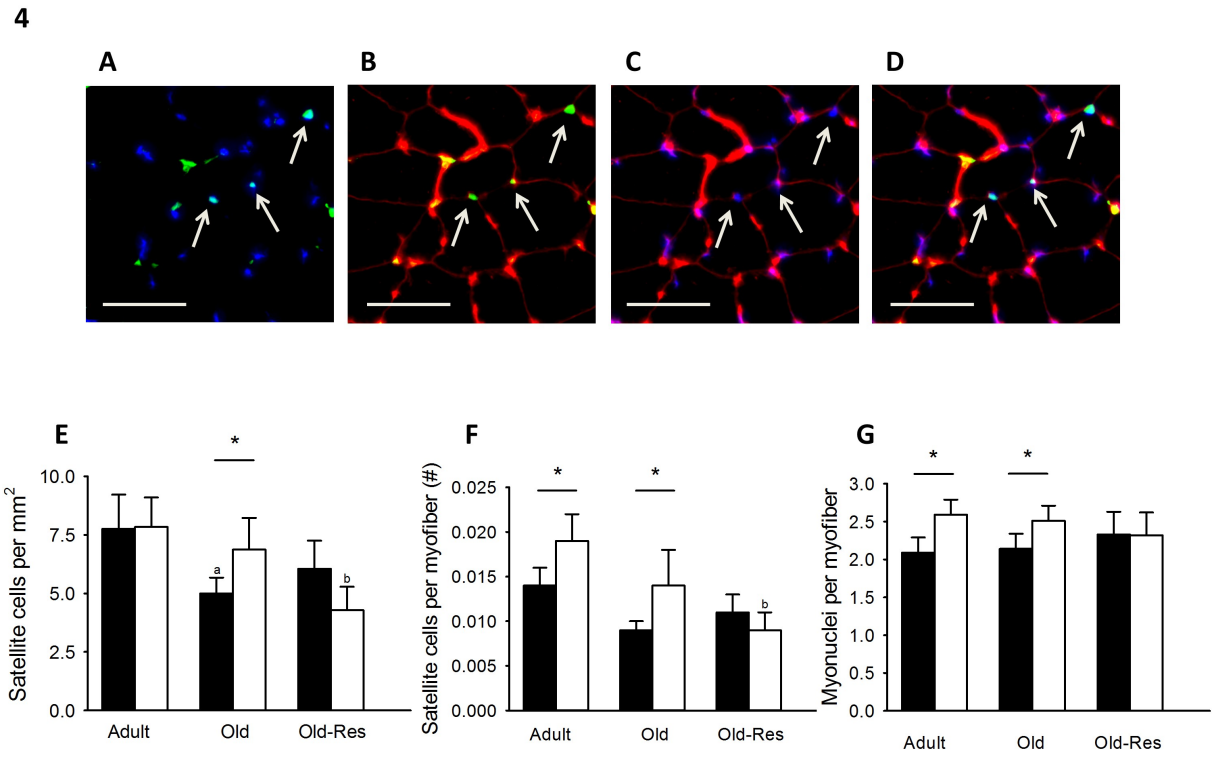


Fig. 4.4. *Effects of aging, overload and resveratrol on SC density. SCs were identified in plantaris muscle cross-sections by co-localization of DAPI and staining of Pax7 of C57Bl/6 mice. Example of a section stained for (A) Pax7 (SC; green) and DAPI (nuclei; blue), (B) Pax7 (green) and WGA (membranes; red), (C) DAPI (blue) and WGA (red) and (D) Pax7 (green), DAPI (blue) and WGA (red) merged. Arrows indicate SCs. Bar represents 50 µm. (E) Number of SC fragments expressed per muscle CSA (mm2) was higher for adult than for old muscle ($P < 0.01$). A significant Age ∗ Overload interaction ($P < 0.05$) is reflected by an increase in the number of SC fragments permm2 for old, but not for adult muscle. Resveratrol decreased the number of SC fragments permm2 after overload (interaction effect Resveratrol ∗ Overload $P < 0.01$). (F) Number of SC fragments expressed per myofiber cross-section tended to be higher in the adult than old group ($P = 0.06$). Overload increased the number of SC fragments per myofiber cross-section in both adult and old, but not in old-res mice (interaction effect Resveratrol ∗ Overload $P > 0.05$). (G) Overload increased the number of myonuclear fragments per myofiber cross-section in adult and old (Fig. 3.4G; $P < 0.01$), but*

not for old-res muscles (P > 0.05). [*]: different from control. [a]: different from adult. [b]: different from old. Values are mean±SEM.

3.3. Effects of age and resveratrol on SC number

The number of SCs per muscle CSA (mm2) or SC density was higher in adult than old muscle (Fig. 4.4E; P = 0.002). The number of SCs per myofiber tended to be higher for adult compared to old muscle (Fig. 4.4F; P = 0.06). Hypertrophy was accompanied by an increase in the number of SCs per myofiber in both adult and old mice (Fig. 4.4F; P < 0.05). However, only in old, but not adult, muscle hypertrophy was accompanied by an increase in SC density (Fig. 4.4E: Age ∗ Overload P = 0.02). In old-res mice, the number of SCs per myofiber (Fig. 4.4F; Resveratrol ∗ Overload P=0.044) and SC density (Resveratrol ∗ Overload P=0.005; Fig. 4.4E) had decreased, rather than increased in the hypertrophied muscles. The number of myonuclei per fiber had increased in overloaded adult and old muscles (Fig. 4.4G; P < 0.01), but not in overloaded old-res muscles.

3.4. Proteins related to SC proliferation and differentiation

MyoD protein expression was not significantly affected by age, overload or resveratrol (Fig. 4.5A). Myogenin and Id2 protein expressions were higher in old compared to adult muscle, but did not significantly change with hypertrophy or resveratrol treatment (P < 0.05; Figs. 4.5B & C). In all groups, overload was accompanied by an increased expression of total caspase-3 (P < 0.05; Fig. 4.5D), while resveratrol induced a decrease in the expression of caspase-3 expression (Fig. 4.5D; P < 0.05). No cleaved caspase-3 was detected. There were no significant effects of age, overload or resveratrol on caspase-9 expression in the *m. plantaris* (Fig. 4.5E; P > 0.05).

5

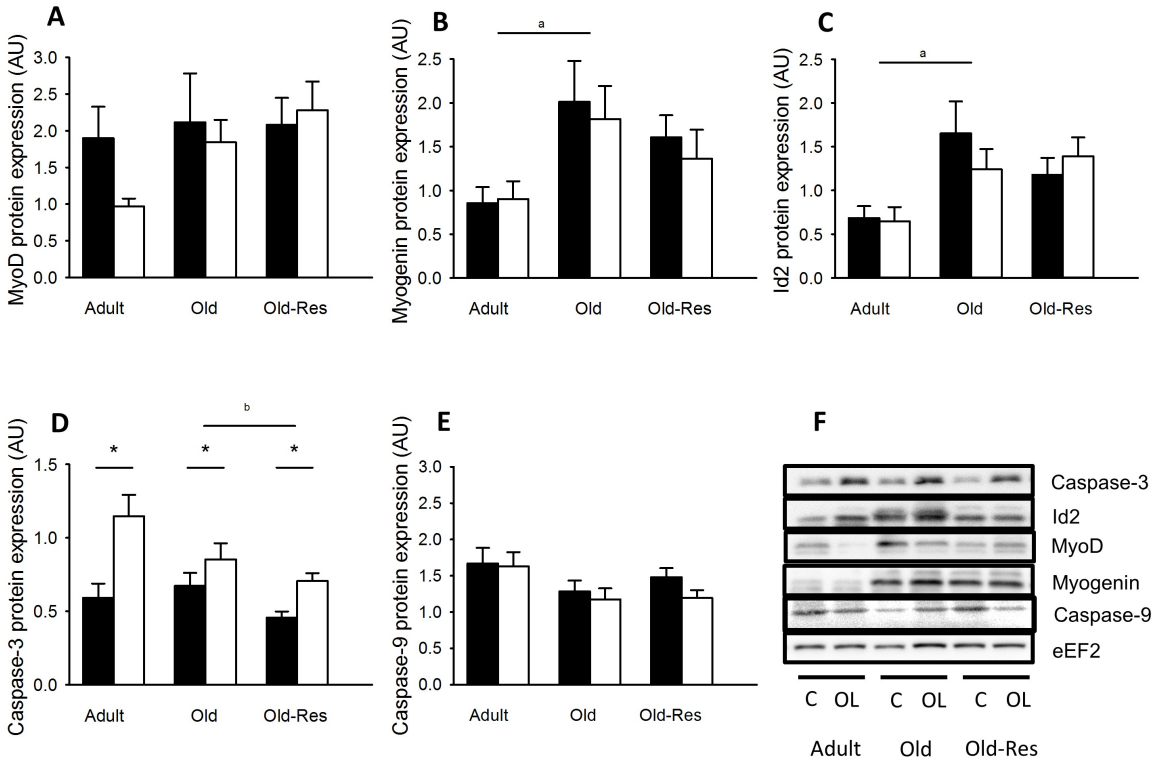


Fig. 4.*5. Protein content in m. plantaris for MyoD, myogenin, Id2 and caspase-3. (A) MyoD protein expression was not affected by age or overload (P > 0.05). (B) Myogenin and (C) Id2 protein content were higher in old muscle than in adult muscle (P < 0.01), but unaffected by resveratrol (P > 0.05). (D) Caspase-3 protein content was increased by overload in all groups (P < 0.05) and decreased by resveratrol (P < 0.05). (E) No age-, overload- or resveratrol-induced changes were observed for caspase-9 expression. (F) Western blot examples for MyoD, myogenin Id2, caspase-3, caspase-9 and eEF2 protein expression.* [*]*: different from control.* [b]*: different from old. Values are mean ± SEM.*

4. Discussion

The main finding of this study is that the *m. plantaris* of 25-month-old male C57BL/6J mice showed a blunted hypertrophic response to a 6-week overload stimulus. In addition, the type IIB to IIA myofiber type transition during hypertrophy was less pronounced in the *m. plantaris* of old than of adult mice. The blunted hypertrophic response was not attributable to

a lower proliferative capacity of SCs in old mice, but may rather be a consequence of the lower SC density in old than in adult muscles. Resveratrol may have decreased proliferation and/or differentiation, as reflected by a lack of increase in response to overload in SC density and number of myonuclei per myofiber in old-res mice. In contrast to our hypothesis, in old muscle resveratrol did not rescue the attenuated hypertrophy to overload.

4.1. The hypertrophic response in adult and old mice

In this study overload was induced in the left *m. plantaris* of 7.5- and 23.5-month-old male mice by denervation of its synergists. This technique has previously been shown to be an effective way to induce hypertrophy in rodents, with less inflammation than in, for instance, overload induced by ablation of synergistic muscles (Lowe and Alway, 2002). Here we show that the anatomical CSA of the *m. plantaris* increased by only 20% in the old, in contrast to the 40% increase in anatomical CSA in adult mice, while fiber numbers were similar in the plantaris muscles in adult and old mice. The blunted hypertrophy in old mice was thus not attributable to a lower number of myofibers, but rather to blunted hypertrophy of the existing myofibers, similar to what has been observed in older people (Kosek et al., 2006). Interestingly, the hypertrophic response in IIA myofibers exceeded that of IIX and IIB myofibers in both adult and old animals. This is in agreement with a report, studying overload in 4-month-old female Wistar rats (Degens et al., 1995b). In addition, overload decreased the proportion of type IIB myofibers, however more so in adult than in old muscle. Thus, not only the development of myofiber hypertrophy, but also the transition of myofiber types during hypertrophy is partly lost in 25-month-old compared to adult mice.

4.2. The effect of mechanical overload on muscle force generating capacity in adult and old mice

Resistance exercise in both adult and old humans is associated with an increase in maximal force that is proportionally larger than the increase in muscle mass (Degens et al., 2009; Erskine et al., 2011; Jones et al., 1989; Morse et al., 2005). Here we did not find such

an increase in specific tension with overload in neither adult nor old mouse muscles, similar to what has been reported before in rats (Degens et al., 1995a; Roy et al., 1982). It remains to be seen what causes this discrepancy, but part of the apparent increase in specific tension in human muscles is attributable to improved maximal voluntary activation (Erskine et al., 2011; Morse et al., 2005), which does not play a role in the maximally stimulated isolated muscle preparation in mice (Degens, 2012).

4.3. SC number during overload in adult and old mice

Many studies have shown that overload-induced hypertrophy is accompanied by the acquisition of new myonuclei, requiring proliferation and differentiation of SCs (Allen et al., 1999; Van der Meer et al., 2011a). An explanation for a blunted myofiber hypertrophy in aged muscle may thus be a lower number, a decreased mechanosensitivity, impaired proliferation and/or differentiation of SCs. Here we found that the SC density was lower in old than adult muscle, which is in accordance with others (Day et al., 2010). Despite the age-related reduction in SC density, overload did induce SC proliferation not only in adult, but also in old muscles. Similarly in old human muscle SC proliferation and hypertrophy have been observed in elderly men after resistance training (Verdijk et al., 2009). In our study, the proliferation was such that the SC density remained constant in adult, while it was even increased in old hypertrophied muscles. The observation that the SC density in old hypertrophied muscles was even elevated above that of the age matched control muscles indicates that SC proliferation was likely not the limiting factor for the development of hypertrophy in the old muscles. Rather, it suggests that the lower absolute number of SCs and/or impaired SC differentiation and SC apoptosis contributed to the impaired hypertrophic response in aged muscle.

4.4. Expression of proteins related to SC proliferation and differentiation

An elevated expression of Id proteins is a crucial step for SC proliferation (Lluís et al., 2006), but expression beyond a certain threshold, depending on cell environment, may cause

apoptosis rather than proliferation (Yokota and Mori, 2002). Even though the number of SCs was lower in old than adult muscle, the expression of Id2 proteins was elevated. This has also been observed in muscles from old rats and in that case elevated Id2 expression levels were associated with apoptosis and blunted hypertrophy (Alway et al., 2002b). Here, however, we did not find evidence of increased apoptosis in old muscles, as reflected by similar caspase-3 expression in adult and old muscles. Therefore, it is possible that the increase in myogenin served to stimulate differentiation to compensate for an impaired function of SCs in the old muscles. Interestingly, it has been observed previously that the most atrophied muscles in old rats exhibited the largest increase in MRF and IGF-I mRNAs, suggesting no problem with the regenerative drive (Edstrom and Ulfhake, 2005), which corresponds with our data.

In contrast to previous observations in overloaded rat muscles (Alway et al., 2002a), we did not observe an overload-induced increase in MyoD and myogenin in the adult mice. It should be noted, however, that in that study (Alway et al., 2002a) the muscles were overloaded only for 2 weeks, the period of muscle growth, while we studied them when they had reached a new steady state at 6 weeks after induction of overload (Degens et al., 1995b).

In contrast to previous studies in humans (Whitman et al., 2005) and rodents (Pistilli et al., 2006; Phillips & Leeuwenburgh, 2005) we did not find evidence for increased apoptosis in muscles of the old mice, as reflected by similar caspase-3 expressions in adult and old mice. Nevertheless, in both adult and old muscles caspase-3 was significantly elevated during hypertrophy. This may reflect previous and repeated activation of the pathway. In addition, overload induced a considerable IIB–IIA myofiber type transition and a concomitant increase in hybrid myofibers in the absence of a change in myofiber number. This implies that in individual myofibers type IIB myosin must be broken down to be replaced by type IIA or IIX myosin. While the ubiquitin proteasome pathway plays an important role in the breakdown of muscle proteins, it is thought that caspase-3 is involved in the breakdown of large myosin molecules (Powers et al., 2007) and first cleaves the myosin molecule in smaller fragments that can subsequently be broken down in the proteasome (Du et al., 2004). It is thus possible that the increase in caspase-3 in hypertrophied muscles is a prerequisite for the breakdown of

type IIB myosin (in this case) and hence the observed myofiber type transition during hypertrophy.

4.5. Resveratrol decreases the SC density after overload

Oxidative stress and systemic inflammation are factors that contribute to the age-related muscle wasting and weakness (Degens, 2010). Resveratrol has been shown to prevent TNF-α induced muscle atrophy (Wang et al., 2014) and to alleviate oxidative stress also in old mice (Jackson et al., 2011). Therefore, we investigated whether resveratrol attenuates the age-related muscle wasting and normalizes muscle hypertrophy in old mice. In the present study, muscle mass, force and specific force were not affected by resveratrol. Furthermore, the hypertrophic response was not rescued by 0.4% resveratrol supplementation in the old mice. This is in accordance with others, who found that 0.05% resveratrol supplementation was not sufficient to attenuate the age-related muscle wasting even though it alleviated oxidative stress (Jackson et al., 2011). It thus appears that resveratrol does not have beneficial effects on muscle mass or quality in 25-month-old mice at a dose of 0.4%.

Resveratrol may have decreased proliferation and/or differentiation, as reflected by a lack of increase in response to overload in SC density and number of myonuclei per myofiber in old-res mice. In contrast to our hypothesis, in old muscle resveratrol did not rescue the attenuated hypertrophic response to overload. Note, however, that despite the lack of increase in myonuclear number in old-res muscle, the hypertrophic response was similar to that in the untreated old muscle. The preserved hypertrophy suggests that the increase in myonuclei was not required to induce a ~20% hypertrophy by overload, which is in line with the observation that even in SC depleted muscles myofiber hypertrophy was not attenuated (McCarthy et al., 2011). Although resveratrol has been reported to stimulate myoblast differentiation (Kaminski et al., 2012; Montesano et al., 2013) and to block the proliferation (Montesano et al., 2013) we did not find an altered expression of Id2, MyoD and myogenin, important regulators of SC proliferation and differentiation. Resveratrol did, however, decrease total caspase-3 protein content, which is in accordance with others where resveratrol decreased mitochondrial caspase-dependent apoptotic signaling pathways (Bennett et al., 2013; Marzetti et al., 2011).

However, the total caspase-9 protein content (mitochondrial dependent apoptotic pathway) was not affected by resveratrol. Therefore, in 25-month-old mice, resveratrol seems to block proliferation and subsequent differentiation of SCs after overload independent of MyoD or myogenin.

5. Conclusion

In conclusion, the *m. plantaris* of 25-month-old male mice showed a blunted hypertrophic response, in terms of an increase in myofiber size and myofiber type transition compared to the *m. plantaris* from 9-month-old mice. The blunted myofiber hypertrophy could partly be explained by an age-related decrease in SC density. Resveratrol did not alleviate the age-related decrease in muscle force, specific tension or mass. Nor did it restore the ability to develop hypertrophy to levels observed in adult mice. On the contrary, resveratrol blunted the SC response to overload in 25-month-old mice, possibly caused by a diminished proliferation and differentiation of SCs.

Acknowledgments

The authors would like to thank Tinelies Busé-Pot, Guus Baan and Glenn Ferris for the excellent technical assistance. Furthermore, we would like to acknowledge Peter Meijer, Evelien Bos, Frank van't Hoff and Joshua Dunnink for their support. This research was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate program (2011–0015). The authors are grateful for 21st Century Alternative, UK, for providing the resveratrol for this study.

Chapter 5

Validation of a new semi-automated technique to evaluate muscle capillarisation

This chapter is accepted as:
Sam B. Ballak, Moi H. Yap, Peter J. Harding, Hans Degens. Validation of a new semi-automated technique to evaluate muscle capillarisation. *Adv Exp Med Bio.*

Abstract

The method of capillary domains has often been used to study capillarization of skeletal and heart muscle. However, the conventional data processing method using a digitizing tablet is an arduous and time-consuming task. Here we compare a new semi-automated capillary domain data collection and analysis in muscle tissue with the standard capillary domain method. The capillary density (1481±59 vs. 1447±54 caps·mm^{-2}; R^2:0.99; P<0.01) and heterogeneity of capillary spacing (0.085±0.002 vs. 0.085±0.002; R^2:0.95; P<0.01) were similar in both methods. The fiber cross-sectional area correlated well between the methods (R^2:0.84; P<0.01) and did not differ significantly (~8% larger in the old than new method at P=0.08). The latter was likely due to differences in outlining the contours between the two methods. In conclusion, the semi-automated method gives quantitatively and qualitatively similar data as the conventional method and saves a considerable amount of time.

1 Introduction

An adequate blood supply to the muscle is not only important for delivery of oxygen to the working muscle, but also for the removal of metabolites and heat. This exchange between blood and muscle fibers takes place in the capillaries and an adequate muscle capillarization is thus crucial for muscle function. The capillary supply to a fiber is determined by the fiber its size, type, mitochondrial content and metabolic activity of surrounding fibers (Ahmed et al., 1997; Degens et al., 1992; Wust et al., 2009a). During hypoxia (Wust et al., 2009b) and hypertrophy (Degens et al., 1992) capillary proliferation ensures adequate muscle oxygenation.

The method of capillary domains has been used to study the capillarization in skeletal (Degens et al., 1992; Wust et al., 2009a) and heart muscle (Hoofd et al., 1985). The strengths of the method are that it not only provides measures of overall capillary supply, such as the capillary density (CD in caps·mm^{-2}) and capillary to fiber ratio, but also the capillary supply to individual fibers. It is also unique in that it gives an indication of the heterogeneity of capillary spacing, which can have a significant impact on muscle oxygenation (Al-Shammari et al., 2012; Degens et al., 2006; Degens et al., 1994; Liu et al., 2012), and is an accurate method to estimate the oxygen supply areas of individual capillaries and is an indirect indicator of tissue oxygenation (Al-Shammari et al., 2014). The data obtained with the method of capillary domain can be fed into models of tissue oxygenation (Al-Shammari et al., 2012; Degens et al., 1994; Wust et al., 2009b). The drawback of the method is, however, that data collection is a manual and time-consuming process. First, pictures have to be printed, fibers and capillaries manually traced on paper and then traced again on a digitizer. The coordinates of the capillaries and fiber outlines are then processed and analyzed with AnaTis (BaLoH Software, www.baloh.nl) (Hoofd and Degens, 2013).

Automation would significantly reduce the data processing time, potentially reduce human errors and improve the accuracy of the data processing. A semi-automated method would also provide the possibility to expand the analysis by introducing new parameters.

Therefore, the aim of this study was to compare the conventional method with a new semi-automated Matlab® based software.

2 Methods

2.1 Immunohistochemistry

The left *m. plantaris* of ten 9-month-old C57BL/6j mice were excised, frozen in liquid nitrogen at optimal length and stored at -80°C for further analysis. Sections of 10 µm were cut in a cryostat at -20°C and stained with biotinylated lectin (*Griffonia simplicifolia*) to identify capillaries as described previously (Wust et al., 2009a).

2.2 Analysis of capillarization

The capillarization was analyzed first as described previously (Degens et al., 1992; Wust et al., 2009a). In short, the coordinates of the capillaries and the outlines of the muscle fibers were manually delineated with a digitizing tablet (Summagraphics MM1201) and the data fed into AnaTis (BaLoH Software, www.baloh.nl) to calculate capillary domains. Domains are areas surrounding a capillary delineated from surrounding capillaries by equidistant boundaries (Hoofd et al., 1985). It also gives an index of the heterogeneity of capillary spacing as the logarithmic standard deviation of the domain radii (Log_RSD) and overall indices of capillarization, such as capillary density (CD; cap·mm^{-2}). In addition, the program calculates the fiber cross-sectional areas (FCSA) and provides indices of the capillary supply to individual fibers: the local capillary to fiber ratio (LCFR). It is calculated as the sum of the fractions of the capillary domains overlapping a given fiber. The capillary fiber density (CFD; cap·mm^{-2} of a given fiber) is the LCFR divided by the FCSA of that fiber. We developed a semi-automated program for capillary domain analysis, as originally described by Hoofd et al. (Hoofd et al., 1985) based on Voronoi tessellations. Two annotation tools were developed using Matlab® libraries. The first annotation tool was used to select the capillary and border coordinates. The second annotation tool delineated the fiber outlines.

Subsequently, a data analysis function was implemented to calculate the capillary domain (Fig. 5.1). Capillary domains or fiber outlines crossing the border were considered border domains or fibers. The domain size, CD, FCSA, LCFR and CFD were calculated with custom Matlab® functions. All statistics and metrics were compiled from these data.

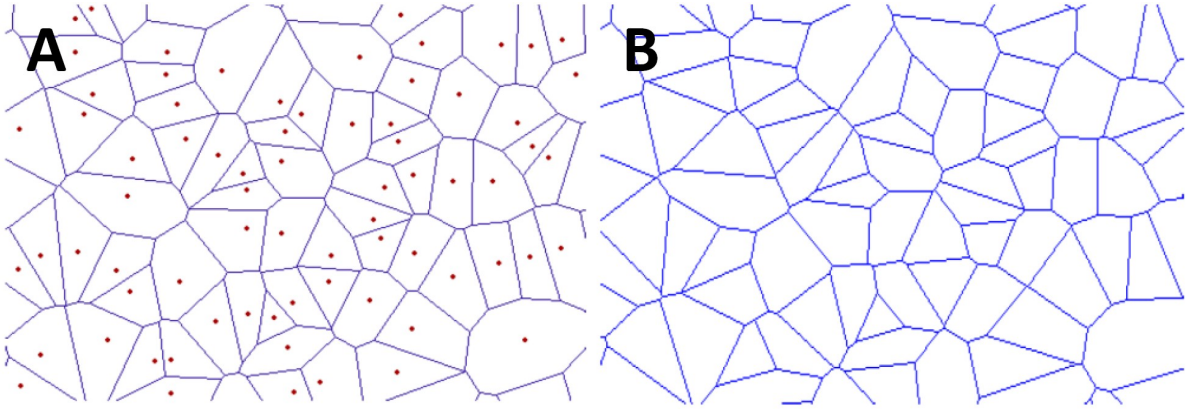


Fig 5.1 Calculated capillary domains using Voronoi tessellations in the conventional (A) and new (B) method.

2.3 Statistical analysis

The data of the two methods were compared with a paired Student's *t* test and correlations (R^2). Data are presented as mean±standard error of the mean (SEM). A value of $P < 0.05$ was considered significant.

3 Results

Figure 4.2A shows a comparison of the CD obtained with the conventional and new method. The CD in the conventional and the new method (1481±59 vs. 1447±54 cap·mm^{-2}) are highly correlated (R^2:0.99, P<0.01). The same applied to the capillary domain areas (R^2:0.97, P<0.01; Fig. 5.2B). Figure 5.2C shows the heterogeneity of capillary spacing (Log_RSD) for the conventional and the new method (0.085±0.002 vs. 0.085±0.002). The Log_RSD for both methods are strongly correlated (R^2:0.95, P<0.01). The correlation for the FCSA between the two methods is strong (R^2:0.87, P<0.01; Fig. 4.2D) but the new method

gives consistently, but not significantly lower FCSAs than the old method (1693±66 vs. 1531±55 µm^2; *P*=0.08).

Fig 5.2 The correlation between the old and new method for CD (cap·mm^{-2}) (R^2=0.99; P<0.01) (A), domain area (µm^2) (R^2=0.97; P<0.01) (B), Log_RSD (R^2=0.95; P<0.01) (C) and FCSA (µm^2) (R^2=0.87; P<0.01) (D) compared between the conventional (x-axis) and new method (y-axis). The line represents the line of identity.

Table 5.1 shows that the percentage of connective tissue (%CT) did correlate between the two methods (R^2:0.43, *P*=0.01). Although the LCFR and the CFD correlated strongly (R^2:0.80 and 0.86; *P*<0.01), the new method gave consistently lower values for both LCFR and CFD than the old method (*P*<0.01 and *P*=0.01).

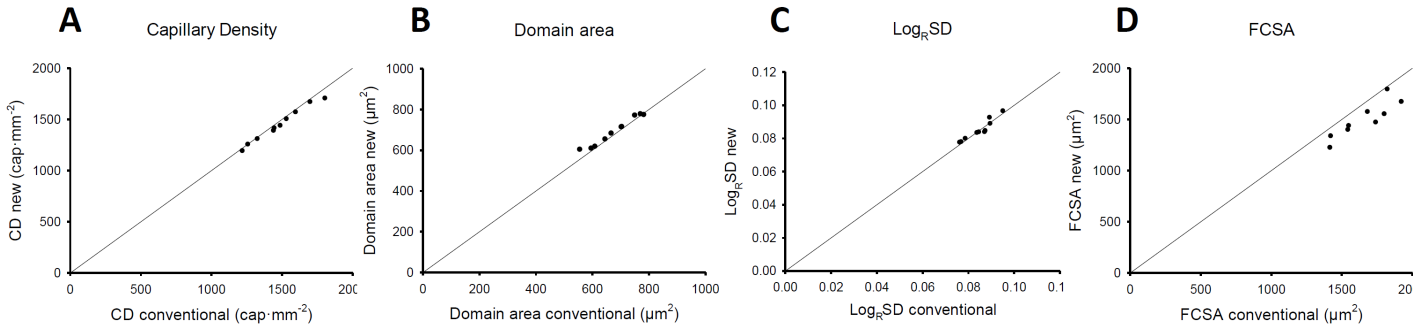


Table 5.1 Correlations and P-values of indices of capillary supply

	Conventional method	New method	R^2	P-value
%CT (%)	11.5±0.6	16.7±1.4	0.43	0.01
LCFR	2.503±0.076	1.998±0.070	0.80	0.00
CFD (mm^{-2})	1564±56	1252±46	0.86	0.00

Correlations and P-values of indices of capillary supply to individual fibers (LCFR: local capillary to fiber ratio; CFD: capillary fiber density) and % connective tissue (%CT) between the two methods in m. plantaris of 9-month-old male C57Bl/6j mice.

4 Discussion

The main finding of this study is that a newly developed Matlab® based semi-automatic method of capillary domains provides a quantitatively and qualitatively similar outcome for parameters of overall capillary supply as the traditional manual method.

Although the FCSA is underestimated in the new method, affecting quantitatively also indices of the capillary supply to individual fibers, this is a systematic underestimate. Therefore, the qualitative outcome is similar for the two methods and the newly developed method can be readily used for comparative studies on changes in muscle or cardiac capillary supply.

The CD and $Log_R SD$ obtained by the two methods were virtually identical. This indicates that the coordinates recorded for each capillary were comparable between the methods. It also indicates that the calculation of the capillary domains is comparable between the two methods, since for the calculation of the $Log_R SD$ the surface areas of the individual domains and the radius of circles with corresponding surface areas have to be calculated.

There was, however, a small difference in the way the fiber outlines were traced; the FCSA was systematically underestimated in the new method. In the conventional method, accidental fiber overlap could be accounted for, while in the Matlab® based software fibers overlapping each other are joined and assessed as one large fiber. Even with good tracing skills, a researcher may avoid tracking the borders too close to each other to prevent this fusion of two separate fibers; in other words, the experimenter may intentionally draw the fibers slightly too small, resulting in a smaller fiber area. On the other hand, the conventional method could lead to a small overestimation of the size of the fibers since overlapping areas will be counted twice. Together, this stresses the importance of good tracing skills in both the new and the old method.

The difference in fiber size, caused by the difference in tracing the outlines of the fibers, also has an impact on the % CT, which was significantly higher in the new method. The differences in fiber size also work through in the LCFR and CFD, which were lower in the new than in the old method, even though the correlation between the two methods was strong. Thus, while there is a systematic qualitative difference between the two methods, the new method is readily applicable in comparative studies.

We are currently working on the tracing algorithm to adjust the systematic underestimation of the FCSA in the Matlab® based software. The main aim of this study was to evaluate the validity of this technique before looking into that issue.

It is clear that the method of capillary domains has limitations, as it does not consider the 3-dimensional structure of the capillary network, differences in flow between capillaries or decrements in oxygen content of the blood from the arteriolar to the venous side of the capillary. Other investigators have tried to address this (Beard and Bassingthwaighte, 2000; Weerappuli and Popel, 1989), but this was outside the scope of the present study. Nevertheless, the method of capillary domains has successfully been used to calculate the domain size in venous and arterial capillaries (Suzuki et al., 1997) and using assumptions of the decrement in capillary oxygen tension from the arteriolar to venular side of the capillary, estimates can be made of the oxygenation in successive planes of tissue (Hoofd, 1995). In addition, in a mathematical model also the impact of flow heterogeneity on tissue oxygenation can be incorporated (Hoofd and Degens, 2009) illustrating the usefulness of the collected data in the 2 dimensional plane to get an estimate of tissue oxygenation under different conditions.

The new Matlab® based program saves on average about 1.5 hour per section/photo. This will increase even more when additional analyses are automated further. In addition, the Matlab® based program makes analysis more flexible and allows the implementation of new variables in the future. This new time-sparing method is the first step in fully automating the process and assessment of capillary domains. Future research should focus on fully automating the sampling of capillary and fiber outline coordinates.

5 Conclusion

In conclusion, the new semi-automated Matlab® based method is highly comparable to the standard method of capillary domain analysis when considering indices of overall muscle capillarization. However, due to differences in the way of tracing the fiber outlines there are small difference in FCSA, which also affect indices of capillary supply to individual fibers. Together, this new method appears to be a valid way to qualitatively and quantitatively analyze the capillarization in cardiac and skeletal muscle.

Acknowledgments

This research was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate program (2011-2015). The Matlab codes are available from the authors on request.

Chapter 6

Blunted angiogenesis and hypertrophy are associated with increased fatigue resistance and unchanged aerobic capacity in old overloaded mouse muscle

This chapter is submitted:
Sam B. Ballak, Richard T. Jaspers, Tinelies Busé-Pot, Peter J. Harding, Moi H. Yap, Louise Deldicque, Arnold de Haan and Hans Degens.

Abstract

Background: We hypothesise that the attenuated hypertrophic response in old mouse muscle 1. is partly due to a reduced capillarisation and angiogenesis, which is 2. accompanied with a reduced oxidative capacity and fatigue resistance in old control and overloaded muscles, that 3. can be rescued by the anti-inflammatory anti-oxidant resveratrol.

Methods: To investigate this, the hypertrophic response, capillarisation, oxidative capacity and fatigue resistance of *m. plantaris* were compared in 9- and 25-month-old non-treated and 25-month-old resveratrol-treated mice.

Results: Capillarisation was not significantly affected by age. Overload increased the local capillary to fibre ratio more in adult than old muscle ($P<0.05$), indicating attenuated angiogenesis in old muscles. Although muscles of old mice had a higher succinate dehydrogenase (SDH) activity ($P<0.05$) and a slower fibre type profile ($P<0.05$), the isometric fatigue resistance was similar in 9- and 25-month-old mice. In both age groups, the fatigue resistance was increased to the same extent after overload ($P<0.01$), without a significant change in SDH activity, but an increased capillary density. Interestingly, integrated SDH activity for a given fibre size was higher in overloaded than control muscles. Resveratrol supplementation had no significant effect on hypertrophy, capillarisation, or fatigue resistance.

Conclusion: Attenuated angiogenesis during overload may contribute to the attenuated hypertrophic response in old age. Neither was rescued by resveratrol supplementation. Changes in fatigue resistance with overload and ageing were dissociated from changes in SDH activity, but paralleled those in capillarisation. This suggests that capillarisation plays a more important role in fatigue resistance than oxidative capacity.

1. Introduction

Ageing is accompanied by a progressive decline in muscle mass (sarcopenia) and power (Roseberg, 1989). These changes not only result in a decreased peak performance, but also in a reduced maximal sustainable power of the muscles as a larger proportion of the available muscle mass has to be recruited for a given task. The latter can be aggravated by a reduced aerobic capacity to generate ATP. Together, these changes will ultimately limit the ability to perform daily life activities and reduce the quality of life (Maden-Wilkinson et al., 2014a; Paterson et al., 2007).

During ageing, maximal whole-body oxygen consumption (VO_{2max}) is reduced (Fleg and Lakatta, 1988), even when corrected for lean leg muscle mass (Fitts et al., 1984), indeed suggesting that the oxidative metabolism is impaired in old muscle. Such a situation can occur due to a lower oxidative enzyme activity and/or a diminished muscle capillarisation, which both have been observed in aged humans (Konopka et al., 2014) (Coggan et al., 1992; Degens, 1998; Hepple et al., 1997) and rats (Degens et al., 1993b; Skorjanc et al., 2001).

It is equivocal whether muscle fatigue resistance decreases with age. Fatigue resistance has been reported to be increased, unaltered or decreased in old age, depending on the protocol used to induce fatigue (Avin and Law, 2011; Callahan and Kent-Braun, 2011; McPhee et al., 2014). Fatigue elicited by intermittent isometric contractions is related to the oxidative capacity of single muscle fibres and motor units (Degens and Veerkamp, 1994). Since aerobic metabolism requires an adequate supply of oxygen via the capillaries, it is likely that fatigue induced by such protocols is also related to the capillarisation of the muscle (Hudlicka et al., 1977). Thus major determinants of the fatigue resistance are, besides muscle mass (Fleg and Lakatta, 1988), the oxidative capacity (Layec et al., 2013; Marzetti et al., 2013) and capillarisation. But how changes in aerobic capacity and capillarisation affect intermittent isometric fatigue resistance during ageing and overload are not well understood.

For a muscle fibre to make use of its oxidative capacity, an adequate capillary network is required to supply the muscle fibre with oxygen. In human *m. vastus lateralis* the number of capillaries per fibre has been reported to be strongly correlated with the oxidative capacity of the fibre (Bekedam et al., 2003). The higher capillary density in oxidative than in more glycolytic regions of a given muscle illustrates that this relationship also applies to capillary supply of a muscle region (Degens et al., 1992). Several studies indicate that the capillary supply to a muscle fibre is positively related to cross-sectional area in both control (Ahmed et al., 1997; Wust et al., 2009a) and overloaded, hypertrophic muscle (Degens et al., 1994). This relationship is tightly regulated as reflected by the similar time course of fibre hypertrophy and capillary proliferation in a rat model of compensatory hypertrophy (Egginton et al., 2011; Plyley et al., 1998). This suggest that a reduction in muscle capillarisation during ageing, which would increase the diffusion distance for oxygen and limit the oxygenation of the muscle (Degens, 2012; van der Laarse et al., 2005), may partly be responsible for the age-related impaired hypertrophic response in aged muscle (Ballak et al., 2015; Degens and Alway, 2003).

Muscle fibre size and oxidative metabolism are inversely related (van der Laarse et al., 1998; van Wessel et al., 2010), which suggests that muscle fibre size and oxidative metabolism are under coordinated control. The coordinated control would be reflected according to this relationship by a decrease in aerobic capacity during muscle (fibre) hypertrophy. However, how this relationship is affected by ageing and hypertrophy is unknown.

If a reduced angiogenic response and impaired mitochondrial function contribute to the attenuated muscle function and hypertrophic response, agents that improve angiogenesis and mitochondrial function may rescue muscle function and the hypertrophic response in old age. Resveratrol (3,5,4’-trihydroxystilbene), a polyphenol with anti-inflammatory properties

(Jackson et al., 2011; Ryan et al., 2010) may be such an agent. Resveratrol has been shown to enhance expression of PGC-1α, improve mitochondrial function in mouse muscle (Jackson et al., 2011) and to increase expression of the mitogenic vascular endothelial growth factor (VEGF) and its receptor Flk-1 (Fukuda et al., 2006), even in old rodents (Leick et al., 2009; Murase et al., 2009). These properties of resveratrol may rescue the hypertrophic response in old muscle.

The objectives of this study were to assess (1) whether the blunted muscle fibre hypertrophy is associated with attenuated angiogenesis, (2) how muscle fatigue resistance changes with age and overload and how this is related to changes in mitochondrial content and/or capillarisation and (3) whether resveratrol improved the oxidative capacity and capillarisation in 25-month-old mice and rescued the hypertrophic response. To investigate the effects of ageing on the hypertrophic response, capillarisation, aerobic capacity and fatigue resistance in *m. plantaris* we used 9- and 25-month-old and 25-month-old resveratrol-treated mice.

2. Methods

2.1 Animals

At the terminal experiment, adult (Adult; $n = 11$) and old (Old: $n = 10$) male C57BL/6J mice (Janvier, France) were 9 and 25 months old, respectively. Mice were kept under specific-pathogen-free conditions and housed individually at 20-22 ^{o}C at a 12-h light/dark cycle. Animals were given free access to water and chow (Ssniff® S8189-S095, the same as provided at the supplier). Another group of old mice (old-res) received 0.4 % resveratrol (98.6 % pure, *Polygonum cuspidatum* extract; 21st Century Alternative, UK) in the chow. At the age of 7.5 or 23.5 months, the *m. gastrocnemius* and *m. soleus* of the left leg were denervated to impose

an overload onto the *m. plantaris* for six weeks as described previously (Ballak et al., 2015; Degens and Alway, 2003). The right leg served as internal control.

All experiments were approved by the local animal use and care committee of the VU University Amsterdam and conformed to the Dutch Research Council's guide for care and use of laboratory animals.

2.2 Preparation for in situ muscle function

Fifteen minutes prior to surgery, mice received a subcutaneous injection of 0.06 mL 1 % Temgesic (Reckitt Benckiser, UK) as an analgesic and were anaesthetized with 4 % isoflurane, 0.1 $L \cdot min^{-1}$ O_2 and 0.2 $L \cdot min^{-1}$ air. After nociceptive responses had ceased, the level of anaesthesia was maintained with 1.5-2.5 % isoflurane. A humidifier moistened the inhaled air to prevent dehydration due to respiration. The mice were placed on a heated pad to maintain body temperature at ~36.5 ^{o}C.

All experiments were performed as described previously (Ballak et al., 2014a; Degens and Alway, 2003). The *m. plantaris* was dissected free from surrounding tissue while maintaining its innervation and blood supply. The sciatic nerve was severed and the proximal end was placed over an electrode for stimulation of the muscle. The distal tendon of the *m. plantaris* was dissected free and tightened with a Kevlar thread via a small steel bar to a force transducer, which was mounted on the lever arm of an isovelocity measuring system (de Haan et al., 1989). The femur was fixed by a clamp on the condyle of the femur. During the experiment, the muscle and its surrounding were kept moist at physiological temperature (34-36 ^{o}C) with a water-saturated airflow.

2.3 Experimental setup and fatigue measurements

The experimental procedures have been described previously (Ballak et al., 2014a). Contractile properties were determined of both the overloaded and contralateral control muscle. The order of the experiments was randomised. Contractions were elicited by supramaximal electrical stimulation of the sciatic nerve at a constant current (2 mA; 200 µs pulse width). Optimal length ($\square_o$) was defined as the muscle length where maximal tetanic isometric force was generated. To set $\square_o$, the length at which the muscle produced maximal twitch force was determined. To fine adjust $\square_o$, tetani (stimulation frequency: 150 Hz, 150 ms) were applied once every 2 min. We adjusted the stimulation frequency for the fatigue test so that the force was ~40 % of the maximal isometric force (test modified for mice from (Degens and Alway, 2003)). This fatigue test consisted of 330-ms stimulation trains once every 2 sec at the pre-determined frequency, for 4 minutes. Potentiation was calculated as $F_{max}/F_1 \cdot 100$, where F_{max} is the maximal force during the protocol and F_1 is the tetanic force during the first contraction. The fatigue index (FI) was calculated as the $F_{120}/_1$, but to correct for the effect of the potentiation we also calculated the $F_{120/max}$, where F_{120} is the force of the last contraction.

2.4 Histology

The *m. plantaris* was embedded at $\square_o$in gelatin-tyrode (NaCl, 128.3 mM; KCl, 4.7 mM; MCl_2, 1.05 mM; NaH_2PO_4, 0.42 mM; $NaHCO_3$, 20.2 mM; EGTA, 15.0 mM; Gelatin 15 % (w/v), pH 7.2) solution and frozen in liquid nitrogen. Subsequently, the *m. soleus* and *m. gastrocnemius medialis* were excised from the hind limbs and weighed. Within a month after the contraction protocol, serial cross-sections (10 µm) were cut from the mid-belly of the *m. plantaris* in a cryostat at -20 ºC. Sections were mounted on glass slides (Menzel-Gläser, superfrost® plus, GER), air-dried and stored at -80 ºC until further use. All chemicals were obtained from Sigma Aldrich (The Netherlands) unless stated otherwise.

One section was stained for succinate dehydrogenase (SDH), a marker of oxidative capacity, according to the protocol described previously (van der Laarse et al., 1989). SDH activity was calculated as the absorbance at 660 nm per µm section thickness per second of staining time ($\Delta A660 \cdot \mu m^{-1} \cdot s^{-1}$). The integrated SDH was calculated as the product of muscle fibre CSA and SDH activity.

Capillaries were depicted by staining sections with biotinylated lectin (*Griffonia simplicifolia*). Sections were fixed in ice-cold acetone for 15 min and washed with HEPES-buffer (HEPES, 5 mM; NaCl, 74.9 mM; $CaCl_2$, 0.1 mM, pH 7.5) for 5 min. After blocking with 0.1 % bovine serum albumin (BSA) in HEPES for 60 min the sections were washed with HEPES again and incubated with hydrogen peroxide for 30 min. Subsequently, sections were washed with HEPES for 5 min, incubated with lectin for an hour (Vector Laboratories, UK) and washed three times with HEPES for 5 min. After incubation with VIB substrate (Vector Laboratories, UK), sections were washed with dH_2O for 5 min before enclosing with glycerin-gelatin.

Serial sections of *m. plantaris* were immunohistochemically stained for type I, IIA, IIX and IIB MHC to distinguish muscle fibre types as described previously (Ballak et al., 2014a). Thereto, monoclonal antibodies specific against type I, IIA, IIX, IIB were used; BAD5, SC-71, 6H1 and BF-F3 (Developmental Studies Hybridoma Bank, USA), respectively. In short, sections were fixated with acetone for 10 min at 4 ºC and washed in phosphate-buffered saline (PBS) plus tween (PBST) three times for 5 min. After blocking with 10 % normal swine serum for 30 min, sections were incubated with the primary antibody. Subsequently, sections were washed in PBST three times for 3 min and incubated in the dark with secondary antibody (Alexa 488 anti-mouse, Molecular Probes) for 30 min. After washing with PBST, incubating with wheat germ agglutinin (WGA) for 20 min, washing with PBST and subsequently washing once more with PBS - all in the dark - sections were

enclosed with Vectashield®-hardset mounting medium with DAPI (Vector Laboratories, USA).

2.4.1 Morphometric analysis

The method of capillary domains has been used and described previously to analyse muscle capillarisation, fibre type composition and relationship between capillarisation and SDH activity (Degens et al., 1992; Hoofd et al., 1985; Wust et al., 2009a). Recently, a semi-automatic domain method has been developed and validated (Ballak et al., 2014c), based on the calculations in AnaTis (BaLoH Software, www.baloh.nl). This method has been shown to reliably assess muscle oxygenation (Al-Shammari et al., 2014). Briefly, coordinates of fibre outlines and capillaries were traced and stored. Capillary domains were constructed by applying Voronoi tessellations, which create equidistant boundaries around the coordinates of each capillary. The fibre cross-sectional area (FCSA) and capillary domain sizes were calculated. Besides the usual indices of capillary density (CD; cap·mm^{-2}) and capillary to fibre ratio (C:F), the method allows the calculation of the capillary supply to individual fibres, even those lacking direct capillary contacts. The capillary supply to a fibre was expressed as the local capillary to fibre ratio (LCFR; sum of the fractions of the capillary domains overlapping a given fibre), domains around fibre (DAF; number of domains overlapping a given fibre) and the capillary fibre density (CFD; LCFR divided by the FCSA of a particular fibre). The logarithmic standard deviation of the radius of the capillary domains provides an index of the heterogeneity of capillary spacing (Log_{rSD}). This analysis was performed for the glycolytic and oxidative regions of the muscle separately.

2.4.2 Western blot

Frozen muscle tissue (~5-10 mg) was homogenised with a Polytron mixer in ice-cold buffer (1:40, w/v) [50 mM Tris-HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 0.1 % Triton-X 100] supplemented with a phosphatase and protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium). Homogenates were then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected and immediately stored at -80 °C. The protein concentration was measured using the DC protein assay kid (Bio-Rad laboratories, Nazareth, Belgium). Forty µg of protein was separated by SDS-PAGE (10-12 % gels) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5 % non-fat milk for 1 h and then incubated overnight (4 °C) with the following antibodies: SDH (1:10,000, Santa Cruz, UK), cytochrome c oxidase subunit 4 (COX-4) (1:1,000, Abcam, Cambridge, UK), Flk-1 (1:500, Santa Cruz, UK) and eukaryotic elongation factor 2 (eEF2) (1:1,000, Cell Signaling Technology, Leiden, The Netherlands). Horseradish peroxidase-conjugated anti-mouse (1:10,000) or anti-rabbit (1:5,000) secondary antibodies (Sigma-Aldrich, Bornem, Belgium) were used for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genetools and Genesnap software (Syngene, Cambridge, UK), respectively. eEF2 was used as a loading control as preliminary experiments showed that eEF2 content was stable across the different treatments and conditions. The results are presented as the ratio protein of interest/eukaryotic elongation factor 2 (eEF2).

Table 6.1. Muscle mass, maximal isometric force, potentiation and fatigue resistance in control and overloaded m. plantaris of adult, old and old-res mice.

	Adult mice (n=7)		**Old mice (n=10)**		**Old-res mice (n=10)**	
	Con	**vs. Ovl**	**Con**	**vs. Ovl**	**Con**	**vs. Ovl**
Muscle mass (mg)	24.2±0.7	32.1±1.2*	22.4±0.7[a]	28.1±1.1*	23.8±0.8	29.8±0.
Max force (mN)	497±34	663±50*	470±22	579±36*	529±30	557±39
Potentiation (%)	31.5±5.6	29.2±6.8	47.8±6.8	35.4±3.9	51.3±4.3	19.3±6.1
$FI_{120/1}$	49.9±1.0	66.2±4.9*	51.6±8.2	70.0±5.6*	58.0±3.8	64.3±5.

$FI_{120/max}$	38.3±1.4	51.8±4.4*	34.2±5.1	52.5±5.5*	38.4±2.5	54.3±4.

*Muscle mass was reported previously (Ballak et al., 2015), Max force: the maximal achieved force during the fatigue protocol. $FI_{120/1}$: Fatigue index for ~40% of tetanic force (not reported here), dividing the force of the 120th contraction by the first. $FI_{120/max}$: Fatigue index for ~40% of tetanic force (not reported here), dividing the force of the 120th contraction by the maximal achieved force during the fatigue protocol. *: different from control condition at P<0.05, [a]: different from adult at P<0.05, [o]: Overload * Resveratrol interaction at P<0.05. Values are mean±SEM*

2.5 Statistics

To test for differences between age and treatment groups we used a univariate ANOVA with factors 1) age, muscle region and overload, or 2) resveratrol, muscle region and overload. The factor 'muscle fibre type' was also added where applicable. Effects were considered significant at P<0.05. Data are expressed as means ± standard error of the mean (SEM). All calculations were performed using IBM SPSS version 20.

Figure 6.1. Effects of age, overload and resveratrol on in situ plantaris muscle fatigue resistance. Plantaris force (% of the force during the first contraction) is expressed as contraction number percentage of first contraction in the m. plantaris for adult (circle), old (triangle) and old-res (square) mice in both control (black) and overloaded (white) conditions.

3. Results

3.1 Effects of age, overload and resveratrol on fatigue resistance and potentiation

Table 6.1 shows that overload resulted in an increase in muscle mass and maximal tetanic force in both adult and old mice ($P<0.05$) (Ballak et al., 2014a). Resveratrol supplementation did not significantly affect muscle mass or the maximal tetanic force.

Figure 6.1 shows tetanic force normalised for the tetanic force of the first contraction during the fatigue test. The potentiation during the fatigue protocol was similar in adult and old muscles. Overload did not significantly alter the potentiation in adult or old muscle. Resveratrol reduced potentiation in overloaded muscle only (Resveratrol*Overload interaction $P<0.05$; Table 6.1).

After the initial potentiation, the force of all control muscles dropped rapidly to ~50 % of the initial value at the end of the test. There was no significant difference in the fatigue resistance between adult and old control muscles. In both adult and old overloaded muscles, fatigue resistance was improved compared to control muscles; the forces at the end of the

protocol were 65-70 % of the initial values (Table 6.1; Fig. 6.1; P<0.05). Resveratrol did not significantly affect fatigue resistance.

Figure 6.2. Effects of age, overload and resveratrol on m. plantaris FCSA. FCSA was smaller in old compared to adult muscle (^{a}; P<0.05), bigger in old-res than old muscle (^{o}; P<0.05) and increased after overload in adult and old (; P<0.01), but not in old-res muscle. Values are mean±SEM.*

Table 6.2. Effects of age, overload and resveratrol supplementation on muscle fibre type area and fibre cross-sectional area.

		FCSA (μm^2)				Fibre type area %			
		ConG	ConO	OvlG	OvlO	ConG	ConO	OvlG	OvlO
Adult	IIA	458±59	642±37^{g}	894±123*	1046±42$^{*/g}$	7.1±3.4	21.0±1.4^{g}	9.3±3.0	30.9±3.3^{g}
	IIAX	503±64	839±83^{g}	1066±188*	1260±49$^{*/g}$	3.0±3.2	5.6±2.0	11.3±1.7*	17.1±1.9*
	IIX	1023±123	1284±65^{g}	1616±196*	1883±125$^{*/g}$	19.0±7.4	29.8±2.7	38.4±5.8*	36.4±4.0
	IIXB	1234±400	1284±334	1770±172*	1875±72$^{*/g}$	0.9±1.2	2.3±1.5	9.4±3.9*	2.6±0.9
	IIB	1790±185	1785±71	2361±221*	1868±173*	70.0±15.2	41.3±2.7^{g}	31.7±9.2*	9.7±3.5$^{g/*}$
Old	IIA	504±49	840±70^{g}	654±98^{a}	918±146^{g}	11.2±2.2	34.1±5.6	7.0±1.7	34.5±6.5
	IIAX	535±86	1064±193^{g}	592±83^{a}	985±106^{g}	1.8±0.1	2.9±1.2^{a}	2.7±1.2^{a}	7.5±2.8$^{*/a}$
	IIX	864±80^{a}	1408±179^{g}	1086±102^{a}	1355±206$^{g/a}$	25.8±1.0	36.0±3.2	27.2±3.7*	34.1±3.4
	IIXB	585±190^{a}	1398±379^{g}	1168±186$^{*/a}$	1543±107$^{*/g}$	1.6±0.5	2.5±0.8	3.0±1.0$^{*/a}$	4.6±2.0
	IIB	1446±201^{a}	1929±263	1726±130^{a}	1676±144	60.4±1.7^{a}	24.6±6.2	60.1±6.0$^{*/a}$	20.2±5.8
Resveratrol	IIA	509±62	757±86^{g}	873±126$^{*/o}$	986±117$^{g/*}$	4.3±2.4^{o}	24.1±3.2$^{o/g}$	6.0±1.6	36.8±3.6^{g}
	IIAX	772±92^{o}	831±129^{g}	986±132^{o}	1116±202^{g}	1.6±0.7	3.5±0.9	5.7±1.4*	9.9±3.0*
	IIX	1270±112^{o}	1557±153^{g}	1608±80$^{*/o}$	1686±134^{g}	19.8±2.7	34.1±4.6	34.1±5.0	31.9±1.8
	IIXB	1547±249^{o}	1498±288	1469±84^{o}	1227±253	2.9±1.1^{o}	3.5±1.7	10.8±4.9$^{*/o}$	3.6±1.9
	IIB	1776±137^{o}	1970±152	2115±188^{o}	1866±183	71.4±5.4^{o}	34.9±6.0^{g}	43.5±11.5	16.4±4.5^{g}

*Glycolytic (ConG) and oxidative (ConO) regions of the m. plantaris in control condition were separated for analysis of FCSA and fibre type area %. The same was done for glycolytic (OvlG) and oxidative (OvlO) regions in overloaded condition. ^{a} significantly different compared to adult muscle (P<0.05), * significantly different compared to control muscle (P<0.05), ^{o} significantly different from old muscle (P<0.05), ^{t} significantly different from other fibre types and ^{g} significantly different from glycolytic region. Values are mean±SEM.*

3.2 Effects of age, overload and resveratrol on muscle fibre size and muscle fibre type composition

For all three groups, FCSA was larger for fast (i.e. IIB and IIX) than for slow fibres (IIA) and in the oxidative region of the *m. plantaris* FCSA for all fibres, expect of IIB, was larger than in the glycolytic part (Table 6.2; $P<0.01$). The FCSA was lower in old than adult muscle, but increased by overload in both groups (Fig. 6.2; $P<0.05$). In type IIA, IIAX and IIX fibres, overload increased the FCSA in both adult and old muscle ($P<0.01$), but more so in adult than old muscle (Age*Overload $P<0.01$; Table 6.2). Resveratrol increased the CSA in both old control and old overloaded pooled (Fig. 6.2; $P<0.05$) and IIAX, IIX, IIXB and IIB fibres (Table 6.2; $P<0.05$). There was no significant Resveratrol*Overload interaction, indicating that resveratrol attenuated or reversed the age-related atrophy, but did not enhance the hypertrophic response.

Figure 6.3. SDH activity and integrated SDH activity per fibre type for adult, old and old-res muscle. SDH activity for adult, old and old-res muscle in control and overloaded condition for type A, X and B fibres in the oxidative (A) and glycolytic (B) region of the muscle. (C) Mean SDH for adult, old and old-res mice in both control (black) and overloaded (white) conditions. SDH activity in old muscle was higher than in adult muscle (^{a}; P<0.05). SDH activity was higher in old compared to adult muscle for all three fibre types (P<0.05). Furthermore, the SDH activity in IIB of the glycolytic region of the muscle was lower than of type IIB fibres in the oxidative region (^{g}; P<0.05). Resveratrol did only increase SDH activity in type IIB fibres of the glycolytic region of the muscle (P<0.05). SDH activity was lower in type IIB compared to IIX fibres (^{x}; P<0.01) and lower in IIX compared to IIA fibres (^{a}; P<0.01). Integrated SDH activity for adult, old and old-res muscle in control and overloaded condition for type A, X and B fibres in the oxidative region (D) and glycolytic (E) region of the muscle. (F) Mean integrated SDH for adult, old and old-res mice in both control (black) and overloaded (white) conditions. Integrated SDH activity increased after overload for all three fibre types (P<0.01). Old muscle showed a higher integrated SDH activity in type IIA fibres, compared to adult (P<0.01). Type IIX fibres had a lower integrated SDH activity in the glycolytic region, compared to the oxidative region (P<0.01). Data shown are mean±SEM.

Age did not significantly affect the percentage area covered by each fibre type. In adult mice, overload did result in an increased proportion of the muscle area covered by IIA, IIAX, IIX and IIXB at the expense of that covered by IIB fibres, but not in old muscle (Age*Overload P<0.05; Table 6.2). Resveratrol supplementation resulted in a larger area occupied by IIB fibres (P<0.05), which was obliterated after overload (Resveratrol*Overload P<0.05).

3.3 Effects of age, overload and resveratrol on SDH activity

Type IIB fibres had the lowest and type IIA the highest SDH activity, with that of type IIX fibres in between (Figs. 6.3A and B; $P<0.01$). Furthermore, SDH activity in IIB fibres in the glycolytic region was lower than that in the oxidative region of the muscle (Figs. 6.3A and B; $P<0.05$). The SDH activity was 20 % higher in old than in adult muscle (Fig 6.3C; $P<0.05$), suggesting a higher oxidative capacity in aged muscle. Overload did not significantly affect the SDH activity ($P>0.05$), while resveratrol increased the SDH activity in type IIB fibres of the glycolytic region only (Fig. 6.3B; $P<0.05$).

Figure 6.4. Flk-1, SDH and COX-4 protein expression in adult, old and old-res muscle. (A) SDH protein expression was similar in adult and old muscle, while COX-4 protein expression was decreased in old muscle (^{a}; $P<0.05$). The SDH/COX-4 ratio (B) was not significantly different in aged muscle. (C) Flk-1 protein expression in m. plantaris, an important receptor for VEGF, was decreased in old compared to adult muscle (^{a}; $P<0.05$), but not affected by overload or resveratrol. The results are presented as the ratio protein of interest/loading control (eEF2).Values are mean±SEM.

*Figure 6.5. Effects of age, overload and resveratrol on CD, C:F and Log_{rSD}. (A) CD was unaffected by age, however increased by overload ($P<0.01$). Resveratrol blunted the overload-induced increase in CD, but only in the glycolytic region of the muscle (Resveratrol*Overload $P<0.05$). (B) C:F was increased by overload ($P<0.01$), but unaffected*

by age or resveratrol. Both CD and C:F were higher in the oxidative region of the m. plantaris compared to the glycolytic region. (C) Log_{rSD} for adult, old and old-res control (black) and overloaded (white) muscle in the oxidative and glycolytic muscle regions. Log_{rSD} was higher in the glycolytic than in the oxidative region of the m. plantaris muscle (^{g}; P<0.01), but not affected by age. Overload reduced Log_{rSD} (; P<0.01), while resveratrol did not affect the Log_{rSD} (P>0.05). Values are mean±SEM.*

Type IIB fibres showed a significantly lower integrated SDH activity than IIX fibres (P<0.01), while the integrated SDH activity of IIA fibres was comparable with that of IIX fibres (P>0.05). Type IIX fibres in the glycolytic region had a lower integrated SDH activity than those in the oxidative region of the muscle (P<0.01). A further analysis per fibre type showed that the integrated SDH activity for type IIA fibres was lower in adult than old muscles (Figs. 6.3D and E; P<0.01). Overload increased the integrated SDH in all groups in all three muscle fibre types (P<0.01). The increase in integrated SDH activity, but not in SDH activity, suggests that mitochondrial biogenesis and fibre hypertrophy during overload were proportional. Resveratrol did not significantly affect the integrated SDH activity (Figs. 6.3D and E).

In contrast to the elevated SDH activity western blot analyses revealed that SDH protein expression was not significantly affected by age (Fig. 6.4A). This suggests that the specific activity of the SDH complex was increased by age. COX-4 protein expression did decrease with age (Fig. 6.4A; P<0.05). Despite the lower COX-4 expression, the SDH/COX-4 ratio was not significantly altered by age (Fig. 6.4B; P>0.05).

3.4 Muscle capillarisation and angiogenesis

Both CD and C:F were significantly higher in the oxidative region of the muscle, compared to the glycolytic (P<0.01; Figs 6.5A and B). CD was not affected by age, but

increased by overload (P<0.01). Resveratrol did not affect CD, however in resveratrol-treated muscle overload only increased CD in the oxidative region (Resveratrol*Overload P<0.05; Fig 6.5A). C:F was unaffected by age, but increased by overload (P<0.01; Fig 6.5B). This increase was higher for adult than for old muscle (Age*Overload P<0.05). Resveratrol, however, did not affect C:F. Log_{rSD} was higher in the glycolytic than in the oxidative region of the *m. plantaris* muscle (P<0.01), indicating a larger heterogeneity in capillary spacing in the glycolytic than in the oxidative region of the muscle. Log_{rSD} was not significantly affected by age (Fig. 6.5). Overload reduced Log_{rSD} indicating a more homogeneous distribution of the capillaries (P<0.01), while resveratrol did not significantly affect Log_{rSD} (P>0.05).

*Figure 6.6. Effects of age, overload, muscle area and resveratrol supplementation on local capillary to fibre ratio and capillary fibre density. LCFR for adult control (black), overloaded (white), old control (dashed) and overloaded (striped) muscle for type A, X and B fibres in the oxidative (A) and glycolytic (B) region of the muscle. LCFR activity for old control (black), overloaded (white), old-res control (dashed) and overloaded (striped) muscle for type A, X and B fibres in the oxidative (C) and glycolytic (D) region of the muscle. Overload increased the LCFR in all fibres types in both regions (P<0.05), except for oxidative type IIB fibres (P>0.05). An interaction effect (Age*Overload) showed that the increase in LFCR was larger for adult than for old muscle (P<0.05). Resveratrol did not affect the LCFR (P>0.05). For both old and old-res muscle the LCFR was higher in the oxidative, compared to the glycolytic part (P<0.01). CFD (mm^{-2}) for adult control (black), overloaded (white), old control (dashed) and overloaded (striped) muscle for type A, X and B fibres in the oxidative (E) and glycolytic (F) region of the muscle. CFD (mm^{-2}) for old control (black), overloaded (white), old-res control (dashed) and overloaded (striped) muscle for type A, X and B fibres in the oxidative (G) and glycolytic (H) region of the muscle. CFD showed an Age*Overload interaction for type IIAX and IIX fibres, only in the glycolytic region (P<0.05). Resveratrol decreased the*

CFD for IIAX, X and B fibres in the glycolytic region of the muscle (P<0.05). For type AX fibres overload increased the CFD in both old and old-res muscle (P<0.05). Furthermore, in IIX and IIB fibres CFD was lower in the glycolytic, compared to the oxidative region (P<0.01). Data shown are mean±SEM.

For all groups LCFR was higher in the oxidative than in the glycolytic region (Fig 6.6; P<0.01). LCFR of all muscle fibre types was not significantly affected by age. However, overload increased LCFR in fibres of all types (Figs. 6.6A and B; P<0.05), except that of type IIB fibres in the oxidative region (Fig. 6.6A). The overload-related increase in LCFR was higher for adult than old muscle (Age*Overload; P<0.05), indicating attenuated angiogenesis in old overloaded muscles. Resveratrol did not affect LCFR (Figs. 6.6C and D).

In both old and old-res, but not adult muscle, CFD was lower in the glycolytic compared to the oxidative region of the muscle for type IIX and IIB fibres (Figs. 6.6E-F; P<0.01). In most cases, the CFD was not significantly affected by age or overload (Figs. 6.6E and F), but there was a significant Age*Overload interaction (P<0.05) for type IIAX and IIX fibres, reflected by a decrease in CFD in old, but not adult, plantaris muscle after overload. Resveratrol decreased CFD of IIAX fibres in the glycolytic region of the muscle (Resveratrol*Region interaction: P<0.05).

Flk-1 protein expression, a receptor for vascular endothelial growth factor (VEGF), was decreased by age (Fig. 6.4C; P<0.05), but unaffected by overload and resveratrol.

3.5 Overload affects inverse relationship between SDH and FCSA

Figure 6.7 shows values for SDH activity of the pooled fibres of each individual muscle expressed as a function of FCSA. For both adult and old there is an inverse relationship between SDH activity and FCSA (R^2 = 0.74 and R^2 = 0.67, respectively, both

P<0.01). Overload induced a right-ward shift of the fitted hyperbola, compared to the control muscle.

The LCFR of adult and old muscle correlated strongly with the FCSA (R^2 = 0.52, P<0.05), but not with the integrated SDH-activity (R^2 = 0.07, P>0.05). Suggesting that the capillary supply to a fibre is more related to the size of the fibre than its oxidative capacity.

Figure 6.7. SDH activity and FCSA are inversely related. The figure shows the relationship between mean SDH activity per muscle and FCSA for adult and old muscles. Overload affects this relationship by inducing a right-ward shift (R^2=0.74; P<0.01), compared to the control (R^2=0.67; P<0.01). The black line represents the best fit hyperbola. Values are mean±SEM.

4. Discussion

Here we investigated whether sarcopenia and the blunted hypertrophic response in old mouse muscle 1. are partly due to a reduced capillarisation and angiogenesis, that are 2. accompanied with a reduced oxidative capacity and fatigue resistance in old control and overloaded muscles, that 3. can be rescued by the anti-inflammatory anti-oxidant resveratrol. The main observations were that both overload-induced hypertrophy and angiogenesis of the *m. plantaris* were attenuated in old mice. Despite the diminished angiogenic response and the unaltered aerobic capacity, the fatigue resistance was increased to a similar extent in adult and old *m. plantaris* of C57BL/6J mice after 6 weeks overload. Interestingly, the aerobic capacity and size of the muscle fibres showed a proportional increase after overload. This was unexpected as the inverse relationship between fibre size and oxidative capacity (van der Laarse et al., 1998) suggests that oxygen diffusion limitations may impose a size constraint on muscle fibres. It is possible that the more homogeneous distribution of capillaries in the overloaded muscles improves muscle oxygenation (Degens et al., 1994; Goldman et al., 2006;

Turek et al., 1989) and makes such an adaptation possible *in vivo*. While resveratrol supplementation did rescue some of the age-related atrophy, it did not affect the fatigue resistance or rescue the angiogenic and hypertrophic response in old age. It is thus possible that impaired angiogenesis contributes to the attenuated hypertrophic response in old age, which cannot be rescued by resveratrol.

4.1.1 Age-related changes in muscle capillarisation and oxidative capacity

An adequate capillarisation is vital for muscle function and any impairment in oxygen delivery could have major implications for fatigue resistance. It has been observed in Wistar rats that the capillary supply to fibres is reduced in old age (Degens et al., 1993b), suggesting an age-related capillary rarefaction. This is, however, not shown in the present study, where the capillary density and capillary to fibre ratio were similar in adult and old mouse *m. plantaris*. The discrepancy maybe related to the different species, but suggests that in 25-month-old mice, the capillary bed is not yet affected by age. Nevertheless, in the current study we did find a reduced Flk-1 expression in old muscle, similar to a previous study in mice (Wagatsuma, 2006), indicative of a reduction in angiogenic signalling in old muscle.

SDH, or complex II, is both part of the citric acid cycle and the respiratory chain. While it has been reported that the SDH activity in the muscle decreases with age (Proctor et al., 1995; Roseberg, 1989) others (Doran et al., 2008) and we found an increased SDH activity in old compared to adult *m. plantaris*. In western blots, however, we observed no age-related change in the expression of SDH, suggesting that the specific activity of the SDH complex was increased. The age-related increase in SDH activity in our study may be a compensation for the decrease in COX expression, which has also been observed by others (Bua et al., 2006; Lee et al., 1998), leading to an increased SDH/COX ratio, in an attempt to maintain aerobic capacity with increasing age.

The absence of any age-related differences in fatigue resistance during a series of intermittent isometric contractions in our study was similar to that observed in the *m. plantaris* of 13- and 25-month-old Wistar rats during a similar test (Degens et al., 1993a). This observation was unexpected, considering the age-related increase in the SDH-activity and the type IIB to type IIA fibre type transition. Interestingly, after overload the fatigue resistance was increased with no change in SDH-activity. The dissociation of changes in fatigue resistance and oxidative capacity suggest that the fatigue test, while useful to distinguish fatigable and fatigue resistant motor units (Larsson et al., 1991) and to predict the aerobic capacity in single muscle fibres, is not as powerful a predictor of aerobic capacity in whole muscle (Degens and Veerkamp, 1994). The reason for this may be related to the nonlinear and more severe increase in metabolites during whole muscle fatigue protocols compared to single motor unit fatigue (Gardiner and Olha, 1987). If indeed lactate accumulation from glycolytic fibres may have a negative impact on the neighbouring fatigue resistant aerobic fibres, one may expect that a higher capillarisation may help enhance the removal of lactate and be associated with a higher fatigue resistance. Consistent with this we found that changes in fatigue resistance during ageing and overload paralleled changes in muscle capillarisation. Nevertheless, in rats older than 26 months there was a significant reduction in fatigue resistance with a similar test (Degens and Alway, 2003). This, and our previous observations that 25-month-old mice already show age-related deteriorations in determinants of muscle force generating capacity similar to 60-70-year-old humans (Ballak et al., 2014b) is in line with our suggestion that these mice present a model for early age-related changes in muscle structure and function.

4.2 Effects of mechanical overload in old age on muscle morphology and fatigue resistance

Overload increased the FCSA more in adult than in old muscle, confirming the blunted hypertrophic response in old age (Ballak et al., 2015; Degens and Alway, 2003). In both adult and old muscle, hypertrophy was accompanied by a proportional increase in the number of capillaries and fibre size as reflected by the maintained capillary fibre density. However, the increase in LCFR, reflecting the number of capillaries per fibre, was less in old than in adult hypertrophied muscles, indicating impaired angiogenesis. Part of the impaired angiogenic response maybe related to the age-related decrease in Flk-1 protein expression, the receptor for the vascular endothelial growth factor (VEGF), an important endothelial mitogenic factor (Wagatsuma, 2006). An impaired angiogenic response has also been seen in ischaemic hind limbs that was due to impaired VEGF expression and HIF-1α activity in muscles of old animals (Rivard et al., 2000; Rivard et al., 1999). Others and we have found a positive relationship between the size of a fibre and the capillary supply in both normal (Ahmed et al., 1997; Wust et al., 2009a) and hypertrophied muscle (Degens et al., 1993b) and a similar time course of hypertrophy and angiogenesis (Egginton et al., 2011; Plyley et al., 1998). Whatever the cause, the impaired angiogenesis we observed in the old muscle may thus well contribute, in addition to a reduced Id2 expression and lower satellite number (Ballak et al., 2015), to the blunted hypertrophic response in old age.

As discussed briefly above, the fatigue resistance of the muscle was elevated after overload despite an unaltered SDH-activity. While this suggests that the fibre hypertrophy and mitochondrial biogenesis are proportional, it also indicates that the used fatigue test is not a powerful indicator of the aerobic capacity of a muscle. In addition, the fibre capillary density was similar in adult and old overloaded and control muscles, indicating that mitochondrial biogenesis, angiogenesis and fibre hypertrophy were proportional, reinforcing the concept of a coordinated control of aerobic capacity, size and capillary supply to a fibre (Ahmed et al., 1997; Wust et al., 2009a).

The elevated fatigue resistance in spite of an unaltered aerobic capacity and capillary density in overloaded muscles may be attributable to an increased proportion of slower fibres (type IIB to IIA shift), that are more economical during isometric contractions than faster fibres (Stienen et al., 1996). The fibre type transition is, however, unlikely to play a significant role, as the overload-induced fast-to-slower shift was less in old than adult muscle, while the overload-induced increase in fatigue resistance was similar in adult in old muscle. In theory, an enhanced myoglobin concentration could facilitate the diffusion of oxygen (Kreuzer and Hoofd, 1987), but this has been shown to not significantly change during overload (Masuda et al., 1997). Interestingly, the CD was increased and the distribution of capillaries became more homogeneous in both adult and old overloaded muscle, which is expected to improve the oxygenation of the muscle (Degens et al., 2006; Turek et al., 1991) and thereby potentially contribute to the enhance fatigue resistance in the overloaded muscles. Further support for the potential significance of the distribution of capillaries in muscle fatigue resistance is the absence of a change in both CD and heterogeneity of capillary spacing on the one hand and fatigue resistance on the other during ageing. It is admittedly a tenuous link that needs further investigation.

We observed a similar fatigue resistance and capillarisation in adult and old muscle, while SDH levels were higher in old muscle. In addition, overload increased both fatigue resistance and capillary density, but not the oxidative capacity. Together this implies that the capillary density may be a more important determinant of fatigue resistance than the oxidative capacity.

4.3 Effect of resveratrol on muscle morphology and muscle fatigue in old overloaded muscle

Resveratrol is thought to induce its effects via Sirt-1 (Yun et al., 2012) and/or AMP-activated protein kinase activation (Park et al., 2012) that controls PGC-1α expression

(Jackson et al., 2011). PGC-1α plays an important role in mitochondrial biogenesis (Goffart and Wiesner, 2003) and has been reported to stimulate angiogenesis (Olesen et al., 2010). In line we this, resveratrol supplementation has been shown to improve mitochondrial function, endurance performance and muscle fatigue resistance in rodents (Hart et al., 2013; Lagouge et al., 2006; Murase et al., 2009; Selsby et al., 2012), without changes in FCSA (Jackson et al., 2011). In contrast to our expectation, resveratrol did not improve capillarisation, mitochondrial function or fatigue resistance, but it did result in an increased FCSA in the old animals. However, a recent study also did not find any beneficial effects of resveratrol on muscle capillarisation in 15-months old mice (Ringholm et al., 2013). In addition, resveratrol was even shown to limit training-induced angiogenesis in old men (Gliemann et al., 2014). The discrepancy in the published literature and our findings may be related to the dose of resveratrol and the degree of oxidative stress in the muscle (Bosutti and Degens, 2015), where we administered 0.4 % resveratrol per g body mass and others a lower dose (0.05 % per g body mass). It is also possible that because in these studies and the present study muscle capillarisation did not show any age-related deteriorations, the beneficial effects of resveratrol on fatigue resistance and the capillary bed may only become apparent when the muscle capillarisation is already impaired.

4.4 Relationship between fibre size and aerobic capacity

The SDH activity correlates strongly with the maximal oxygen uptake of a muscle fibre (van der Laarse et al., 1989). The inverse relationship between SDH activity and fibre cross-sectional area was suggested to be determined by diffusion limitations (van der Laarse et al., 1998). Such a relationship was also observed in our study, and did not change during ageing. To our surprise, however, the relationship in overloaded muscles was shifted upwards, indicating that the maximal oxygen uptake for a fibre with a given FCSA was larger in

overloaded than control muscles. It is likely that the improved homogeneity of capillary distribution that improves muscle oxygenation (Degens and Veerkamp, 1994; Turek et al., 1991), has made this adaptation possible.

5 Conclusion

In conclusion, even though capillarisation in muscles from old and adult mice was similar, overload-induced angiogenesis was blunted. The impaired angiogenic response may at least partly contribute to the blunted hypertrophic response in old age. Despite the attenuated angiogenesis, the fibre hypertrophy and mitochondrial biogenesis in response to overload were all proportional, so that the relationships between fibre capillarisation, aerobic capacity and size were similar in control and overloaded muscle. The increased maximal oxygen uptake for a fibre of a given size and the increased fatigue resistance of overloaded muscles may be partly attributable to an increased capillary density and a more homogeneous distribution of capillaries, while the SDH activity was unchanged with overload. Resveratrol supplementation attenuated age-related muscle wasting, but did not improve fatigue resistance or capillarisation.

Acknowledgments

This research was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate programme (2011-0015). The authors are grateful for 21st Century Alternative, UK, for providing the resveratrol for this study. The authors would also like to acknowledge Sreeda Chalil, Eva Peters, Evelien Bos, Frank van ‘t Hoff and Joshua Dunnink for their support in the western blot and histological measurements.

Chapter 7

General Discussion

General discussion

Age-related muscle wasting is the main cause for a decreased independence in older people. It is also related to an increased incidence of falls and related injuries, greatly decreasing the quality of life of the older person and causing a burden on healthcare systems. The aim of this thesis was therefore to investigate in a mouse model, the effects of age on determinants of muscle force generating and endurance capacity, as well as muscle hypertrophy. This chapter provides an overview of what this thesis added to the literature and gives some suggestions for future investigations.

We completed a literature review about the effects of age on determinants of muscle force generating capacity, in which we compared data of different male rodent models with men at similar relative lifespans (**chapter 2**). In **chapter 3, 4 and 6** male 9- and 25-month-old mice were used. In the strain of mice we used, 9 months is an adult age and 25 months is the average lifespan. Overall, the effects of age on determinants of muscle force showed similar directions, but were relatively smaller.

We used 9- and 25-month-old mice as models for young adults and older people to study age-related changes in muscle structure and function. It was shown, that the lower force generating capacity of muscles from 25- than 9-month-old mice was mainly attributable to a reduction in specific force, rather than myofibre atrophy and loss of myofibres (**Chapter 3)**. Part of the decline in muscle specific force was due to an age-related increase in connective tissue, but not to an increased proportion of non-functional, denervated (type IIB) myofibres. In **chapter 4** we showed, that 25-month-old mice had an impaired hypertrophic response to a 6-week overload stimulus, compared to 9-month-old mice. The attenuated hypertrophic response was characterised by an attenuated myofibre hypertrophy, but not by fewer myofibres. The attenuated myofibre atrophy was associated with a lower SC density in old muscle. Resveratrol, an anti-oxidant, was not effective in alleviating age-related decreases in

muscle mass and function and even abolished the increase in SC density seen in non-resveratrol-treated old mice after overload. In **chapter 5** a semi-automated method of capillary domain analysis was validated, which was subsequently used in **chapter 6**. In chapter 6 we observed that an attenuated angiogenesis may contribute to the attenuated hypertrophic response in old age, while neither one of them was rescued by resveratrol supplementation.

Subsequently, the effects of age and overload on SC content and fatigue will be discussed and how this may be modulated with resveratrol supplementation. Based on the observations of this thesis some recommendations are given regarding possible intervention programs to improve muscle function, power and endurance, in (pre-) sarcopenic subjects. The discussion closes with recommendations for future research.

Ageing

It is well-known that ageing is accompanied with a reduction in the force generating capacity of a muscle. It is, however, difficult if not impossible, to simultaneously assess in humans to what extent ageing affects myofibre size, myofibre number and specific tension of myofibres, which are the key factors that determine the force generating capacity. This can, however, be studied systematically in mice. In our mice, the 25-month-old animals reflected the initial stages of sarcopenia. In this model, we were able to establish that the quality of the muscle declines before quantity. Therefore, it is likely that the loss of muscle mass comes more into play with the progression of sarcopenia. A situation where the age-related loss of muscle mass affects muscle function is to be expected in 26-28 month-old mice . Therefore, studying those old animals would be an important continuation of the research done in **chapter 3**.

It would also be relevant to study at what age specific muscle force starts to decline in both humans and rodents. In addition, measurement of the contractile force characteristics of

skinned single myofibres of aged mouse and human muscle may help to explain the decrease in specific muscle force.

Overload model

To study the hypertrophic response of the plantaris muscle in adult and old mice we denervated its synergists. This model induces a 30-35% hypertrophy of rat plantaris muscle in 6 weeks' time and we observed a similar magnitude of hypertrophy in mice. The hypertrophy was accompanied with an increase in muscle force and endurance capacity. Although it is a chronic intervention, dissimilar of traditional strength training interventions, it has been suggested to be an adequate model of resistance exercise and for the study of the hypertrophic response , even in old muscle. The results from **chapter 4 and 6** show that the hypertrophic response in old muscle was attenuated compared to adult muscle. This was associated with a lower SC density, without evidence for a lower capacity for SC proliferation, as well as an attenuated angiogenesis.

SCs ageing and hypertrophy

In **chapter 4** of this thesis we showed that the SC density was less in old than in adult muscle. This observation is in accordance with results of other studies which reported a loss of SCs with increasing age (Timson, 1990), with greater losses in faster myofibres . Since SCs play an important role in myofibre growth and repair (Timson, 1990), a loss of SCs could lead to a decreased adaptive response to injury or anabolic stimuli. SC loss has even been shown to precede age-related myofibre atrophy (Kadi et al., 2004). Therefore, we hypothesized that at old age the hypertrophic response was attenuated, and if so that this would be due to a lower satellite cell content. Indeed, we showed that although SC content increased after overload, lower levels of SCs in old control muscle were associated with an impaired hypertrophic

response in overloaded old compared to adult muscle. This is in accordance with another study, which observed that the degree of hypertrophy correlated positively with the number of SCs before training . These findings support the idea that SC content is a key factor for FCSA size regulation and that an age-related loss of SCs leads to muscle atrophy and a diminished myofibre hypertrophy.

Apart from the SC content, it is important to know whether there is an effect of age on the proliferation and differentiation of SCs. We found no change in MyoD, but an age-related increase in myogenin protein expression, which is a factor involved in SC differentiation . In addition, we also observed an age-related increase in Id2 protein expression, which when overexpressed may induce apoptosis and inhibition of differentiation . Together this suggests that both proliferation and differentiation were increased to compensate for the loss of myonuclei. Interestingly, it has been observed that regenerative factors are increased most in muscles of most severely affected sarcopenic rodents, suggesting an upregulation of the regenerative drive to combat the atrophy at old age (Hawke and Garry, 2001). Therefore, it seems that the capacity to adapt to a stimulus, remains even at old age, only the magnitude and the efficiency of the adaptation decrease.

The effect of resveratrol on hypertrophy and SCs

Since low-grade systemic inflammation and oxidative stress in old age may contribute to muscle wasting (Brack et al., 2005), the presumption was that treatment with 0.4% resveratrol, an anti-inflammatory anti-oxidant, would at least partly counteract the effects of ageing on muscle function and mass. However, except from an attenuation of atrophy in **chapter 6,** in this series of experiments, we could not find a beneficial effect of resveratrol on the magnitude of overload-induced hypertrophy, force generating capacity or fatigue resistance. The lack of effect of resveratrol on the above mentioned variables is in contrast to

studies reporting an increased aerobic performance even in old , but are in agreement with those of other studies also reporting no effects of resveratrol on muscle contractile properties . Resveratrol does seem to protect against oxidative stress in aged rodent muscle . In line with this, we found a reduced caspase-3 protein expression in old mouse plantaris muscle after resveratrol supplementation, but this was not sufficient to ameliorate age-related reduction in muscle strength, hypertrophy and angiogenesis. Rather than having beneficial effects on muscle function, resveratrol even abolished the overload-induced increase in SC number in old mice. The reason for this remains elusive, since no changes in myogenin, Id2 and MyoD protein expression were observed. Yet, the degree of hypertrophy was not decreased, suggesting that the hypertrophy was attained by an increase in the myonuclear domain size as was indeed observed in **chapter 4**. That SCs are not essential for hypertrophy is supported by a study that showed that inactivation of SCs by tamoxifen did not attenuate the hypertrophic response in adult mice . Thus, resveratrol does not ameliorate age-related changes in muscle function and mass in 25-month-old mice. Therefore, the efficacy of resveratrol supplementation as a sarcopenic intervention remains questionable.

Fully automating the technique of capillary domain

In this thesis we automatized the capillary domain method, which has been used extensively in our lab . We developed a software package that was validated in **chapter 5**. To further develop this semi-automated method to a fully automated system, several additional steps have to be taken. The automatic location of capillaries is the first challenge. It proved difficult to identify capillaries in grey-scaled images automatically, but this may be enhanced by fluorescently staining the capillaries. A second step is the fully automated tracing of complete myofibre outlines. At the moment adjacent myofibres are occasionally seen as one myofibre. Also here fluorescent staining of myofibres may help to improve this. The next step

is combining the fluorescence staining for both capillaries and myofibre outlines. When this works, the next step is to automatically distinguish different myofibre types.

Relation between attenuated angiogenesis and hypertrophy

The capillary bed becomes less dense with age, therewith decreasing the oxygen supply to and removal of waste products from the muscle. In **chapter 6** we observed that reduced angiogenesis may partly explain the attenuated hypertrophic response. In support of this, a previous study observed a strong relationship between fibre hypertrophy and angiogenesis (Hawke and Garry, 2001). The impaired angiogenic response in old muscle may at least partly be attributable to a reduced protein expression of the VEGF receptor Flk-1.

Another factor which may have contributed to the blunted hypertrophy is the reduced activity of old mice. Old mice show a reduced daily activity compared to adult mice (Edstrom and Ulfhake, 2005). The lower daily activity level may explain the 5% lower muscle mass in the old mice (chapter 2). It is, however, unlikely that this explains the age-related attenuated hypertrophic response. Due to the lower muscle mass and the lower muscle mass/body mass ratio in old mice (chapter 2), the overload stimulus in old muscle was likely higher than in adult muscle. In addition, it is conceivable that the *m. plantaris* also replaced the postural function of the *m. soleus*. Since overload consisted of a chronic stimulus, comparable to resistance training (Degens, 2010), combined with the limited time needed to induce a significant hypertrophic response, the possibility that a reduced activity pattern in old muscle may have caused the attenuated hypertrophic response in old age, is negligible. In addition, if old muscles are less activated, because of decreased daily activity levels, than the chronic overload stimulus, which mimics resistance training, may actually be more interspersed with postural activity in old muscle. This may be considered as concurrent training, which may limit muscle hypertrophy (Degens, 2010), more so in old than in adult mice. These factors

should be considered in future research, to better establish the relationship between attenuated angiogenesis and hypertrophy in old age.

Previously, SDH activity of muscle fibres from a wide variety of species expressed as a function of the FCSA revealed an inverse hyperbolic relation . From this relation it has been calculated that over the range of species the critical interstitial oxygen tension for a muscle fibre to attain its VO_{2max} was 14 mmHg. The observation that muscle fibres of the mice described in this thesis were below the hyperbola suggests that mitochondrial function was not limited by the oxygen supply. However, after the overload, FCSA and SDH activity of muscle fibres of both adult and old mice fitted with the previously described hyperbola, which suggests that in the hypertrophic conditions maximal oxygen diffusion was limited by the diffusion distance for oxygen. Given the FCSA and SDH activities of the old and adult overloaded muscles, it is conceivable that a reduced LCFR indicates an insufficient supply of oxygen and hence a blunted hypertrophic response (see for review).

Practical implications and future research

Muscle mass and function decline with age, causing not only a decreased quality of life, but also social and economic problems. Therefore, the aim of this thesis was to investigate factors that contribute to the impaired muscle function and hypertrophy in old age. We addressed this by studying 9- and 25-month-old mice. Interventions to counteract age-related muscle wasting are needed and the findings of this thesis provide indications where such interventions should be aiming at.

First of all, the results of chapter 3 show that a decline in muscle quality, in terms of force generating capacity per unit muscle mass occurred without large decreases in muscle mass. This may suggest that a decrease in muscle quality precedes the age-related loss of

muscle mass in 25-month old mice. Therefore, training programs for the elderly should aim specifically at increasing or at least maintaining specific muscle force. Furthermore, when the age-related effects on muscle mass and force are still modest, old muscle is still capable of showing a robust hypertrophic response. Thus, the timing and start of an intervention is crucial. Since the fatigue resistance in old muscle was not impaired, increasing muscle force may have beneficial effects on the absolute fatigue resistance as the older person has to recruit a smaller proportion of the available muscle mass for a given task.

The results of our studies show that the attenuated myofibre hypertrophy in old muscles was associated with a lower SC density and impaired angiogenesis. Future studies should focus on the question whether an age-related reduction in SCs may contribute to (myofibre) atrophy. This can be done through studies using more age groups, particularly around the threshold age, the age where age-related muscle wasting starts to develop. There is also the open question as to what causes the age-related loss of muscle specific force and how it develops over time. To study this, the specific tension should be determined in mice of many ages between 9- and 25-month of age, with additional studies on skinned myofibres.

This thesis provides a first start of a fully automated capillary domain analysis. Further work is needed to make this system fully automatic. Immunofluorescent labelling of capillaries and myofibre outlines may greatly facilitate the automatic detection of these morphological structures.

Lastly, the results of chapter 5 indicates that an age-related reduced angiogenesis may explain part of the blunted hypertrophic response observed in the old animals. Therefore, to improve their effectiveness, future interventions counteracting sarcopenia should also focus on maintaining a fully functional capillary bed.

Therefore, the findings presented in this thesis lead to the following questions, which should be addressed in future studies:

- What is the contribution of a decrease in specific force, myofibre loss and atrophy to the age-related loss in animals of different ages?
- At what age does the muscle specific force decline and what causes this reduction in specific tension?
- Does an age-related reduction in SC density lead to muscle (fibre) atrophy over time?
- How does resveratrol abolish the overload-induced increase in SC content, observed in non-treated old animals, and what does happen with the myonuclear domain size?
- Could the use of immunofluorescence staining help to fully automate the capillary domain analysis?
- Does age-related attenuated angiogenesis cause blunted hypertrophy observed in old muscle?

The original aim of this thesis was to answer the question what causes the impaired muscle function and hypertrophy at old age. Although this thesis has shed some light on this topic and provides new information, there is still much to explore about the aetiology of muscle ageing. To further expand our knowledge more studies should be carried out in different rodent models in which all determinants of muscle force generating capacity, endurance capacity and hypertrophy are taken into account, to attain a profound understanding about processes of muscle degeneration during the ageing process. What this thesis adds to the existing knowledge, is that although with increasing age mice lose muscle mass and function, the ability of muscle to adapt to stimuli remains until old age. This ability of old mouse muscles to adapt to overload stimulates development of novel interventions in

humans, which will increase the number of years with an independent life style and quality of life, simultaneously decreasing the burden on healthcare systems.

References.

Aagaard P, Suetta C, Caserotti P, Magnusson SP and Kjaer M, (2010). Role of the nervous system in sarcopenia and muscle atrophy with aging: strength training as a countermeasure. Scand J Med Sci Sports. 20, 49-64.

Ahmed SK, Egginton S, Jakeman PM, Mannion AF and Ross HF, (1997). Is human skeletal muscle capillary supply modelled according to fibre size or fibre type? Exp Physiol. 82, 231-4.

Al-Shammari AA, Gaffney EA and Egginton S, (2012). Re-evaluating the use of Voronoi Tessellations in the assessment of oxygen supply from capillaries in muscle. Bull Math Biol. 74, 2204-31.

Al-Shammari AA, Gaffney EA and Egginton S, (2014). Modelling capillary oxygen supply capacity in mixed muscles: Capillary domains revisited. J Theor Biol. 356c, 47-61.

Allen DG, Lamb GD and Westerblad H, (2008). Impaired calcium release during fatigue. J Appl Physiol (1985). 104, 296-305.

Alway SE, Siu PM, Murlasits Z and Butler DC, (2005). Muscle hypertrophy models: applications for research on aging. Can J Appl Physiol. 30, 591-624.

Andersen JL, (2003). Muscle fibre type adaptation in the elderly human muscle. Scand J Med Sci Sports. 13, 40-7.

Andersson DC, Betzenhauser MJ, Reiken S, Meli AC, Umanskaya A, Xie W, Shiomi T, Zalk R, Lacampagne A and Marks AR, (2011). Ryanodine receptor oxidation causes intracellular calcium leak and muscle weakness in aging. Cell Metab. 14, 196-207.

Aniansson A, Hedberg M, Henning GB and Grimby G, (1986). Muscle morphology, enzymatic activity, and muscle strength in elderly men: a follow-up study. Muscle Nerve. 9, 585-91.

Ansved T and L. L, (1990). Quantitative and qualitative morphological properties of the soleus motor nerve and the L5 ventral root in young and old rats. Relation to the number of soleus muscle fibers. J Neurol Sci. 96, 269-82.

Ansved T and Larsson L, (1989). Effects of ageing on enzyme-histochemical, morphometrical and contractile properties of the soleus muscle in the rat. J Neurol Sci. 93, 105-24.

Augusto V, Padovani CR and Campos GER, (2004). Skeletal muscle fiber types in C57BL6J mice. Braz J Morphol Sci. 21(2), 89-94.

Avin KG and Law LA, (2011). Age-related differences in muscle fatigue vary by contraction type: a meta-analysis. Phys Ther. 91, 1153-65.

Ballak SB, Degens H, Busé-Pot T, de Haan A and Jaspers RT, (2014a). Plantaris muscle weakness in old mice: relative contributions of changes in specific force, muscle mass, myofiber cross-sectional area, and number. AGE. 36, 1-11.

Ballak SB, Degens H, de Haan A and Jaspers RT, (2014b). Aging related changes in determinants of muscle force generating capacity: a comparison of muscle aging in men and male rodents. Ageing Res Rev. 14, 43-55.

Ballak SB, Jaspers RT, Deldicque L, Chalil S, Peters EL, de Haan A and Degens H, (2015). Blunted hypertrophic response in old mouse

muscle is associated with a lower satellite cell density and is not alleviated by resveratrol. Exp Gerontol.
Ballak SB, Yap MH, Harding PJ and Degens H, (2014c). Validation of a new semi-automated technique to evaluate muscle capillarisation, ISOTT. London.
Beard DA and Bassingthwaighte JB, (2000). Advection and diffusion of substances in biological tissues with complex vascular networks. Ann Biomed Eng. 28, 253-68.
Bekedam MA, van Beek-Harmsen BJ, Boonstra A, van Mechelen W, Visser FC and van der Laarse WJ, (2003). Maximum rate of oxygen consumption related to succinate dehydrogenase activity in skeletal muscle fibres of chronic heart failure patients and controls. Clin Physiol Funct Imaging. 23, 337-43.
Blackwell BN, Bucci TJ, Hart RW and Turturro A, (1995). Longevity, body weight, and neoplasia in ad libitum-fed and diet-restricted C57BL6 mice fed NIH-31 open formula diet. Toxicol Pathol. 23, 570-82.
Blough ER and Linderman JK, (2000a). Lack of skeletal muscle hypertrophy in very aged male Fischer 344 x Brown Norway rats. Journal of Applied Physiology. 88, 1265-70.
Blough ER and Linderman JK, (2000b). Lack of skeletal muscle hypertrophy in very aged male Fischer 344 x Brown Norway rats. J Appl Physiol (1985). 88, 1265-70.
Bosutti A and Degens H, (2015). The impact of resveratrol and hydrogen peroxide on muscle cell plasticity shows a dose-dependent interaction. Sci Rep. 5, 8093.
Bottinelli R, Canepari M, Pellegrino MA and Reggiani C, (1996). Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. J Physiol. 495 (Pt 2), 573-86.
Bottinelli R, Pellegrino MA, Canepari M, Rossi R and Reggiani C, (1999). Specific contributions of various muscle fibre types to human muscle performance: an in vitro study. Journal of Electromyography & Kinesiology. 9, 87-95.
Brack AS, Bildsoe H and Hughes SM, (2005). Evidence that satellite cell decrement contributes to preferential decline in nuclear number from large fibres during murine age-related muscle atrophy. J Cell Sci. 118, 4813-21.
Brooks SV and Faulkner JA, (1988). Contractile properties of skeletal muscles from young, adult and aged mice. J Physiol. 404, 71-82.
Brooks SV and Faulkner JA, (1991). Maximum and sustained power of extensor digitorum longus muscles from young, adult, and old mice. J Gerontol. 46, B28-33.
Brooks SV and Faulkner JA, (1994a). Isometric, shortening, and lengthening contractions of muscle fiber segments from adult and old mice. American Journal of Physiology. 267, C507-13.
Brooks SV and Faulkner JA, (1994b). Skeletal muscle weakness in old age: underlying mechanisms. Med Sci Sports Exerc. 26, 432-9.
Brooks SV and Faulkner JA, (1994c). Skeletal muscle weakness in old age: underlying mechanisms. Medicine & Science in Sports & Exercise. 26, 432-9.

Brown M, (1987). Change in fibre size, not number, in ageing skeletal muscle. Age Ageing. 16, 244-8.
Brown M and Hasser EM, (1996). Complexity of age-related change in skeletal muscle. The Journals of Gerontology Series A Biological Sciences and Medical Sciences. 51, B117-23.
Brown WF, (1972). A method for estimating the number of motor units in thenar muscles and the changes in motor unit count with ageing. Journal of Neurology, Neurosurgery & Psychiatry. 35, 845-52.
Bua E, Johnson J, Herbst A, Delong B, McKenzie D, Salamat S and Aiken JM, (2006). Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. Am J Hum Genet. 79, 469-80.
Bua EA, McKiernan SH, Wanagat J, McKenzie D and Aiken JM, (2002). Mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia. Journal of Applied Physiology. 92, 2617-24.
Burke RE, Levine DN, Tsairis P and Zajac FE, 3rd, (1973). Physiological types and histochemical profiles in motor units of the cat gastrocnemius. J Physiol. 234, 723-48.
Callahan DM and Kent-Braun JA, (2011). Effect of old age on human skeletal muscle force-velocity and fatigue properties. J Appl Physiol (1985). 111, 1345-52.
Campbell MJ, McComas AJ and Petito F, (1973). Physiological changes in ageing muscles. Journal of Neurology, Neurosurgery & Psychiatry. 36, 174-82.
Cartee GD, (1995). What insights into age-related changes in skeletal muscle are provided by animal models? The Journals of Gerontology Series A Biological Sciences and Medical Sciences. 50 Spec No, 137-41.
Carter EE, Thomas MM, Murynka T, Rowan SL, Wright KJ, Huba E and Hepple RT, (2010). Slow twitch soleus muscle is not protected from sarcopenia in senescent rats. Exp Gerontol. 45, 662-70.
Chakkalakal JV, Jones KM, Basson MA and Brack AS, (2012). The aged niche disrupts muscle stem cell quiescence. Nature. 490, 355-60.
Chan S and Head SI, (2010). Age- and gender-related changes in contractile properties of non-atrophied EDL muscle. PLoS One. 5, e12345.
Chen Scarabelli C, McCauley RB, Yuan Z, Di Rezze J, Patel D, Putt J, Raddino R, Allebban Z, Abboud J, Scarabelli GM, Chilukuri K, Gardin J, Saravolatz L, Faggian G, Mazzucco A and Scarabelli TM, (2008). Oral administration of amino acidic supplements improves protein and energy profiles in skeletal muscle of aged rats: elongation of functional performance and acceleration of mitochondrial recovery in adenosine triphosphate after exhaustive exertion. American Journal of Cardiology. 101, 42E-48E.
Chopek JW and Gardiner PF, (2010). Life-long caloric restriction: Effect on age-related changes in motoneuron numbers, sizes and apoptotic markers. Mech Ageing Dev. 131, 650-9.
Clemmons DR, (2009). Role of IGF-I in skeletal muscle mass maintenance. Trends Endocrinol Metab. 20, 349-56.

Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM and Holloszy JO, (1992). Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. J Gerontol. 47, B71-6.
Conley KE, Amara CE, Jubrias SA and Marcinek DJ, (2007). Mitochondrial function, fibre types and ageing: new insights from human muscle in vivo. Exp Physiol. 92, 333-9.
D'Antona G, Pellegrino MA, Adami R, Rossi R, Carlizzi CN, Canepari M, Saltin B and Bottinelli R, (2003). The effect of ageing and immobilization on structure and function of human skeletal muscle fibres. J Physiol. 552, 499-511.
D'Antona G, Ragni M, Cardile A, Tedesco L, Dossena M, Bruttini F, Caliaro F, Corsetti G, Bottinelli R, Carruba MO, Valerio A and Nisoli E, (2010). Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. Cell Metab. 12, 362-72.
Dasgupta B and Milbrandt J, (2007). Resveratrol stimulates AMP kinase activity in neurons. Proc Natl Acad Sci U S A. 104, 7217-22.
Davies CT, Thomas DO and White MJ, (1986). Mechanical properties of young and elderly human muscle. Acta Medica Scandinavica Supplementum. 711, 219-26.
Daw CK, Starnes JW and White TP, (1988). Muscle atrophy and hypoplasia with aging: impact of training and food restriction. Journal of Applied Physiology. 64, 2428-32.
de Haan A, Jones DA and Sargeant AJ, (1989). Changes in velocity of shortening, power output and relaxation rate during fatigue of rat medial gastrocnemius muscle. Pflugers Arch. 413, 422-8.
Deerberg F, Rapp K, Rehm S and Pittermann W, (1980). Genetic and environmental influences on lifespan and diseases in Han:Wistar rats. Mech Ageing Dev. 14, 333-43.
Degens H, (1998). Age-related changes in the microcirculation of skeletal muscle. Adv Exp Med Biol. 454, 343-8.
Degens H, (2010). The role of systemic inflammation in age-related muscle weakness and wasting. Scand J Med Sci Sports. 20, 28-38.
Degens H, (2012). Determinants of skeletal muscle hypertrophy and the attenuated hypertrophic response at old age. Sports Medicine & Doping Studies. S1:1-8.
Degens H and Alway SE, (2003). Skeletal muscle function and hypertrophy are diminished in old age. Muscle Nerve. 27, 339-47.
Degens H and Alway SE, (2006). Control of muscle size during disuse, disease, and aging. Int J Sports Med. 27, 94-9.
Degens H, Deveci D, Botto-van Bemden A, Hoofd LJ and Egginton S, (2006). Maintenance of heterogeneity of capillary spacing is essential for adequate oxygenation in the soleus muscle of the growing rat. Microcirculation. 13, 467-76.
Degens H, Erskine RM and Morse CI, (2009a). Disproportionate changes in skeletal muscle strength and size with resistance training and ageing. J Musculoskelet Neuronal Interact. 9, 123-9.

Degens H, Hoofd L and Binkhorst RA, (1995). Specific force of the rat plantaris muscle changes with age, but not with overload. Mech Ageing Dev. 78, 215-9.
Degens H and Korhonen MT, (2012). Factors contributing to the variability in muscle ageing. Maturitas. 73, 197-201.
Degens H and Larsson L, (2007). Application of skinned single muscle fibres to determine myofilament function in ageing and disease. J Musculoskelet Neuronal Interact. 7, 56-61.
Degens H, Morse CI and Hopman MT, (2009b). Heterogeneity of capillary spacing in the hypertrophied plantaris muscle from young-adult and old rats. Adv Exp Med Biol. 645, 61-6.
Degens H, Ringnalda BE and Hoofd LJ, (1994). Capillarisation, fibre types and myoglobin content of the dog gracilis muscle. Adv Exp Med Biol. 361, 533-9.
Degens H, Turek Z and Binkhorst RA, (1993a). Compensatory hypertrophy and training effects on the functioning of aging rat m. plantaris. Mech Ageing Dev. 66, 299-311.
Degens H, Turek Z, Hoofd L, van't Hof MA and Binkhorst RA, (1993b). Capillarisation and fibre types in hypertrophied m. plantaris in rats of various ages. Respir Physiol. 94, 217-26.
Degens H, Turek Z, Hoofd LJ, Van't Hof MA and Binkhorst RA, (1992). The relationship between capillarisation and fibre types during compensatory hypertrophy of the plantaris muscle in the rat. J Anat. 180 (Pt 3), 455-63.
Degens H and Veerkamp JH, (1994). Changes in oxidative capacity and fatigue resistance in skeletal muscle. Int J Biochem. 26, 871-8.
Degens H, Veerkamp JH, van Moerkerk HT, Turek Z, Hoofd LJ and Binkhorst RA, (1993c). Metabolic capacity, fibre type area and capillarization of rat plantaris muscle. Effects of age, overload and training and relationship with fatigue resistance. Int J Biochem. 25, 1141-8.
Delbono O, (2002). Molecular mechanisms and therapeutics of the deficit in specific force in ageing skeletal muscle. Biogerontology. 3, 265-70.
Delbono O, Renganathan M and Messi ML, (1997). Excitation-Ca2+ release-contraction coupling in single aged human skeletal muscle fiber. Muscle Nerve Suppl. 5, S88-92.
Delmonico MJ, Harris TB, Visser M, Park SW, Conroy MB, Velasquez-Mieyer P, Boudreau R, Manini TM, Nevitt M, Newman AB and Goodpaster BH, (2009). Longitudinal study of muscle strength, quality, and adipose tissue infiltration. Am J Clin Nutr. 90, 1579-85.
Deschenes MR, (2004). Effects of aging on muscle fibre type and size. Sports Medicine. 34, 809-24.
Doherty TJ, (2003). Invited review: Aging and sarcopenia. Journal of Applied Physiology. 95, 1717-27.
Doherty TJ and Brown WF, (1993). The estimated numbers and relative sizes of thenar motor units as selected by multiple point stimulation in young and older adults. Muscle Nerve. 16, 355-66.
Doherty TJ and Brown WF, (1997). Age-related changes in the twitch contractile properties of human thenar motor units. Journal of Applied Physiology. 82, 93-101.

Doherty TJ, Vandervoort AA and Brown WF, (1993). Effects of ageing on the motor unit: a brief review. Canadian Journal of Applied Physiology. 18, 331-58.
Dolinsky VW, Jones KE, Sidhu RS, Haykowsky M, Czubryt MP, Gordon T and Dyck JR, (2012). Improvements in skeletal muscle strength and cardiac function induced by resveratrol during exercise training contribute to enhanced exercise performance in rats. J Physiol. 590, 2783-99.
Doran P, O'Connell K, Gannon J, Kavanagh M and Ohlendieck K, (2008). Opposite pathobiochemical fate of pyruvate kinase and adenylate kinase in aged rat skeletal muscle as revealed by proteomic DIGE analysis. Proteomics. 8, 364-77.
Eddinger TJ, Moss RL and Cassens RG, (1985). Fiber number and type composition in extensor digitorum longus, soleus, and diaphragm muscles with aging in Fisher 344 rats. Journal of Histochemistry & Cytochemistry. 33, 1033-41.
Edstrom E and Ulfhake B, (2005). Sarcopenia is not due to lack of regenerative drive in senescent skeletal muscle. Aging Cell. 4, 65-77.
Edstrom L and Larsson L, (1987). Effects of age on contractile and enzyme-histochemical properties of fast- and slow-twitch single motor units in the rat. J Physiol. 392, 129-45.
Egginton S, Badr I, Williams J, Hauton D, Baan GC and Jaspers RT, (2011). Physiological angiogenesis is a graded, not threshold, response. J Physiol. 589, 195-206.
Erskine RM, Jones DA, Maffulli N, Williams AG, Stewart CE and Degens H, (2011). What causes in vivo muscle specific tension to increase following resistance training? Exp Physiol. 96, 145-55.
Essen-Gustavsson B and Borges O, (1986). Histochemical and metabolic characteristics of human skeletal muscle in relation to age. Acta Physiol Scand. 126, 107-14.
Faulkner JA and Brooks SV, (1995). Muscle fatigue in old animals. Unique aspects of fatigue in elderly humans. Adv Exp Med Biol. 384, 471-80.
Faulkner JA, Larkin LM, Claflin DR and Brooks SV, (2007a). Age-related changes in the structure and function of skeletal muscles. Clin Exp Pharmacol Physiol. 34, 1091-6.
Faulkner JA, Larkin LM, Claflin DR and Brooks SV, (2007b). Age-related changes in the structure and function of skeletal muscles. Clinical and Experimental Pharmacology and Physiology. 34, 1091-6.
Fiatarone MA, Marks EC, Ryan ND, Meredith CN, Lipsitz LA and Evans WJ, (1990). High-intensity strength training in nonagenarians. Effects on skeletal muscle. Jama. 263, 3029-34.
Fielding RA, Vellas B, Evans WJ, Bhasin S, Morley JE, Newman AB, Abellan van Kan G, Andrieu S, Bauer J, Breuille D, Cederholm T, Chandler J, De Meynard C, Donini L, Harris T, Kannt A, Keime Guibert F, Onder G, Papanicolaou D, Rolland Y, Rooks D, Sieber C, Souhami E, Verlaan S and Zamboni M, (2011). Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. J Am Med Dir Assoc. 12, 249-56.

Fitts RH, Troup JP, Witzmann FA and Holloszy JO, (1984). The effect of ageing and exercise on skeletal muscle function. Mech Ageing Dev. 27, 161-72.
Fleg JL and Lakatta EG, (1988). Role of muscle loss in the age-associated reduction in VO2 max. J Appl Physiol (1985). 65, 1147-51.
Flurkey KC, JM. Harrison, DE., 2007. The mouse in biomedical research, 2nd Edition ed. American College Laboratory Animal Medicine (Elsevier), Burlington, MA.
Frontera WR, Hughes VA, Fielding RA, Fiatarone MA, Evans WJ and Roubenoff R, (2000a). Aging of skeletal muscle: a 12-yr longitudinal study. Journal of Applied Physiology. 88, 1321-6.
Frontera WR, Hughes VA, Lutz KJ and Evans WJ, (1991). A cross-sectional study of muscle strength and mass in 45- to 78-yr-old men and women. Journal of Applied Physiology. 71, 644-50.
Frontera WR, Reid KF, Phillips EM, Krivickas LS, Hughes VA, Roubenoff R and Fielding RA, (2008a). Muscle fiber size and function in elderly humans: a longitudinal study. Journal of Applied Physiology. 105, 637-42.
Frontera WR, Reid KF, Phillips EM, Krivickas LS, Hughes VA, Roubenoff R and Fielding RA, (2008b). Muscle fiber size and function in elderly humans: a longitudinal study. J Appl Physiol. 105, 637-42.
Frontera WR, Suh D, Krivickas LS, Hughes VA, Goldstein R and Roubenoff R, (2000b). Skeletal muscle fiber quality in older men and women. American Journal of Physiology - Cell Physiology. 279, C611-8.
Fryer MW, Owen VJ, Lamb GD and Stephenson DG, (1995). Effects of creatine phosphate and P(i) on Ca2+ movements and tension development in rat skinned skeletal muscle fibres. J Physiol. 482 (Pt 1), 123-40.
Fukuda S, Kaga S, Zhan L, Bagchi D, Das DK, Bertelli A and Maulik N, (2006). Resveratrol ameliorates myocardial damage by inducing vascular endothelial growth factor-angiogenesis and tyrosine kinase receptor Flk-1. Cell Biochem Biophys. 44, 43-9.
Fulgoni VL, 3rd, (2008). Current protein intake in America: analysis of the National Health and Nutrition Examination Survey, 2003-2004. Am J Clin Nutr. 87, 1554S-1557S.
Gardiner PF and Olha AE, (1987). Contractile and electromyographic characteristics of rat plantaris motor unit types during fatigue in situ. J Physiol. 385, 13-34.
Gilliver SF, Jones DA, Rittweger J and Degens H, (2010a). Effects of oxidation on the power of chemically skinned rat soleus fibres. J Musculoskelet Neuronal Interact. in press.
Gilliver SF, Jones DA, Rittweger J and Degens H, (2010b). Effects of oxidation on the power of chemically skinned rat soleus fibres. J Musculoskelet Neuronal Interact. 10, 267-73.
Gliemann L, Olesen J, Bienso RS, Schmidt J, Akerstrom T, Nyberg M, Lindqvist A, Bangsbo J and Hellsten Y, (2014). Resveratrol Modulates the Angiogenic Response to Exercise Training in Skeletal Muscle of Aged Men. Am J Physiol Heart Circ Physiol.

Goffart S and Wiesner RJ, (2003). Regulation and co-ordination of nuclear gene expression during mitochondrial biogenesis. Exp Physiol. 88, 33-40.
Goldman D, Bateman RM and Ellis CG, (2006). Effect of decreased O2 supply on skeletal muscle oxygenation and O2 consumption during sepsis: role of heterogeneous capillary spacing and blood flow. Am J Physiol Heart Circ Physiol. 290, H2277-85.
Gonzalez E, Messi ML and Delbono O, (2000). The specific force of single intact extensor digitorum longus and soleus mouse muscle fibers declines with aging. Journal of Membrane Biology. 178, 175-83.
Grimby G, Aniansson A, Zetterberg C and Saltin B, (1984). Is there a change in relative muscle fibre composition with age? Clinical Physiology. 4, 189-94.
Grimby G and Saltin B, (1983). The ageing muscle. Clinical Physiology. 3, 209-18.
Gutmann E and Carlson BM, (1976). Regeneration and transplantation of muscles in old rats and between young and old rats. Life Sci. 18, 109-14.
Hakkinen K, Newton RU, Gordon SE, McCormick M, Volek JS, Nindl BC, Gotshalk LA, Campbell WW, Evans WJ, Hakkinen A, Humphries BJ and Kraemer WJ, (1998). Changes in muscle morphology, electromyographic activity, and force production characteristics during progressive strength training in young and older men. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 53, B415-23.
Harris BA, (2005). The influence of endurance and resistance exercise on muscle capillarization in the elderly: a review. Acta Physiol Scand. 185, 89-97.
Hart N, Sarga L, Csende Z, Koltai E, Koch LG, Britton SL, Davies KJ, Kouretas D, Wessner B and Radak Z, (2013). Resveratrol enhances exercise training responses in rats selectively bred for high running performance. Food Chem Toxicol. 61, 53-9.
Hashizume K and Kanda K, (1990). Neuronal dropout is greater in hindlimb motor nuclei than in forelimb motor nuclei in aged rats. Neurosci Lett. 113, 267-9.
Hashizume K, Kanda K and Burke RE, (1988a). Medial gastrocnemius motor nucleus in the rat: age-related changes in the number and size of motoneurons. J Comp Neurol. 269, 425-30.
Hashizume K, Kanda K and Burke RE, (1988b). Medial gastrocnemius motor nucleus in the rat: age-related changes in the number and size of motoneurons. Journal of Comparative Neurology. 269, 425-30.
Hawke TJ and Garry DJ, (2001). Myogenic satellite cells: physiology to molecular biology. J Appl Physiol (1985). 91, 534-51.
Heath GW, Hagberg JM, Ehsani AA and Holloszy JO, (1981). A physiological comparison of young and older endurance athletes. J Appl Physiol Respir Environ Exerc Physiol. 51, 634-40.
Hepple RT, Mackinnon SL, Goodman JM, Thomas SG and Plyley MJ, (1997). Resistance and aerobic training in older men: effects on VO2peak and the capillary supply to skeletal muscle. J Appl Physiol (1985). 82, 1305-10.

Hoofd L, (1995). Calculation of oxygen pressures in tissue with anisotropic capillary orientation. II. Coupling of two-dimensional planes. Math Biosci. 129, 25-39.
Hoofd L and Degens H, (2009). The influence of flow redistribution on working rat muscle oxygenation. Adv Exp Med Biol. 645, 55-60.
Hoofd L and Degens H, (2013). Statistical treatment of oxygenation-related data in muscle tissue. Adv Exp Med Biol. 789, 137-42.
Hoofd L, Turek Z, Kubat K, Ringnalda BE and Kazda S, (1985). Variability of intercapillary distance estimated on histological sections of rat heart. Adv Exp Med Biol. 191, 239-47.
Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B and Sinclair DA, (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 425, 191-6.
Hudlicka O, Brown M, Cotter M, Smith M and Vrbova G, (1977). The effect of long-term stimulation of fast muscles on their blood flow, metabolism and ability to withstand fatigue. Pflugers Arch. 369, 141-9.
Ingram DK, (2000). Age-related decline in physical activity: generalization to nonhumans. Medicine & Science in Sports & Exercise. 32, 1623-9.
Ishihara A and Araki H, (1988). Effects of age on the number and histochemical properties of muscle fibers and motoneurons in the rat extensor digitorum longus muscle. Mech Ageing Dev. 45, 213-21.
Ishihara A, Naitoh H and Katsuta S, (1987). Effects of ageing on the total number of muscle fibers and motoneurons of the tibialis anterior and soleus muscles in the rat. Brain Res. 435, 355-8.
Ivey FM, Tracy BL, Lemmer JT, NessAiver M, Metter EJ, Fozard JL and Hurley BF, (2000). Effects of strength training and detraining on muscle quality: age and gender comparisons. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 55, B152-7; discussion B158-9.
Jackson JR, Ryan MJ and Alway SE, (2011). Long-term supplementation with resveratrol alleviates oxidative stress but does not attenuate sarcopenia in aged mice. J Gerontol A Biol Sci Med Sci. 66, 751-64.
Janssen I, Heymsfield SB, Wang ZM and Ross R, (2000). Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. Journal of Applied Physiology. 89, 81-8.
Jones DA, (1996). High-and low-frequency fatigue revisited. Acta Physiol Scand. 156, 265-70.
Jones DA, (2010). Changes in the force-velocity relationship of fatigued muscle: implications for power production and possible causes. J Physiol. 588, 2977-86.
Jones DA, Round J and De Haan A, 2004. Skeletal muscle from molecules to movement: a textbook of muscle physiology for sport, exercise, physiotherapy and medicine, Elsevier.
Jubrias SA, Odderson IR, Esselman PC and Conley KE, (1997). Decline in isokinetic force with age: muscle cross-sectional area and specific force. Pflugers Archiv. 434, 246-53.

Kadhiresan VA, Hassett CA and Faulkner JA, (1996). Properties of single motor units in medial gastrocnemius muscles of adult and old rats. J Physiol. 493 (Pt 2), 543-52.
Kadi F, Charifi N, Denis C and Lexell J, (2004). Satellite cells and myonuclei in young and elderly women and men. Muscle Nerve. 29, 120-7.
Kamel HK, (2003). Sarcopenia and aging. Nutr Rev. 61, 157-67.
Kanda K, (2002). Effects of food restriction on motoneuronal loss with advancing age in the rat. Microsc Res Tech. 59, 301-5.
Kanda K, Hashizume K, Miwa T and Miwa Y, (1996). Overloading a muscle does not alter the rate of motoneuronal loss in aged rats. Neurobiol Aging. 17, 613-7.
Kawamura Y, O'Brien P, Okazaki H and Dyck PJ, (1977). Lumbar motoneurons of man II: the number and diameter distribution of large- and intermediate-diameter cytons in "motoneuron columns" of spinal cord of man. Journal of Neuropathology & Experimental Neurology. 36, 861-70.
Kayani AC, Close GL, Dillmann WH, Mestril R, Jackson MJ and McArdle A, (2010a). Overexpression of HSP10 in skeletal muscle of transgenic mice prevents the age-related fall in maximum tetanic force generation and muscle Cross-Sectional Area. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 299, R268-76.
Kayani AC, Close GL, Dillmann WH, Mestril R, Jackson MJ and McArdle A, (2010b). Overexpression of HSP10 in skeletal muscle of transgenic mice prevents the age-related fall in maximum tetanic force generation and muscle Cross-Sectional Area. Am J Physiol Regul Integr Comp Physiol. 299, R268-76.
Kirkeby S and Garbarsch C, (2000). Aging affects different human muscles in various ways. An image analysis of the histomorphometric characteristics of fiber types in human masseter and vastus lateralis muscles from young adults and the very old. Histol Histopathol. 15, 61-71.
Klein CS, Marsh GD, Petrella RJ and Rice CL, (2003). Muscle fiber number in the biceps brachii muscle of young and old men. Muscle Nerve. 28, 62-8.
Klein CS, Rice CL and Marsh GD, (2001). Normalized force, activation, and coactivation in the arm muscles of young and old men. Journal of Applied Physiology. 91, 1341-9.
Klitgaard H, Mantoni M, Schiaffino S, Ausoni S, Gorza L, Laurent-Winter C, Schnohr P and Saltin B, (1990). Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds. Acta Physiol Scand. 140, 41-54.
Konopka AR and Sreekumaran Nair K, (2013). Mitochondrial and skeletal muscle health with advancing age. Mol Cell Endocrinol. 379, 19-29.
Konopka AR, Suer MK, Wolff CA and Harber MP, (2014). Markers of human skeletal muscle mitochondrial biogenesis and quality control: effects of age and aerobic exercise training. J Gerontol A Biol Sci Med Sci. 69, 371-8.

Kosek DJ, Kim JS, Petrella JK, Cross JM and Bamman MM, (2006). Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. J Appl Physiol (1985). 101, 531-44.
Kreuzer F and Hoofd L, 1987. Facilitated diffusion of oxygen and carbon dioxide, Handbook of Physiology. American Physiological Society, pp. 89-111.
Kunstyr I and Leuenberger HG, (1975). Gerontological data of C57BL/6J mice. I. Sex differences in survival curves. J Gerontol. 30, 157-62.
Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P and Auwerx J, (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell. 127, 1109-22.
Lamboley CR, Wyckelsma VL, Dutka TL, McKenna MJ, Murphy RM and Lamb GD, (2015). Contractile properties and sarcoplasmic reticulum calcium content in type I and type II skeletal muscle fibres in active aged humans. J Physiol.
Larkin LM, Kuzon WM and Halter JB, (2003). Effects of age and nerve-repair grafts on reinnervation and fiber type distribution of rat medial gastrocnemius muscles. Mech Ageing Dev. 124, 653-61.
Larsson L, (1978). Morphological and functional characteristics of the ageing skeletal muscle in man. A cross-sectional study. Acta Physiologica Scandinavica Supplementum. 457, 1-36.
Larsson L and Ansved T, (1995). Effects of ageing on the motor unit. Prog Neurobiol. 45, 397-458.
Larsson L, Ansved T, Edstrom L, Gorza L and Schiaffino S, (1991). Effects of age on physiological, immunohistochemical and biochemical properties of fast-twitch single motor units in the rat. J Physiol. 443, 257-75.
Larsson L and Edstrom L, (1986). Effects of age on enzyme-histochemical fibre spectra and contractile properties of fast- and slow-twitch skeletal muscles in the rat. J Neurol Sci. 76, 69-89.
Larsson L, Grimby G and Karlsson J, (1979). Muscle strength and speed of movement in relation to age and muscle morphology. Journal of Applied Physiology. 46, 451-6.
Larsson L, Li X and Frontera WR, (1997a). Effects of aging on shortening velocity and myosin isoform composition in single human skeletal muscle cells. American Journal of Physiology. 272, C638-49.
Larsson L, Li X, Yu F and Degens H, (1997b). Age-related changes in contractile properties and expression of myosin isoforms in single skeletal muscle cells. Muscle Nerve Supplement. 5, S74-8.
Larsson L and Moss RL, (1993). Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles. J Physiol. 472, 595-614.
Layec G, Haseler LJ and Richardson RS, (2013). Reduced muscle oxidative capacity is independent of O2 availability in elderly people. Age (Dordr). 35, 1183-92.

Lee CM, Lopez ME, Weindruch R and Aiken JM, (1998). Association of age-related mitochondrial abnormalities with skeletal muscle fiber atrophy. Free Radic Biol Med. 25, 964-72.
Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Nilwik R and van Loon LJ, (2013). Elderly men and women benefit equally from prolonged resistance-type exercise training. J Gerontol A Biol Sci Med Sci. 68, 769-79.
Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JF, Hidalgo J and Pilegaard H, (2009). PGC-1alpha mediates exercise-induced skeletal muscle VEGF expression in mice. Am J Physiol Endocrinol Metab. 297, E92-103.
Lexell J, (1995). Human aging, muscle mass, and fiber type composition. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 50 Spec No, 11-6.
Lexell J and Downham D, (1992). What determines the muscle cross-sectional area? J Neurol Sci. 111, 113-4.
Lexell J, Downham D and Sjostrom M, (1986). Distribution of different fibre types in human skeletal muscles. Fibre type arrangement in m. vastus lateralis from three groups of healthy men between 15 and 83 years. J Neurol Sci. 72, 211-22.
Lexell J, Taylor C and Sjostrom M, (1985). Analysis of sampling errors in biopsy techniques using data from whole muscle cross sections. Journal of Applied Physiology. 59, 1228-35.
Lexell J, Taylor CC and Sjostrom M, (1988). What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men. J Neurol Sci. 84, 275-94.
Lhotellier L and Cohen-Salmon C, (1989). Genetics and senescence. I. Age-related changes in activity and exploration in three inbred strains of mice. Physiol Behav. 45, 491-3.
Linderman JK and Blough ER, (2002). Aging does not attenuate plantaris muscle hypertrophy in male Fischer 344 rats. Medicine & Science in Sports & Exercise. 34, 1115-9.
Lipman RD, Chrisp CE, Hazzard DG and Bronson RT, (1996). Pathologic characterization of brown Norway, brown Norway x Fischer 344, and Fischer 344 x brown Norway rats with relation to age. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 51, B54-9.
Liu G, Mac Gabhann F and Popel AS, (2012). Effects of fiber type and size on the heterogeneity of oxygen distribution in exercising skeletal muscle. PLoS One. 7, e44375.
Lodder MA, De Haan A, Lind A and Sargeant AJ, (1993). Changes in morphological and functional characteristics of male rat EDL muscle during growth. J Muscle Res Cell Motil. 14, 47-53.
Lowe DA, Surek JT, Thomas DD and Thompson LV, (2001). Electron paramagnetic resonance reveals age-related myosin structural changes in rat skeletal muscle fibers. American Journal of Physiology - Cell Physiology. 280, C540-7.
Lushaj EB, Johnson JK, McKenzie D and Aiken JM, (2008). Sarcopenia accelerates at advanced ages in Fisher 344xBrown Norway rats. The

Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 63, 921-7.
Lynch GS, Hinkle RT, Chamberlain JS, Brooks SV and Faulkner JA, (2001). Force and power output of fast and slow skeletal muscles from mdx mice 6-28 months old. J Physiol. 535, 591-600.
Macaluso A, Nimmo MA, Foster JE, Cockburn M, McMillan NC and De Vito G, (2002). Contractile muscle volume and agonist-antagonist coactivation account for differences in torque between young and older women. Muscle Nerve. 25, 858-63.
Maden-Wilkinson TM, McPhee JS, Jones DA and Degens H, (2014a). Age Related Loss of Muscle Mass, Strength and Power and their Association With Mobility in Recreationally Active UK Older Adults. J Aging Phys Act.
Maden-Wilkinson TM, McPhee JS, Rittweger J, Jones DA and Degens H, (2014b). Thigh muscle volume in relation to age, sex and femur volume. Age (Dordr). 36, 383-93.
Manini TM, Hong SL and Clark BC, (2013). Aging and muscle: a neuron's perspective. Current Opinion in Clinical Nutrition & Metabolic Care. 16, 21-6.
Marchal J, Pifferi F and Aujard F, (2013). Resveratrol in mammals: effects on aging biomarkers, age-related diseases, and life span. Ann N Y Acad Sci. 1290, 67-73.
Marzetti E, Calvani R, Bernabei R and Leeuwenburgh C, (2011). Apoptosis in Skeletal Myocytes: A Potential Target for Interventions against Sarcopenia and Physical Frailty - A Mini-Review. Gerontology.
Marzetti E, Calvani R, Cesari M, Buford TW, Lorenzi M, Behnke BJ and Leeuwenburgh C, (2013). Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. Int J Biochem Cell Biol. 45, 2288-301.
Masoro EJ, (1980). Mortality and growth characteristics of rat strains commonly used in aging research. Exp Aging Res. 6, 219-33.
Masuda K, Kano Y and Katsuta S, (1997). Adaptation of myoglobin in compensatory hypertrophied rat muscle. Acta Physiol Scand. 160, 327-31.
Mayhew DL, Kim JS, Cross JM, Ferrando AA and Bamman MM, (2009). Translational signaling responses preceding resistance training-mediated myofiber hypertrophy in young and old humans. J Appl Physiol (1985). 107, 1655-62.
McArdle A, Dillmann WH, Mestril R, Faulkner JA and Jackson MJ, (2004). Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction. FASEB J. 18, 355-7.
McKiernan SH, Bua E, McGorray J and Aiken J, (2004). Early-onset calorie restriction conserves fiber number in aging rat skeletal muscle. The FASEB Journal. 18, 580-1.
McPhee JS, Maden-Wilkinson TM, Narici MV, Jones DA and Degens H, (2014). Knee extensor fatigue resistance of young and older men and women performing sustained and brief intermittent isometric contractions. Muscle Nerve.

Mittal KR and Logmani FH, (1987). Age-related reduction in 8th cervical ventral nerve root myelinated fiber diameters and numbers in man. J Gerontol. 42, 8-10.
Morley JE, (2003). Anorexia and weight loss in older persons. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 58, 131-7.
Morley JE, Baumgartner RN, Roubenoff R, Mayer J and Nair KS, (2001). Sarcopenia. J Lab Clin Med. 137, 231-43.
Morley JE, Thomas DR and Wilson MM, (2006). Cachexia: pathophysiology and clinical relevance. Am J Clin Nutr. 83, 735-43.
Morse CI, Thom JM, Reeves ND, Birch KM and Narici MV, (2005). In vivo physiological cross-sectional area and specific force are reduced in the gastrocnemius of elderly men. Journal of Applied Physiology. 99, 1050-5.
Murase T, Haramizu S, Ota N and Hase T, (2009). Suppression of the aging-associated decline in physical performance by a combination of resveratrol intake and habitual exercise in senescence-accelerated mice. Biogerontology. 10, 423-34.
Murray MP, Duthie EH, Jr., Gambert SR, Sepic SB and Mollinger LA, (1985). Age-related differences in knee muscle strength in normal women. J Gerontol. 40, 275-80.
Murray MP, Gardner GM, Mollinger LA and Sepic SB, (1980). Strength of isometric and isokinetic contractions: knee muscles of men aged 20 to 86. Phys Ther. 60, 412-9.
National institute for public health and the environment Mohwas, (2011). Dutch national food consumption survey.
Newman AB, Yanez D, Harris T, Duxbury A, Enright PL and Fried LP, (2001). Weight change in old age and its association with mortality. J Am Geriatr Soc. 49, 1309-18.
NIA, (2012). National Institute of Aging, http://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/strain-survival-information.
Nilwik R, Snijders T, Leenders M, Groen BB, van Kranenburg J, Verdijk LB and van Loon LJ, (2013). The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. Exp Gerontol. 48, 492-8.
Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I and Brownlee M, (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature. 404, 787-90.
O'Connor KG, Tobin JD, Harman SM, Plato CC, Roy TA, Sherman SS and Blackman MR, (1998). Serum levels of insulin-like growth factor-I are related to age and not to body composition in healthy women and men. J Gerontol A Biol Sci Med Sci. 53, M176-82.
Olesen J, Kiilerich K and Pilegaard H, (2010). PGC-1alpha-mediated adaptations in skeletal muscle. Pflugers Arch. 460, 153-62.
Olesen J, Ringholm S, Nielsen MM, Brandt CT, Pedersen JT, Halling JF, Goodyear LJ and Pilegaard H, (2013). Role of PGC-1alpha in exercise training- and resveratrol-induced prevention of age-associated inflammation. Exp Gerontol. 48, 1274-84.

Opalach K, Rangaraju S, Madorsky I, Leeuwenburgh C and Notterpek L, (2010). Lifelong calorie restriction alleviates age-related oxidative damage in peripheral nerves. Rejuvenation Res. 13, 65-74.
Overend TJ, Cunningham DA, Paterson DH and Lefcoe MS, (1992). Thigh composition in young and elderly men determined by computed tomography. Clinical Physiology. 12, 629-40.
Pagala MK, Ravindran K, Namba T and Grob D, (1998). Skeletal muscle fatigue and physical endurance of young and old mice. Muscle Nerve. 21, 1729-39.
Pansarasa O, Flati V, Corsetti G, Brocca L, Pasini E and D'Antona G, (2008). Oral amino acid supplementation counteracts age-induced sarcopenia in elderly rats. American Journal of Cardiology. 101, 35E-41E.
Park SJ, Ahmad F, Philp A, Baar K, Williams T, Luo H, Ke H, Rehmann H, Taussig R, Brown AL, Kim MK, Beaven MA, Burgin AB, Manganiello V and Chung JH, (2012). Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. Cell. 148, 421-33.
Paterson DH, Jones GR and Rice CL, (2007). Ageing and physical activity: evidence to develop exercise recommendations for older adults. Can J Public Health. 98 Suppl 2, S69-108.
Pearson SJ, Young A, Macaluso A, Devito G, Nimmo MA, Cobbold M and Harridge SD, (2002). Muscle function in elite master weightlifters. Med Sci Sports Exerc. 34, 1199-206.
Petrella JK, Kim JS, Cross JM, Kosek DJ and Bamman MM, (2006). Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. Am J Physiol Endocrinol Metab. 291, E937-46.
Petrella JK, Kim JS, Mayhew DL, Cross JM and Bamman MM, (2008). Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. J Appl Physiol (1985). 104, 1736-42.
Pettigrew FP and Gardiner PF, (1987). Changes in rat plantaris motor unit profiles with advanced age. Mech Ageing Dev. 40, 243-59.
Phillips SK, Bruce SA and Woledge RC, (1991). In mice, the muscle weakness due to age is absent during stretching. J Physiol. 437, 63-70.
Phillips SK, Wiseman RW, Woledge RC and Kushmerick MJ, (1993). Neither changes in phosphorus metabolite levels nor myosin isoforms can explain the weakness in aged mouse muscle. J Physiol. 463, 157-67.
Plyley MJ, Olmstead BJ and Noble EG, (1998). Time course of changes in capillarization in hypertrophied rat plantaris muscle. J Appl Physiol (1985). 84, 902-7.
Porter MM, Vandervoort AA and Lexell J, (1995). Aging of human muscle: structure, function and adaptability. Scand J Med Sci Sports. 5, 129-42.
Powers SK and Lennon SL, (1999). Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. Proc Nutr Soc. 58, 1025-33.

Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, Agarwal B, Ye L, Ramadori G, Teodoro JS, Hubbard BP, Varela AT, Davis JG, Varamini B, Hafner A, Moaddel R, Rolo AP, Coppari R, Palmeira CM, de Cabo R, Baur JA and Sinclair DA, (2012). SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab. 15, 675-90.
Proctor DN, Sinning WE, Walro JM, Sieck GC and Lemon PW, (1995). Oxidative capacity of human muscle fiber types: effects of age and training status. J Appl Physiol (1985). 78, 2033-8.
Quinn R, (2005). Comparing rat's to human's age: how old is my rat in people years? Nutrition. 21, 775-7.
Ramamurthy B, Hook P, Jones AD and Larsson L, (2001). Changes in myosin structure and function in response to glycation. Faseb J. 15, 2415-22.
Rice CL, Cunningham DA, Paterson DH and Lefcoe MS, (1989). Arm and leg composition determined by computed tomography in young and elderly men. Clinical Physiology. 9, 207-20.
Rice KM, Linderman JK, Kinnard RS and Blough ER, (2005). The Fischer 344/NNiaHSd X Brown Norway/BiNia is a better model of sarcopenia than the Fischer 344/NNiaHSd: a comparative analysis of muscle mass and contractile properties in aging male rat models. Biogerontology. 6, 335-43.
Ringholm S, Olesen J, Pedersen JT, Brandt CT, Halling JF, Hellsten Y, Prats C and Pilegaard H, (2013). Effect of lifelong resveratrol supplementation and exercise training on skeletal muscle oxidative capacity in aging mice; impact of PGC-1alpha. Exp Gerontol. 48, 1311-8.
Rivard A, Berthou-Soulie L, Principe N, Kearney M, Curry C, Branellec D, Semenza GL and Isner JM, (2000). Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem. 275, 29643-7.
Rivard A, Fabre JE, Silver M, Chen D, Murohara T, Kearney M, Magner M, Asahara T and Isner JM, (1999). Age-dependent impairment of angiogenesis. Circulation. 99, 111-20.
Roseberg IH, (1989). Summary comments. Am J Clin Nutr. 50: 1231-1233.
Roubenoff R and Hughes VA, (2000). Sarcopenia: current concepts. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 55, M716-24.
Rowan SL, Purves-Smith FM, Solbak NM and Hepple RT, (2011). Accumulation of severely atrophic myofibers marks the acceleration of sarcopenia in slow and fast twitch muscles. Exp Gerontol. 46, 660-9.
Rowan SL, Rygiel K, Purves-Smith FM, Solbak NM, Turnbull DM and Hepple RT, (2012). Denervation causes fiber atrophy and myosin heavy chain co-expression in senescent skeletal muscle. PLoS One. 7, e29082.
Runge M, Rittweger J, Russo CR, Schiessl H and Felsenberg D, (2004). Is muscle power output a key factor in the age-related decline in physical performance? A comparison of muscle cross section, chair-

rising test and jumping power. Clin Physiol Funct Imaging. 24, 335-40.
Ryan MJ, Jackson JR, Hao Y, Williamson CL, Dabkowski ER, Hollander JM and Alway SE, (2010). Suppression of oxidative stress by resveratrol after isometric contractions in gastrocnemius muscles of aged mice. J Gerontol A Biol Sci Med Sci. 65, 815-31.
Sato T, Akatsuka H, Kito K, Tokoro Y, Tauchi H and Kato K, (1984). Age changes in size and number of muscle fibers in human minor pectoral muscle. Mech Ageing Dev. 28, 99-109.
Sattler FR, (2013). Growth hormone in the aging male. Best Pract Res Clin Endocrinol Metab. 27, 541-55.
Scelsi R, Marchetti C and Poggi P, (1980). Histochemical and ultrastructural aspects of m. vastus lateralis in sedentary old people (age 65--89 years). Acta Neuropathol. 51, 99-105.
Schuenke MD, Brooks NE and Hikida RS, (2011). Interactions of aging, overload, and creatine supplementation in rat plantaris muscle. J Aging Res. 2011, 393416.
Selsby JT, Morine KJ, Pendrak K, Barton ER and Sweeney HL, (2012). Rescue of dystrophic skeletal muscle by PGC-1alpha involves a fast to slow fiber type shift in the mdx mouse. PLoS One. 7, e30063.
Sheard PW and Anderson RD, (2012). Age-related loss of muscle fibres is highly variable amongst mouse skeletal muscles. Biogerontology. 13, 157-67.
Shimokawa I, Higami Y, Hubbard GB, McMahan CA, Masoro EJ and Yu BP, (1993). Diet and the suitability of the male Fischer 344 rat as a model for aging research. J Gerontol. 48, B27-32.
Singh MA, Ding W, Manfredi TJ, Solares GS, O'Neill EF, Clements KM, Ryan ND, Kehayias JJ, Fielding RA and Evans WJ, (1999). Insulin-like growth factor I in skeletal muscle after weight-lifting exercise in frail elders. American Journal of Physiology. 277, E135-43.
Skorjanc D, Dunstl G and Pette D, (2001). Mitochondrial enzyme defects in normal and low-frequency-stimulated muscles of young and aging rats. J Gerontol A Biol Sci Med Sci. 56, B503-9.
Slivka D, Raue U, Hollon C, Minchev K and Trappe S, (2008). Single muscle fiber adaptations to resistance training in old (>80 yr) men: evidence for limited skeletal muscle plasticity. Am J Physiol Regul Integr Comp Physiol. 295, R273-80.
Smith DWE, 1993. Human Longevity, Oxford University Press, New York.
Snow LM, Fugere NA and Thompson LV, (2007). Advanced glycation end-product accumulation and associated protein modification in type II skeletal muscle with aging. J Gerontol A Biol Sci Med Sci. 62, 1204-10.
Sohal RS, Agarwal S, Candas M, Forster MJ and Lal H, (1994). Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mech Ageing Dev. 76, 215-24.
Spangler EL, Waggie KS, Hengemihle J, Roberts D, Hess B and Ingram DK, (1994). Behavioral assessment of aging in male Fischer 344 and brown Norway rat strains and their F1 hybrid. Neurobiol Aging. 15, 319-28.

Sprott RL, (1991). Development of animal models of aging at the National Institute of Aging. Neurobiol Aging. 12, 635-8.
Sprott RL and Ramirez I, (1997). Current Inbred and Hybrid Rat and Mouse Models for Gereontological Research. ILAR J. 38, 104-109.
Stienen GJ, Kiers JL, Bottinelli R and Reggiani C, (1996). Myofibrillar ATPase activity in skinned human skeletal muscle fibres: fibre type and temperature dependence. J Physiol. 493 (Pt 2), 299-307.
Storer JB, (1966). Longevity and gross patholgoy at death in 22 inbred strains of mice. J Gerontol. 404-409.
Sugiura M and Kanda K, (2004). Progress of age-related changes in properties of motor units in the gastrocnemius muscle of rats. J Neurophysiol. 92, 1357-65.
Suzuki J, Gao M, Batra S and Koyama T, (1997). Effects of treadmill training on the arteriolar and venular portions of capillary in soleus muscle of young and middle-aged rats. Acta Physiol Scand. 159, 113-21.
Takeuchi H, Sekine S, Noguchi O, Murano Y, Aoyama T and Matsuo T, (2009). Effect of life-long dietary n-6/n-3 fatty acid ratio on life span, serum lipids and serum glucose in Wistar rats. J Nutr Sci Vitaminol (Tokyo). 55, 394-9.
Thompson LV and Brown M, (1999). Age-related changes in contractile properties of single skeletal fibers from the soleus muscle. Journal of Applied Physiology. 86, 881-6.
Thurman JD, Bucci TJ, Hart RW and Turturro A, (1994). Survival, body weight, and spontaneous neoplasms in ad Libitum-fed and food-restricted Fischer-344 rats. Toxicol Pathol. 22, 1-9.
Timmers S, Auwerx J and Schrauwen P, (2012). The journey of resveratrol from yeast to human. Aging (Albany NY). 4, 146-58.
Timson BF, (1990). Evaluation of animal models for the study of exercise-induced muscle enlargement. J Appl Physiol (1985). 69, 1935-45.
Tisdale MJ, (2005). The ubiquitin-proteasome pathway as a therapeutic target for muscle wasting. J Support Oncol. 3, 209-17.
Tomlinson BE and Irving D, (1977). The numbers of limb motor neurons in the human lumbosacral cord throughout life. J Neurol Sci. 34, 213-9.
Tomonaga M, (1977). Histochemical and ultrastructural changes in senile human skeletal muscle. J Am Geriatr Soc. 25, 125-31.
Turek Z, Olders J, Hoofd L, Egginton S, Kreuzer F and Rakusan K, (1989). PO2 histograms in various models of tissue oxygenation in skeletal muscle. Adv Exp Med Biol. 248, 227-37.
Turek Z, Rakusan K, Olders J, Hoofd L and Kreuzer F, (1991). Computed myocardial PO2 histograms: effects of various geometrical and functional conditions. J Appl Physiol (1985). 70, 1845-53.
Turturro A, Duffy P, Hass B, Kodell R and Hart R, (2002). Survival characteristics and age-adjusted disease incidences in C57BL/6 mice fed a commonly used cereal-based diet modulated by dietary restriction. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 57, B379-89.
Turturro A, Witt WW, Lewis S, Hass BS, Lipman RD and Hart RW, (1999). Growth curves and survival characteristics of the animals used in the Biomarkers of Aging Program. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 54, B492-501.

Um JH, Park SJ, Kang H, Yang S, Foretz M, McBurney MW, Kim MK, Viollet B and Chung JH, (2010). AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. Diabetes. 59, 554-63.
United Nations ESA, (2011). World Population Prospects: The 2010 Revision. United Nations.
Urbanchek MG, Picken EB, Kalliainen LK and Kuzon WM, Jr., (2001). Specific force deficit in skeletal muscles of old rats is partially explained by the existence of denervated muscle fibers. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. 56, B191-7.
van der Laarse WJ, Des Tombe AL, Lee-De Groot MBE and Diegenbach PC, (1998). Size principle of striated muscle cells. Neth J Zool. 48(3), 213-23.
van der Laarse WJ, des Tombe AL, van Beek-Harmsen BJ, Lee-de Groot MB and Jaspers RT, (2005). Krogh's diffusion coefficient for oxygen in isolated Xenopus skeletal muscle fibers and rat myocardial trabeculae at maximum rates of oxygen consumption. J Appl Physiol (1985). 99, 2173-80.
van der Laarse WJ, Diegenbach PC and Elzinga G, (1989). Maximum rate of oxygen consumption and quantitative histochemistry of succinate dehydrogenase in single muscle fibres of Xenopus laevis. J Muscle Res Cell Motil. 10, 221-8.
van der Laarse WJ, van Noort P and Diegenbach PC, (1992). Calibration of quantitative histochemical methods: estimation of glycogen content of muscle fibers using the PAS reaction. Biotech Histochem. 67, 303-8.
Van der Meer SF, Jaspers RT and Degens H, (2011a). Is the myonuclear domain size fixed? J Musculoskelet Neuronal Interact. 11, 286-97.
van der Meer SF, Jaspers RT, Jones DA and Degens H, (2011b). Time-course of changes in the myonuclear domain during denervation in young-adult and old rat gastrocnemius muscle. Muscle Nerve. 43, 212-22.
van Wessel T, de Haan A, van der Laarse WJ and Jaspers RT, (2010). The muscle fiber type-fiber size paradox: hypertrophy or oxidative metabolism? Eur J Appl Physiol. 110, 665-94.
Vandervoort AA, (2002). Aging of the human neuromuscular system. Muscle Nerve. 25, 17-25.
Vandervoort AA and McComas AJ, (1986). Contractile changes in opposing muscles of the human ankle joint with aging. Journal of Applied Physiology. 61, 361-7.
Velasco B, Cacicedo L, Escalada J, Lopez-Fernandez J and Sanchez-Franco F, (1998). Growth hormone gene expression and secretion in aging rats is age dependent and not age-associated weight increase related. Endocrinology. 139, 1314-20.
Verdijk LB, Dirks ML, Snijders T, Prompers JJ, Beelen M, Jonkers RA, Thijssen DH, Hopman MT and J. vLL, (2012). Reduced satellite cell numbers with spinal cord injury and aging in humans. Medicine & Science in Sports & Exercise. 44, 2322-30.
Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH and van Loon LJ, (2007). Satellite cell content is specifically reduced in type II skeletal

muscle fibers in the elderly. Am J Physiol Endocrinol Metab. 292, E151-7.
Visser M and Schaap LA, (2011). Consequences of sarcopenia. Clin Geriatr Med. 27, 387-99.
Wagatsuma A, (2006). Effect of aging on expression of angiogenesis-related factors in mouse skeletal muscle. Exp Gerontol. 41, 49-54.
Wanagat J, Cao Z, Pathare P and Aiken JM, (2001). Mitochondrial DNA deletion mutations colocalize with segmental electron transport system abnormalities, muscle fiber atrophy, fiber splitting, and oxidative damage in sarcopenia. The FASEB Journal. 15, 322-32.
Wang ZM, Messi ML and Delbono O, (2000). L-Type Ca(2+) channel charge movement and intracellular Ca(2+) in skeletal muscle fibers from aging mice. Biophys J. 78, 1947-54.
Weerappuli DP and Popel AS, (1989). A model of oxygen exchange between an arteriole or venule and the surrounding tissue. J Biomech Eng. 111, 24-31.
Westerterp KR, (2000). Daily physical activity and ageing. Current Opinion in Clinical Nutrition & Metabolic Care. 3, 485-8.
WHO, (2013). World Health Statistics. World Health Organization, http://www.who.int/gho/publications/world_health_statistics/EN_WHS2013_Full.pdf.
Widrick JJ, Trappe SW, Blaser CA, Costill DL and Fitts RH, (1996a). Isometric force and maximal shortening velocity of single muscle fibers from elite master runners. American Journal of Physiology. 271, C666-75.
Widrick JJ, Trappe SW, Blaser CA, Costill DL and Fitts RH, (1996b). Isometric force and maximal shortening velocity of single muscle fibers from elite master runners. Am J Physiol. 271, C666-75.
Wineinger MA, Sharman RB, Stevenson TR, Carlsen RC and McDonald RB, (1995). Peripheral nerve and muscle function in the aging Fischer 344/brown-Norway rat. Growth, development and aging. 59, 107-19.
Wolf NS, Li Y, Pendergrass W, Schmeider C and Turturro A, (2000). Normal mouse and rat strains as models for age-related cataract and the effect of caloric restriction on its development. Exp Eye Res. 70, 683-92.
Wust RC, Gibbings SL and Degens H, (2009a). Fiber capillary supply related to fiber size and oxidative capacity in human and rat skeletal muscle. Adv Exp Med Biol. 645, 75-80.
Wust RC, Jaspers RT, van Heijst AF, Hopman MT, Hoofd LJ, van der Laarse WJ and Degens H, (2009b). Region-specific adaptations in determinants of rat skeletal muscle oxygenation to chronic hypoxia. Am J Physiol Heart Circ Physiol. 297, H364-74.
Young A, Stokes M and Crowe M, (1984). Size and strength of the quadriceps muscles of old and young women. Eur J Clin Invest. 14, 282-7.
Young A, Stokes M and Crowe M, (1985). The size and strength of the quadriceps muscles of old and young men. Clinical Physiology. 5, 145-54.
Yu BP, Masoro EJ, Murata I, Bertrand HA and Lynd FT, (1982). Life span study of SPF Fischer 344 male rats fed ad libitum or restricted diets:

longevity, growth, lean body mass and disease. J Gerontol. 37, 130-41.
Yu F, Hedstrom M, Cristea A, Dalen N and Larsson L, (2007). Effects of ageing and gender on contractile properties in human skeletal muscle and single fibres. Acta Physiol (Oxf). 190, 229-41.
Yuan R, Tsaih SW, Petkova SB, Marin de Evsikova C, Xing S, Marion MA, Bogue MA, Mills KD, Peters LL, Bult CJ, Rosen CJ, Sundberg JP, Harrison DE, Churchill GA and Paigen B, (2009). Aging in inbred strains of mice: study design and interim report on median lifespans and circulating IGF1 levels. Aging Cell. 8, 277-87.
Yun JM, Chien A, Jialal I and Devaraj S, (2012). Resveratrol up-regulates SIRT1 and inhibits cellular oxidative stress in the diabetic milieu: mechanistic insights. J Nutr Biochem. 23, 699-705.
Zerba E, Komorowski TE and Faulkner JA, (1990a). Free radical injury to skeletal muscles of young, adult, and old mice. Am J Physiol. 258, C429-35.
Zerba E, Komorowski TE and Faulkner JA, (1990b). Free radical injury to skeletal muscles of young, adult, and old mice. American Journal of Physiology. 258, C429-35.
Zurcher C and Van Zweiten M, 1982. Aging research: In the mouse in biomedical research: Experimental biology and oncology, Academic Press, New York.

About the author

Sam Ballak was born on April 10th 1986 in Sittard, The Netherlands. He attended high school at Trevianum in Sittard and graduated from the Gymnasium (VWO+) in 2004. After successfully acquiring his Bachelor degree Health Sciences with major Movement Sciences and minor Bioregulation and Health, he obtained his Master's degree in the Biology of Human Performance and Health in 2009, both at the faculty of Human Movement Sciences at Maastricht University. His Master internship was about the impact of caffeine and protein on post exercise muscle glycogen synthesis.

Later, Sam worked as a research assistant at the faculty of Human Movement Sciences on a project about protein supplementation during resistance-type exercise training old men and women (2010-2011). To further follow his interest about muscle ageing and training he started his PhD in 2011, investigating the effect of ageing on determinants of muscle force generating and endurance capacity. This project was part of the European Move-Age consortium, including the Catholic University Leuven, VU University Amsterdam and the Manchester Metropolitan University. During his PhD, Sam was based in Manchester and had mobility periods in both Amsterdam and Leuven.

Currently Sam works as a sport scientist and exercise physiologist for InnoSportLab Papendal and NOC*NSF. He tests and monitors the performance of athletes training at the National Olympic centre Papendal.