Alterations in Skeletal Muscle Plasticity with Smoking, Smoking Cessation and Vitamin D Deficiency in Mice

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PhD 2020

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

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2020

The work presented in this thesis was carried out as part of a joint doctorate at the Research Group for Rehabilitation in Internal Disorders, Department of Rehabilitation sciences, Katholieke Universiteit Leuven, Belgium and Department of Life Sciences, Research Centre for Musculoskeletal Science and Sports Medicine, Faculty of Science and Engineering, Manchester Metropolitan University, United Kingdom. This research was funded by the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate Program (2011-2015).

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General acknowledgements

I wish to express my profound and heartfelt gratitude to my supervising team, Prof. Ghislaine Gayan-Ramirez, Prof. Thierry Troosters and Prof. Hans Degens for securing the funding for this project and selecting me to be part of it. I am extremely grateful for your immense knowledge, motivation, encouragement, confidence, enthusiasm, patience, hard work, close supervision and follow up and for taking time off your busy schedules to personally oversee my day to day progress.

Special thanks to the examination committee at KU Leuven. Thank you, Prof. Sabine Verschueren, Prof. Benoit Nemery and Prof. Peter Hespel for following up my scientific and academic progress and keeping me on track all through my PhD training. Thank you, Prof. Maria Cordero for coming on board at short notice as internal committee member from MMU.

Thank you, Prof. Hieronymus Van Hees for accepting our invitation and coming on board as external jury member.

I am grateful to Prof. Wim Janssens, the senior staff and technicians of the Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), KU Leuven, for giving me office space and allowing me to carry out my laboratory work there.

Special thanks to Dr Karen Maes and Prof. Ghislaine Gayan-Ramirez for supervising my daily activities and ensuring the smooth running of my experiments. Your open-door policy, technical advice and scientific assistance were priceless.

Thank you very much, Prof. Thierry Troosters for always thinking out of the box and for your constant constructive criticism.

Thank you, Anita and Eline for assisting with administrative procedures and purchases.

Special thanks to Prof. Hans Degens, Guy, Paul and our colleagues of the Research Centre for Musculoskeletal Science & Sports Medicine, Manchester Metropolitan University, for allowing me to use the labs and facilities there. Thank you, Hans, for your particular interest in my welfare, making me feel at home and the numerous official and unofficial meetings. Thank you, Guy, for

accompanying me all through the time spent in Manchester and our lifelong friendship that started from there.

Thank you, Dr Rob W. for your technical and practical help and deep scientific insights.

Special thanks to Jef, David, Toby, Caro, Annelore, Anke, Birger, Siva, Nilakash, Manosij, Daniel, and all my lab mates for the memorable time spent together doing science and numerous extracurricular activities.

I also wish to thank Prof. Jaap van Dieen, Sjouke and the MOVE-AGE administrative team for their constant support, without which it would have been much more difficult for me. Thank you particularly for the start-up course in Manchester and annual conferences in Manchester and Amsterdam which contributed significantly to our training.

My heartfelt gratitude goes to my friends Mike, Kwe Claude, Stephanie, Nadine, Derick, Luchuo (just to name a few), and Papa Benjamen, Mama Carine and my CMFI Brussels family for their constant moral and psychological support which made me press on against all odds.

I am equally immensely grateful to my wife Andy Blondel Ajime for creating a conducive environment at home and supporting me through the last moments.

My heartfelt gratitude goes to my biological parents, Ajime Moses and Ajime Rachel and my sisters for the strict work ethic they instilled in me and their numerous calls and messages through which I still feel their presence thousands of miles away from home.

Most importantly, I wish to express special thanks to God almighty, the custodian of all knowledge and wisdom for his guidance.

List of abbreviations

Akt	Protein kinase B
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
BAL	Bronchoalveolar Lavage
Ca2+	Calcium ion
CD	Capillary Density
CFD	Capillary Fiber Density
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette smoke
DEXA	Dual-energy X-ray Absorptiometry
EDL	Extensor Digitorum Longus
ERK	Extracellular Signal–Regulated Kinase
FCSA	Fiber Cross Sectional Area
FoxO	Forkhead box O
HIF-1α	Hypoxia-inducible factor-1α
HRP	Horseradish Peroxidase
IGF1	Insulin-like Growth Factor 1
IL-6	Interleukin 6
LCFR	Local Capillary-to-Fiber Ratio
$Log_D SD$	Logarithmic standard deviation of the domain areas

MAFBx	Muscle Atrophy F-Box protein
MAPK	Mitogen-Activated Protein Kinase
MHC	Myosin Heavy Chain
MT-CO1	Mitochondrially encoded cytochrome c oxidase I
mTOR	Mammalian Target of Rapamycin
MURF-1	Muscle Ring Finger protein-1
NDUFB8	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide-3-kinase
SDH	Succinate Dehydrogenase
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
TBS	Tris-buffered Saline
TNF-α	Tumor Necrosis Factor Alpha
T-tubule	Transverse Tubule
UQCRC2	Ubiquinol-cytochrome c reductase core protein 2
USP-19	Ubiquitin-Specific Protease-19
VDR	Vitamin D Receptor
WHO	World Health Organization

CHAPTER 1

General Introduction

1 General introduction

Despite several decades of rigid measures to curb the consumption of tobacco worldwide, cigarette smoking remains a major public health problem killing up to half of its consumers [1]. Though the main route of consumption of cigarette smoke is through the lungs, the effects of smoking go beyond the respiratory system. We were intrigued by the extrapulmonary deleterious effects of cigarette smoking, particularly on skeletal muscles, which has been an area of rising research interest in the last decades. Recent research has provided evidence in favor of direct deleterious effects of cigarette smoke on respiratory and limb skeletal muscles [2–6]. However, the mechanisms involved are not completely understood. The devastating effects of smoking on health usually develop slowly and finally manifest only later in life, a phenomenon which is sometimes called '*the smoking time-bomb*'. A better understanding of the etiology of smoking-induced skeletal muscle dysfunction may serve as an incentive to stop smoking before the development of debilitating effects of '*the smoking time-bomb*'. Restoration of skeletal muscle structure and function after long term smoking cessation, as demonstrated by increased fat and lean mass and restored contractility, have been described [7–9]. However, whether this restoration is already present within a few days or weeks of stopping smoking has not been explored yet.

Skeletal muscles normally respond to hypertrophic stimuli such as resistance exercise or overload by increasing the mass and cross-sectional area of their fibers – the hypertrophic response. This hypertrophic response is usually accompanied by adaptations such as increased angiogenesis, mitochondrial biogenesis, and enhanced protein synthesis [10]. However, these adaptations could be impaired by constituents of cigarette smoke, thereby attenuating the hypertrophic response. This has, however, not been investigated hitherto. On the other hand, vitamin D is essential for the maintenance of skeletal muscle structure and physiology. Its deficiency, which is highly prevalent worldwide has been linked to skeletal muscle dysfunction, producing effects such as atrophy and reduced strength which are similar to the smoking-induced effects on skeletal muscles. A recent study from our research group showed that vitamin D deficiency aggravated smoking-induced muscle wasting in mice [11]. Therefore, vitamin D deficiency alone or in

combination with cigarette smoking may impair the skeletal muscle hypertrophic response to overload. All experiments in this project were done using a smoking mouse model.

1.1 Skeletal muscle structure and physiology

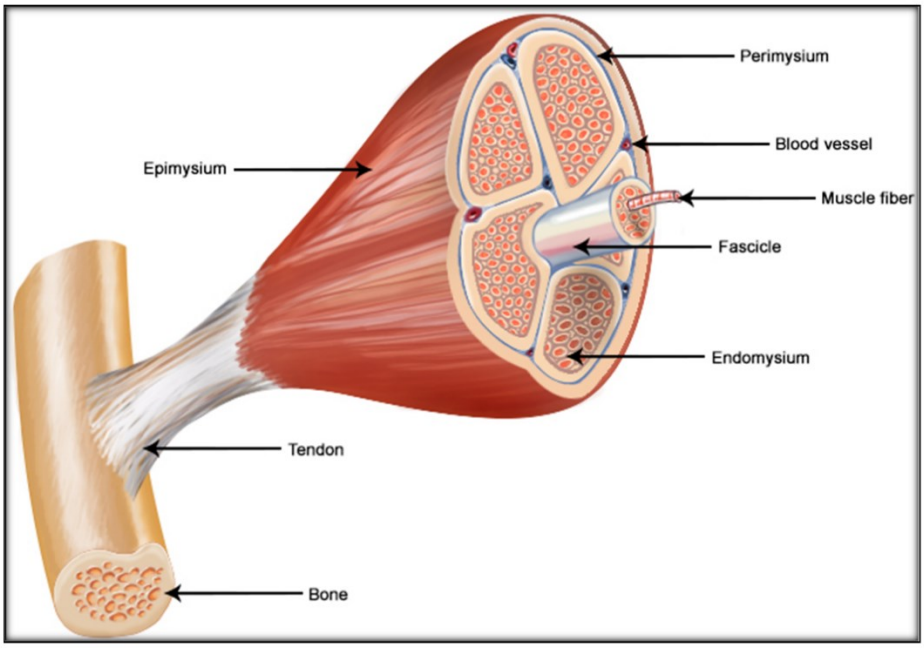
Skeletal muscle is the muscle which is attached to the skeleton through tendons. It makes up approximately 40% of human body mass. Muscle tissue consists of 75% water, 20% protein, and 5% organic salts, fat, minerals and carbohydrates [12]. Skeletal muscles contribute to several physical and metabolic processes in the body. Their main function is to generate power and force to maintain body posture and enable movement, thereby playing a major role in daily life activities. The metabolic roles of skeletal muscles include the maintenance of body temperature through the production of heat, a storage pool for carbohydrates and amino acids and the production of myokines [12,13].

Skeletal muscle is composed of muscle tissue, connective tissue, blood vessels and nerves. Its basic structure consists of numerous bundles of muscle fibers (muscle cells) within a connective tissue sheath (epimysium). Parts of the epimysium project inwardly and divide the muscle into compartments containing smaller bundles of muscle fibers called fascicles surrounded by the perimysium (Fig.1.1A). Each muscle fiber is composed of several myofibrils (Fig.1.1B) arranged within the endomysium (Fig.1.1A). Individual myofibrils consist of sarcomeres arranged in series. The sarcomere is the fundamental functional unit for muscle contraction comprised of a highly organized arrangement of the contractile proteins myosin and actin, and other molecules such as titin, extending from one Z-line to another [14,15] (Fig.1.1C) .

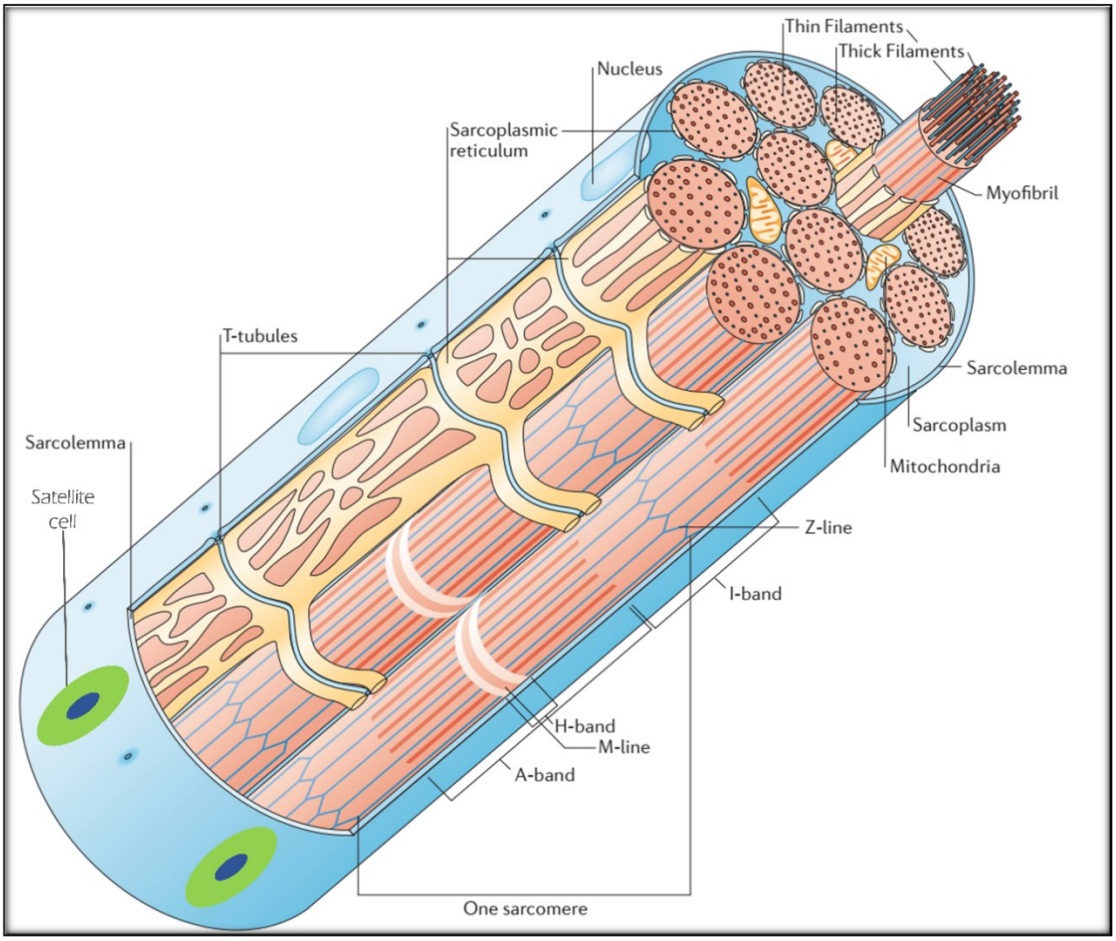
Skeletal muscle fibers are long cylindrical cells with numerous peripherally located nuclei which enable the supply of large amounts of proteins required for muscle function (Fig.1.1B). Each muscle fiber is surrounded by a plasma membrane called the sarcolemma. The sarcoplasm is the name given to their specialized cytoplasm which, like in other cells, encompasses other organelles such as mitochondria and a modified endoplasmic reticulum referred to as the sarcoplasmic reticulum which forms a dense network around the myofibrils and plays the key role of providing and storing calcium ions (Ca^{2+}) which are vital for contraction. The sarcolemma invaginates to form transverse tubules (T-tubules) which facilitate the transmission of action

potentials into the cell and trigger the release of Ca^{2+} from sarcoplasmic reticulum. Skeletal muscles also contain myogenic stem cells called satellite cells located beneath the basement membrane directly above the sarcolemma of the muscle fibers which play a major role in muscle growth, regeneration, differentiation and repair. Myofibrils consist of numerous longitudinal myofilaments which are either thin filaments made up of actin or thick filaments made up of myosin [16] (Fig.1.1B). Together, these filaments cause movement by contracting. Myosin molecules comprise myosin heavy chains (MHC, ~200 kDa) and myosin light chains (~20 kDa) [17]. The head portion of the MHC is critical for muscle contraction and force generation through the sliding filament model. Each skeletal muscle comprises diverse fiber types which are generally distinguished based on their specific isoforms of MHC [18,19].

A)


Perimysium
Blood vessel
Epimysium
Muscle fiber
Fascicle
Endomysium
Tendon
Bone

B)


Thin Filaments
Thick Filaments
Nucleus
Sarcoplasmic reticulum
Myofibril
T-tubules
Sarcolemma
Sarcoplasm
Mitochondria
Sarcolemma
Satellite cell
Z-line
I-band
H-band
M-line
A-band
One sarcomere

C)

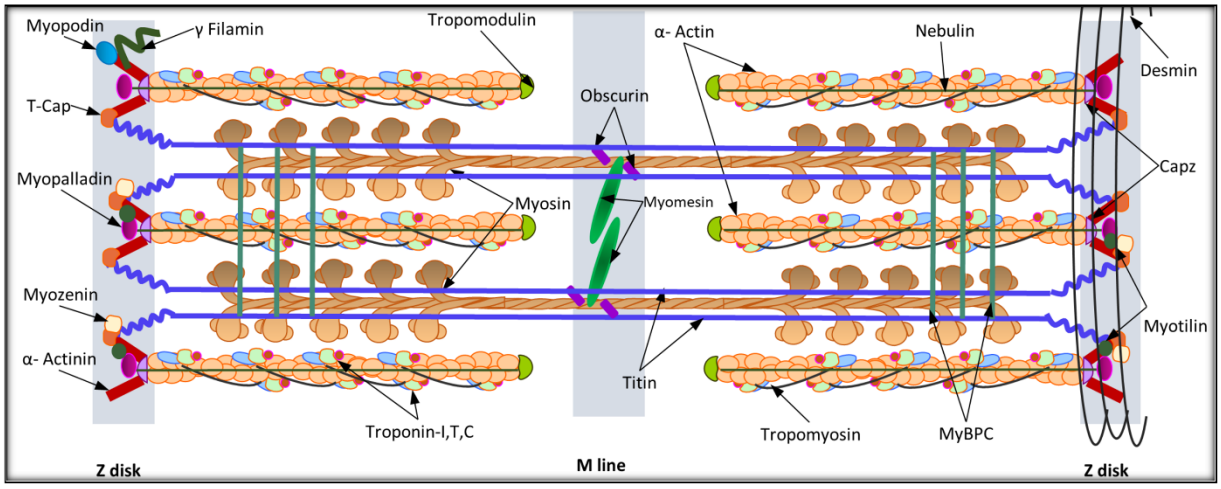


Figure 1.1: *General structure of skeletal muscle (A), skeletal muscle fiber (B) and Sarcomere showing actin and myosin filaments (C) which form cross bridges to generate force as described in text. Adapted from SEER training modules, muscular system [15]; Davies and Nowak., 2006 [16] and Mukund and Subramaniam., 2019 [20].*

1.2 Muscle fiber, fiber types, capillarization and mitochondrial function

Mammalian skeletal muscle is composed of different muscle fibers depending on its function. The diversity of fiber types in skeletal muscle makes it possible to use a muscle for various motor tasks such as maintaining posture, locomotion and maximal contractions [21]. The different fiber types are primarily classified based on their myosin heavy chain (MHC) isoform composition and currently, four major fiber types are distinguished: the type I express the MHC-I while the type IIA, IIX or IIB express the MHC-IIA, MHC-IIX or MHC-IIB respectively (Fig. 1.2). Other classifications have been reported based on oxidative capacity and resistance to fatigue [22–24] (Table 1.1). Adult human muscles do not express MHC isoform IIB, therefore their main fiber types are I, IIA and IIX [25].

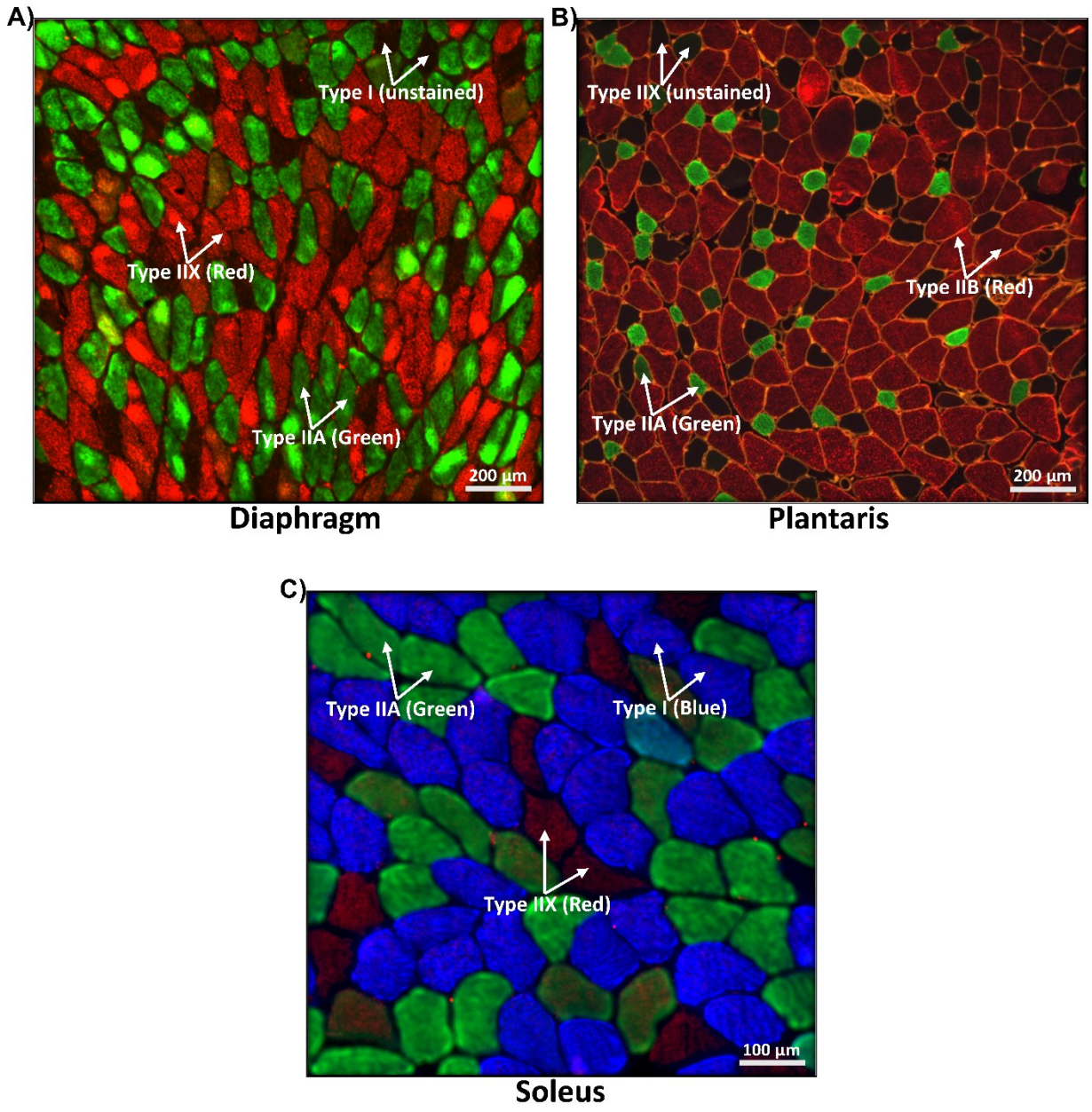


Figure 1.2: *Fiber type composition in skeletal muscles. Cross sections of the* ***diaphragm muscle [A]*** *(a constantly active respiratory muscle composed mainly of type IIA, IIX and a few type I fibers);* ***plantaris muscle [B]*** *(a limb muscle primarily involved in movement composed mainly of type II fibers); and* ***Soleus muscle [C]*** *(a limb muscle mainly involved in maintaining posture composed primarily of type I fibers) stained for different myosin heavy chain isoforms.*

Phenotypically, type I fibers (MHC-I) have a high mitochondrial content, high vascularization, high levels of myoglobin and are rich in oxidative enzyme content with very low glycolysis. These properties make them more resistant to fatigue as they rely on energy produced by oxidative metabolism while contracting slowly and generating little force over long periods of time. Type I fibers are more abundant in the leg muscles of endurance athletes such as marathon runners [20]. Type II fibers (MHC-IIA, MHC-IIX or MHC-IIB), have a lower mitochondrial content and high glycolytic activity which enables them to support short rapid bursts of high intensity activity, which is usually accompanied by an acceleration of adenosine triphosphate (ATP) hydrolysis. However, compared to type I fibers, they fatigue more easily while contracting faster and generating high forces over short periods of time. Type II fibers are more abundant in power and strength athletes such as sprinters [20]. Types IIA and IIX fibers have intermediate metabolic properties while type IIB fibers are the fastest contracting, mainly glycolytic, and easily fatigable. Type IIA fibers are similar to type I since they depend more on oxidative metabolism and have more myoglobin than the other subtypes (Table 1.1). Physiologically, type I (slow) and type II (fast) muscles can be differentiated based on their difference in calcium kinetics, molecular motor activity and twitch parameters such as half relaxation time and time to peak tension. Generally, type II fibers are less sensitive to Ca^{2+} concentration, show shorter twitch parameters, rapid sarcomere contraction velocity (high velocity of shortening) and generate higher mechanical power than type I [20,26,27].

Table 1.1: *Summary of the characteristics of the four major skeletal muscle fiber types*

Characteristic	**Fiber type**			
	I	**IIA**	**IIX**	**IIB**
Speed of contraction	Slow	Moderately fast	Fast	Very fast
Mitochondrial density	High	High	Low	Low
Force/Power produced	Low	Medium	High	Very high
MHC isoform	MHC-I	MHC-IIA	MHC-IIX	MHC-IIB
Capillary density	High	Intermediate	Low	Low
Glycolytic capacity	Low	High	High	Very high
Oxidative capacity	High	High	Low	Low
Fatigue resistance	High	Fairly high	Intermediate	Low
Twitch tension	Low	Moderate	High	High
Myoglobin content	High	Medium	Low	Low
Fiber diameter	Small	Intermediate	Large	Large
Muscle color	Red	Red	White	White

Muscles that need to generate sustained force, such as those involved in the maintenance of posture or balance, usually have a high percentage of oxidative fibers while those needed for fast, short contractions have a predominantly glycolytic phenotype. This is well illustrated by the muscles of the mouse calf complex where the soleus, which is mainly involved in maintenance of posture, is composed mainly of type I fibers (Fig. 1.2C), while the plantaris, involved in movement, is predominantly composed of type II fibers (Fig. 1.2B) and the gastrocnemius which controls both movement and posture is a mixed muscle [23]. The diaphragm, which is a constantly active

respiratory muscle, and requires a high resistance to fatigue is composed of, in order of abundance, types IIA, IIX, and I with a few hybrid fibers (Fig. 1.2A) [17,28].

The presence and spatial distribution of capillaries is crucial for the delivery of hormones, nutrients and oxygen, and disposal of waste, heat, and metabolites from muscle cells [10]. In agreement with the postulate that capillaries mainly supply oxygen [29,30] and mindful of the positive relationship between mitochondrial density and number of capillaries per fiber [31], oxidative muscles which have a higher demand for oxygen and more mitochondria tend to have a higher capillary density than glycolytic muscles [32].

Mitochondria are ubiquitous organelles that play major roles in skeletal muscle structure and function. They participate in ATP production through the electron transport chain (Fig. 1.3) and signaling via reactive oxygen species, and also are important for calcium homeostasis and apoptosis [33]. Most of the energy required for muscle contraction is produced by oxidative phosphorylation through the mitochondrial electron transport chain and released in the form of ATP, which serves as fuel for most contractile processes.

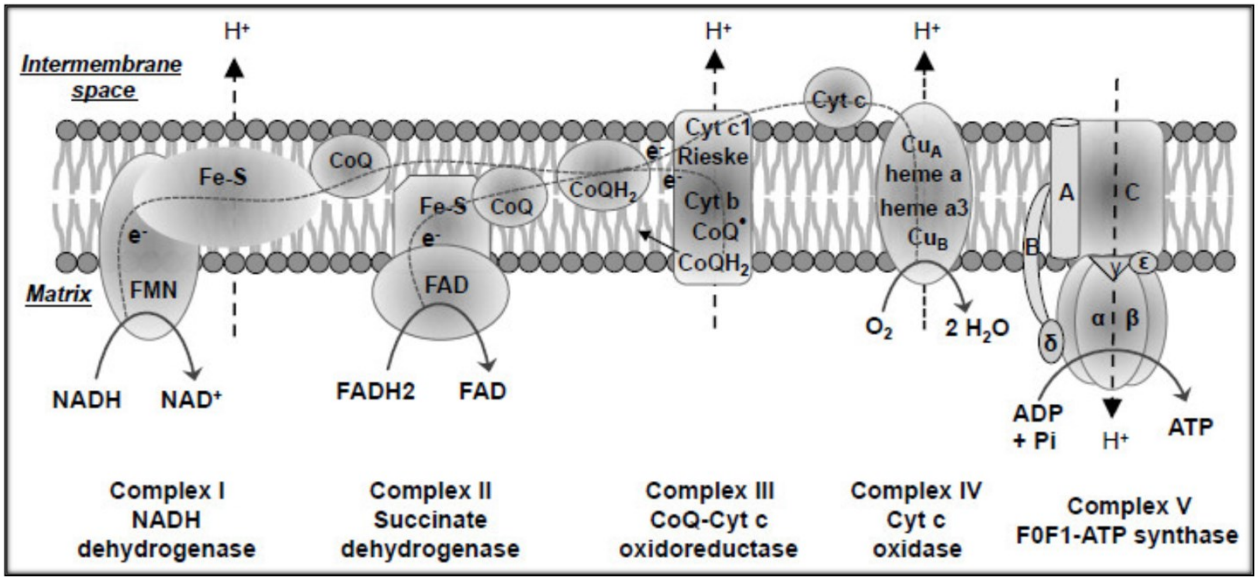


Figure 1.3: *Schematic representation of the mitochondrial electron transport chain. It is organized into five enzyme complexes (I – V) which, through the transfer of electrons in the presence of oxygen culminates in the production of ATP. Adapted from Luo, Ma and Lou., 2020 [34].*

Muscle fibers differ in the availability of various substrates and enzymes responsible for ATP turnover, and the diversity of fibers in skeletal muscles help to maintain homeostasis. In healthy muscles, resting ATP content and consumption is similar in fast and slow fibers, but, during exercise, the rate of consumption of ATP is much higher for fast fibers, though they also produce higher power and efficiency remains constant [35,36].

1.3 Skeletal muscle force and fatigue

To carry out their mechanical functions, skeletal muscles transform chemical energy into mechanical energy. The generation of force in the muscle occurs through a process of excitation-contraction coupling whereby the action potential from a nerve stimulates the release of calcium from the sarcoplasmic reticulum which leads to the interaction between myosin and actin to form cross-bridges which generate force through the sliding of actin and myosin filaments [37,38] (Fig. 1.1C). The force generated by each cross bridge is transmitted laterally and longitudinally within a fiber through the sarcomeres in series and movement is produced when the force reaches the tendons and joints. This implies that larger fibers with a higher content of myosin and actin will generate more cross bridges and produce more force than smaller fibers, indicating that force production depends to a large extent on fiber cross-sectional area. The contractile process requires ATP obtained from the breakdown of carbohydrates, fats and to a lesser extent, amino acids [39]. The breakdown of these fuels to generate ATP occurs through oxidative phosphorylation in muscle mitochondria, anaerobic glycolysis and reserves of creatine phosphate and ATP within the muscle [39].

Skeletal muscle fatigue refers to a decrease in maximal power or force generating capacity in response to sustained contractile activity or increased neural stimulation [40]. Adequate blood supply and optimal mitochondrial function are essential for a continuous regeneration of ATP needed for cross-bridge cycling and hence to produce force and dissipate heat and metabolites in the muscle. Central fatigue results from a decrease in neural stimulation of the muscle, while peripheral fatigue comes from alterations at the neuromuscular junction or distally [41]. Skeletal muscle fatigue also depends on fiber type composition, whereby muscles containing greater proportions of glycolytic (type II) fibers would be less resistant to fatigue and vice versa [21]. Ageing, chronic cigarette smoking and pathologies such as chronic obstructive pulmonary disease

(COPD), muscular dystrophies and cancer cachexia are associated with selective atrophy of different fiber types, fiber type shifts, decreased fatigue resistance and decreased force production due to decreased efficiency of contraction and reductions in oxidative capacity [42–44].

1.4 Skeletal muscle plasticity

Thanks to their plasticity, skeletal muscles can adapt their contractile and metabolic properties and modify their size or fiber types and proportions in response to a variety of stimuli like metabolic and hormonal influences, neuronal activity, mechanical stress, altitude and exercise/disuse within genetically defined boundaries [45]. Simple shifts in fiber type and proportion can lead to significant functional changes such as differences in tension and tissue level contractile characteristics, as slower contracting MHCs (I < IIA < IIX < IIB) produce less tension and vice versa which implies that phenotypic variations in single myofilaments can alter whole muscle performance [44,46,47]. Therefore, the quantity and type of MHC expressed in a muscle is crucial for the maintenance of adequate plasticity.

Muscle fiber cross-sectional area and mass are regulated by a tight equilibrium between protein synthesis and breakdown, both of which depend on diet, hormonal balance, neural stimulation, physical activity and disease or injury [12,14]. Skeletal muscle hypertrophy results from an increase in fiber cross-sectional area (FCSA) and mass at the cellular and whole tissue level which occurs naturally during postnatal growth and in response to neuronal activation, resistance exercise or overload whereby it is referred to as the hypertrophic response [48]. It occurs when muscle protein synthesis exceeds catabolism. Likewise, skeletal muscle atrophy is defined as a decline in FCSA and mass at the cellular and whole tissue level due to ageing, diet, disuse, denervation, or disease [48].

The insulin-like growth factor 1 (IGF1)–phosphoinositide-3-kinase (PI3K)–protein kinase B (Akt)–mammalian target of rapamycin (mTOR) pathway serves as a positive regulator in favor of protein synthesis and muscle growth while the myostatin–Smad3 pathway serves as a negative regulator in favor of protein degradation and muscle shrinking [49–53]. Hypertrophy occurs when the activities of the IGF1-PI3K-Akt-mTOR pathway exceed those of the myostatin–Smad3 pathway. Muscle atrophy involves the interplay between several atrophy-related genes whose down- or up-

regulation activate proteolytic systems. Most of the atrophy-related genes which are upregulated are involved in the autophagy–lysosome, the calcium-dependent and ubiquitin–proteasome systems. This up-regulation which is usually blocked by Akt through inhibition of Forkhead box O (FoxO) transcription factors negatively regulates protein synthesis and enhances protein catabolism and apoptosis [54–56].

1.5 The burden of cigarette smoking

Cigarette smoking is a long-standing worldwide epidemic and a major public health threat. Seven million smokers die every year, while 1.2 million deaths result from the exposure of non-smokers to second-hand smoke [1]. About 1.1 billion people are current smokers worldwide, 80% of whom are residents of low- and middle-income countries with limited access to health care [1]. In addition to the highly addictive effect of nicotine, cigarette smoke contains more than 5000 noxious chemicals which exert deleterious effects on the lungs and other organ systems [57,58]. Smoking cessation is possibly the most inexpensive and efficient way to combat this pandemic, though the addiction to nicotine makes it difficult. In fact, the earlier smoking cessation is attained, the longer the life expectancy of smokers [59]. Governments worldwide are making huge efforts to reduce cigarette smoking by advertising the dangers of smoking, imposing graphic health warnings on packages, increasing sales taxes and taxes on tobacco companies, organizing various campaigns with individuals and groups and telephone counseling just to name a few. Nicotine replacement therapy with nicotine and its derivatives in various forms (gum, patches, nasal spray, oral spray, inhalers and tablets) are also widely used and have shown 1-year cigarette smoking cessation success rates of 24% to 33% [60–62]. Despite the widespread awareness of the negative effects of smoking and accessibility of smoking cessation programs, the global prevalence of cigarette smoke exposure is still high.

The effects of smoking on the lungs, which are the main route of entry of cigarette smoke into the body, have been extensively described. Smoking has been identified as a causative factor for respiratory disorders such as COPD and lung cancer among others [63,64]. In addition, several direct smoking-induced extrapulmonary defects have been identified. Cigarette smoking has indeed been identified as a major risk factor for the development of cardiovascular diseases, various forms of cancer and sarcopenia [57]. Recent evidence has identified respiratory and peripheral

skeletal muscles as direct targets of cigarette smoke [2]. Considering the importance of skeletal muscles in the maintenance of posture, movement, and vital functions such as respiration, discovery and proper understanding of the mechanisms and manifestations of smoking-induced skeletal muscle dysfunction is crucial to avert the ensuing morbidities and mortality. Research in humans and animal models has shown that the damage induced by cigarette smoke in muscles results from increased oxidative stress and inflammation, impaired muscle metabolic processes, activation of various proteolytic pathways, inhibition of various protein synthesis pathways and overexpression of atrophy related genes [2,58].

1.5.1 Composition of cigarette smoke, potentially toxic ingredients on skeletal muscles

More than 5000 constituents have been discovered in CS and the number of unidentified components could be as many as 100000 [65,66]. Mainstream CS, the smoke which is voluntarily drawn from the mouth-end of a cigarette during a puff, consists of a vapor phase and a particulate phase [67]. From a clinical and biological perspective, the major components of CS can be classified as follows: (A) Free radicals such as superoxide, hydroxyl radicals, nitric oxide and hydrogen peroxide, which are present in both phases; (B) Vapor phase components consisting of Carbon monoxide (CO) and other constituents such as formaldehyde, acrolein, acetaldehyde, carbon dioxide and nitrogen oxides; (C) Tar and nicotine which are the main components of the particulate phase [65,67–69].

The vapor and particulate phases of CS contain billions of highly reactive free radicals which can directly induce oxidative stress in circulation and macromolecular damage [69]. The CS-induced increase in circulating oxidative stress coupled to the fact that active smokers and animal models exposed to CS have lower levels of antioxidants could lead to increased oxidative stress in skeletal muscles [68,70]. Some of these reactive oxygen species and reactive nitrogen species could get into the blood and cause oxidative damage in skeletal muscles. In fact, this has been observed in the quadriceps muscles of smokers and the limb and respiratory muscles of CS-exposed animals [3,71]. Increased oxidative stress could, in the long run leads to increased muscle proteolysis and atrophy [58].

The constituents of the vapor phase of CS could impair skeletal muscle protein turnover and metabolism. Smoking a single cigarette can increase the acetaldehyde levels in circulation by up to 9.5 µM [72]. Exposure of human myocytes to acetaldehyde, which is also a major metabolite of alcohol metabolism, led to reductions in basal protein synthesis [73]. Also, reductions in protein synthesis by up to 15% have been observed in the soleus and plantaris muscles of rats injected with acetaldehyde [74]. These studies show that acetaldehyde, which is a major constituent of CS vapor phase, inhibits skeletal muscle protein synthesis. Similarly, exposure of mouse skeletal myotubes to acrolein, another toxic aldehyde which is present in CS, led to degradation of myosin heavy chain, activation of p38 MAPK and catabolic signaling, increased production of free radicals, upregulation of muscle-specific E3 ligases and myotube atrophy [75]. This indicates that acrolein from CS promotes skeletal muscle catabolism resulting in muscle atrophy.

CO, which is present in high concentrations in CS has a 210-fold higher affinity for hemoglobin than oxygen [76]. Therefore, even in relatively low concentrations, CO can be very toxic. Its binding to hemoglobin leads to the formation of carboxyhemoglobin which reduces the oxygen carrying capacity of the blood and could impair skeletal muscle oxygenation. Moreover, the binding of CO to myoglobin leads to its partial inactivation through the formation of carboxy-myoglobin which could also reduce muscle oxygenation and impair muscle function [77]. A study in isolated mitochondria from human skeletal muscles showed that CO also directly inhibits cytochrome c oxidase, one of the enzyme complexes in the mitochondrial respiratory chain [78]. Exposure to CO alone and CS have been linked to increased skeletal muscle fatigability in healthy humans [79,80]. This implies that CO can indeed impair muscle function.

CS can also lead to increased systemic inflammatory activity through the activation of inflammatory cascades which gives rise to the production of pro-inflammatory cytokines [81]. Exposure to CS has been shown to decrease the production of anti-inflammatory cytokines like IL-10 and increase the production of pro-inflammatory cytokines such as IL-6, IL-1, IL-8 and TNF-α which enhance muscle proteolysis and inhibit protein synthesis and can thereby induce muscle wasting [68,81–84].

Nicotine, the main component of the particulate phase of CS, is the major pharmacologically active ingredient of CS. It is also the main chemical responsible for addiction to CS. It induces insulin resistance by decreasing the release of insulin by the beta cells of the pancreas and together with other components of CS , it impairs the action of insulin which may contribute to the reduction in protein turnover and promote catabolic signaling in skeletal muscles and other organs [85].

Although several components of CS are present in very small quantities, dissipate rapidly and may not get to skeletal muscles, other components are relatively stable and are transported through the bloodstream to skeletal muscles where they impair skeletal muscle homeostasis leading to metabolic dysfunction and deleterious structural changes in the long run.

1.5.2 Smoking-induced skeletal muscle dysfunction

As indicated earlier, cigarette smoking has been identified as a risk factor for the development of skeletal muscle dysfunction. Just like many other pathologies, the development of smoking-induced skeletal muscle dysfunction is progressive and multifactorial. The identified consequences of cigarette smoking on skeletal muscles as observed in empirical and epidemiological studies in humans and animal models include: atrophy [86,87], weakness [88–90], decreased mitochondrial function and bioenergetics [91–93], reduced force and fatigue resistance [3,80,88], type II fiber atrophy [93,94], decreased protein synthesis [84,95], increased proteolysis [96,97], increased inflammation and oxidative stress [3,71], capillary regression [5,98], reduced vasodilation and decreased perfusion [84,99]. Most of these studies focused on limb muscles. Surprisingly, few studies have evaluated the deleterious alterations induced by CS in the diaphragm which is the main respiratory muscle and is of particular relevance to patients who develop lung diseases as a consequence of smoking.

A study by Montes de Oca et al. [86] showed that cigarette smoking led to vastus lateralis atrophy as indicated by a decrease in mass and reduced cross-sectional area of type I and, more so, type II fibers, fiber type shifts towards a more glycolytic phenotype and decreased protein synthesis indicated by decreased expression of nitric oxide synthases in healthy smokers compared to non-smoking control subjects. Similar observations have been made in mice exposed to cigarette

smoke whereby a decrease in the mass and fiber dimensions of the soleus and extensor digitorum longus (EDL) muscles and EDL muscle fiber type shift towards a more glycolytic phenotype after 24 weeks of exposure were reported [87]. Though other factors such as physical inactivity, disuse and diet changes, which are also common in smokers [100]and smoke-exposed animal models [4]could induce skeletal muscle atrophy, the fact that decreased mass and fiber atrophy are still observed in smokers and smoke-exposed animals as compared to age, diet and physical activity matched controls indicates that smoking is indeed linked to muscle atrophy in smokers.

Muscle wasting resulting from decreased synthesis of essential contractile proteins and increased protein breakdown has been observed in smokers compared to non-smokers. In a study in humans, the quadriceps femoris muscle of smokers showed decreased protein fractional synthesis rate and increased expression of negative regulators of protein synthesis such as myostatin and muscle atrophy F-box (MAFBx) protein, a ubiquitin-mediated proteolytic marker specific to muscles [95]. Likewise, 8 – 16 weeks of exposure to cigarette smoke in mice led to gastrocnemius, plantaris and soleus muscle atrophy and a simultaneous increase in the expression of MAFBx and muscle ring finger-1 (MURF-1) another enhancer of proteolysis [84]. Another study in rats exposed to cigarette smoke for 4 – 12 weeks and L6 myoblasts showed that smoking leads to over-expression of ubiquitin-specific protease-19 (USP-19) and reduction of myosin heavy chain levels resulting in muscle wasting through activation of the p38/mitogen-activated protein kinase (MAPK)/ extracellular signal–regulated kinase (ERK) pathway [96]. Smoking-induced muscle wasting can also result from chronic increases in circulating proinflammatory cytokines. Increased levels of interleukin 6 (IL-6) have been reported in human smokers [95]and tumor necrosis factor (TNF)-α in smoking animal models [84], both of which enhance muscle proteolysis and inhibit protein anabolism which leads to muscle wasting and consequent atrophy [101]. Some circulating components of cigarette smoke and the cytokines they induce lead to muscle wasting and consequent atrophy by reducing protein anabolism and enhancing proteolysis leading to a shift of protein turnover in favor of proteolysis and net loss of muscle mass.

Smoking has also been associated with muscle weakness in humans and animal models. A 15 year longitudinal study in young healthy subjects by Kok et al. [89] showed that cigarette smoking

reduced quadriceps muscle strength in male and female smokers over time independently of physical activity. Another study in healthy male smokers revealed smoking-induced reductions in maximum isometric grip strength and fast fatigability compared to non-smokers [88]. Likewise, reduced forelimb grip strength has been reported in mice exposed to smoke for 16 – 24 weeks [90]. In addition to adverse biochemical changes, muscle weakness could also be attributable to atrophy since force development is dependent on muscle fiber cross sectional area and mass.

Skeletal muscle mitochondrial dysfunction and impaired bioenergetics have been identified in smokers. Studies in mice show that exposure of male C57BL6 mice to cigarette smoke for 6 – 14 weeks impaired mitochondrial function in the gastrocnemius and soleus muscles as indicated by the reductions in ATP production, decreased oxygen consumption rates and reductions in protein levels of complexes I, III and IV [91,92]. In healthy smokers, a decrease in complex IV (cytochrome c oxidase) activity has also been reported [93]. Likewise, in mitochondria extracted from healthy human skeletal muscles, carbon monoxide, a major component of cigarette smoke decreased the activity of cytochrome c oxidase [78]. Several components of cigarette smoke such as cyanides, aldehydes and carbon monoxide could directly impair the activity of mitochondrial enzyme complexes or indirectly through smoke-induced lung-secreted ceramides. These metabolic alterations may underlie some of the smoking-induced increases in fatigue and in the long run muscle atrophy and fiber type shift.

Reductions in skeletal muscle contractile properties have also been observed as a result of smoking. In a study by Barreiro et al [3], significant reductions in quadriceps muscle force were reported in healthy smokers together with increased oxidative modification of proteins involved in contraction, energy production, and glycolysis. A significant increase in protein oxidation in the diaphragm and gastrocnemius muscles of guinea pigs exposed to cigarette smoke was also reported in the same study [3]. Increased quadriceps muscle fatigue has also been reported in healthy male and female smokers compared to controls matched for age and physical activity [80]. Similar observations have been reported in mice whereby eight weeks of smoking led to 50% reduction in soleus and EDL muscle fatigue resistance associated with altered intracellular calcium handling, capillary regression, and depletion of satellite cells independently of lung function changes [5]. These observations prior to the development of characteristic respiratory

changes show that components of cigarette smoke and smoking-induced defects in calcium homeostasis and increased protein oxidation could contribute to the loss of muscle force, muscle wasting, fiber atrophy and other deleterious modifications.

An adequate quantity and quality of blood transported through capillaries to the muscle is vital for the supply of oxygen and nutrients and elimination of waste. However, the interference of various substances in cigarette smoke with muscle blood supply and capillarization may lead to impaired muscle mitochondrial function and deranged homeostasis[5,98]. Increased vascular stiffness and decreased vasodilatory capacity has been reported in active and passive smokers [99,102]. Also decreased skeletal muscle capillarization coupled with muscle fiber atrophy, reduced exercise tolerance and increased fatigability have been reported in the gastrocnemius, EDL and soleus muscles of mice after 8 – 24 weeks of smoking [5,98]. Therefore, smoking leads to reduced blood flow to the muscles and capillary rarefaction, which could also partially explain the increased fatigability. Moreover, cigarette smoke-induced reductions in the delivery of oxygen to the mitochondria, or the failure of the mitochondria to use oxygen due to indirect smoking-induced impairments may contribute to skeletal muscle dysfunction [2]. Furthermore, components of cigarette smoke have been reported to have a direct inhibitory effect on some muscle mitochondrial respiratory chain enzyme complexes [78]. The fact that skeletal muscle dysfunction is observed in healthy smokers [3,80,93]and smoke-exposed mice [5]independently of lung function decline indicates that skeletal muscle dysfunction is independent of the development of pulmonary disease and could be readily reversible.

1.6 Vitamin D and muscle

Apart from its classical effects in maintaining calcium and phosphorus homeostasis and bone mineralization, vitamin D plays an essential role in skeletal muscle growth, contractility and myogenesis. The identification of the vitamin D receptor (VDR) in skeletal muscles has highlighted the importance of the effects of vitamin D on skeletal muscles.

The actions of vitamin D in skeletal muscles mainly occur through the binding of its active form, 1α,25-dihydroxyvitamin D, to its cytoplasmic or nuclear receptor [103]. Its binding to nuclear VDR leads to the expression of some genes and consequent production of proteins involved in skeletal

muscle cell proliferation, myogenesis, and muscle calcium and phosphate homeostasis [104–107]. The binding of vitamin D to its cytoplasmic receptor leads to the activation of protein phosphorylation and signaling pathways regulating skeletal muscle growth, mitochondrial function, inflammation, calcium flux and muscle contraction [108–111]. Vitamin D stimulates muscle cell differentiation and myogenesis [112,113]. It has also been reported to enhance skeletal muscle hypertrophy and repair indicating a positive role in muscle plasticity [113,114]. It also acts indirectly on the muscle through its influence on phosphorus and calcium homeostasis [115]. Vitamin D plays a vital role in the maintenance of skeletal muscle trophic status and emerging evidence shows that it is also involved in the process of muscle regeneration and remodeling [116,117].

Vitamin D deficiency, which is defined in humans as 25-hydroxyvitamin D levels below 20 ng/ml (50 nmol per liter) in serum is highly prevalent worldwide, especially among the elderly [118]. More than 1 billion people are estimated to be vitamin D-deficient worldwide [119]. In recent years, the impact of vitamin D deficiency on skeletal muscle function and pathophysiology has been of rising interest. In humans and animal models, low vitamin D levels in serum have been linked to significant reductions in muscle mass [120], decreased strength [121], type II muscle fiber atrophy [115], decreased protein synthesis [114], increased proteolysis [122], impaired mitochondrial function and energy production [108], impaired capillarization, reduced vasodilation and decreased perfusion [113]. A study in rats revealed that vitamin D deficiency induces atrophy in the tibialis anterior muscle, a predominantly type II muscle, through attenuation of the notch pathway which plays a central role in the maintenance of satellite cells and muscle regeneration [120]. This effect was very mild in the soleus muscles, indicating preferential atrophy of type II muscle fibers with vitamin D deficiency [120]. Considering that the type II fast twitch muscles produce higher force and strength and are usually the first to be recruited in the maintenance of balance, it is not surprising that reduced skeletal muscle strength as demonstrated by reduced grip strength has been reported in vitamin D-deficient humans and animal models together with increased frequency of falls, especially in the elderly [115,121,122]. Increased levels of myostatin, enhanced expression of the atrophy marker MuRF1 and down-regulation of genes involved in calcium and phosphorus homeostasis have been reported in the quadriceps muscles of vitamin D-deficient mice [122]. Therefore, vitamin D deficiency, like cigarette smoking, leads to skeletal muscle wasting, fiber

atrophy and contractile dysfunction. In a study by Ryan et al [108], treatment of muscle cells and mitochondria extracted from human biopsies with 1 α,25-dihydroxyvitamin D3 led to increased mitochondrial oxygen consumption, increased mitochondrial volume and fusion, enhanced ATP production and increased expression of proteins involved in mitochondrial biogenesis. This indicates that vitamin D regulates muscle mitochondrial function. Consequently, the myopathy and muscle weakness observed in vitamin D-deficient skeletal muscles could be in part due to mitochondrial dysfunction.

The impact of chronic vitamin D deficiency on skeletal muscles is remarkably similar to cigarette smoke-induced skeletal muscle dysfunction and includes decreased muscle strength, reduced mass, muscle mitochondrial dysfunction, decreased vitamin D receptor expression, and impaired regeneration [11,120,121]. This indicates a possibility for synergistic deleterious effects of cigarette smoking and vitamin D deficiency. In fact, a recent study from our group showed that vitamin D deficiency worsened the cigarette smoke-induced muscle wasting in a smoking mouse model [11].

1.7 Smoking cessation

Smoking cessation is the best and most efficient way to avert the deleterious consequences of smoking on the respiratory system, other organ systems and skeletal muscles. A prospective cohort study with 1.2 million US adults showed that smoking cessation significantly increases life expectancy even in the elderly [59]. Though the smoking-induced deleterious changes in the lungs are irreversible [123], skeletal muscles could, thanks to their plasticity and regenerative capacity, recover from smoking-induced atrophy, wasting, weakness, decreased mitochondrial function and decreased fatigue resistance through smoking cessation. In a study in non-smoking and ex-smokers pairs of monozygotic twins the absence of vastus lateralis atrophy and absence of muscle mitochondrial dysfunction supports the notion that cessation of smoking can reverse skeletal muscle dysfunction [93]. Also, the observation of increased lean mass, higher functional muscle mass and fat mass after 16 months of smoking cessation in post-menopausal women suggests that the functional capacity of skeletal muscles is restored after smoking cessation [124]. In line with the aforementioned observations, the fact that both lean body mass and skeletal muscle force generating capacity of former smokers is not different from never smoking controls,

indicates that the recovery of muscle mass after smoking cessation is accompanied by a normalized muscle function [9]. Similarly, 2 months of smoking cessation in mice led to the reversal of soleus muscle atrophy and normalization of gastrocnemius and soleus muscle cell signaling after 24- weeks of cigarette smoke exposure [125]. These studies showcase the benefits of long-term smoking cessation on skeletal muscles. However, little is known about the mechanisms involved in the restoration of muscle structure and function after smoking cessation. A study in human lymphocytes showed that positive metabolic changes such as improved mitochondrial function are already evident within 24 hours of smoking cessation [126]. However, whether these early improvements also occur in skeletal muscles is not yet known. Demonstrating that skeletal muscle metabolism already improves within hours to a few days could serve as an additional incentive for smoking cessation.

1.8 Smoking, vitamin D deficiency and the hypertrophic response

The skeletal muscle hypertrophic response is an increase in muscle mass due to an increase in fiber size or fiber branching in response to exercise training or overloading a muscle by denervation or elimination of synergist muscles in mice and resistance training in humans [127–131]. As indicated earlier, muscle mass is maintained by a tight balance between protein synthesis and proteolysis. In a normal situation, the skeletal muscle hypertrophic response is characterized by increased synthesis of contractile and functional proteins and reduced proteolysis which leads to muscle hypertrophy [132]. On the other hand, cigarette smoking and vitamin D deficiency are characterized by increased proteolysis and reduced protein synthesis leading to muscle wasting and atrophy [95,122], implying that skeletal muscle hypertrophic response could be impaired by smoking or vitamin D deficiency or both. Acute reduction in oxygen supply and impaired blood supply to peripheral skeletal muscles after each cigarette smoke exposure may cause recurrent hypoxia, especially in chronic smokers. The normal reaction of the muscle to this recurrent hypoxia in a bid to maintain homeostasis could include adaptations like increased capillary proliferation, mitochondrial biogenesis and enhanced vasodilation [133,134]. Similar adaptations are also known to occur during skeletal muscle hypertrophy where an increase in size leads to increased demand for oxygen and energy which prompts increased mitochondrial biogenesis, enhanced angiogenesis and protein synthesis [127]. However, some studies show that the

aforementioned adaptations to intermittent hypoxia and hypertrophy are impaired by vitamin D deficiency and toxic constituents of cigarette smoke [11,92,98,120,121,135]. Satellite cells are mitotically quiescent muscle stem cells which, in adults, are activated to proliferate and fuse with their associated muscle fibers in response to increased protein and energy demands as a result of muscle damage, exercise and overload [136,137]. During the hypertrophic response, skeletal muscle satellite cells, which are crucial for fiber maintenance, remodeling, repair and regeneration, are activated to proliferate and fuse with their associated fibers, thereby providing additional nuclei required to cater for the increased metabolism and protein turnover [136,137]. Cigarette smoking been shown to cause depletion of satellite cells [5] and could potentially attenuate the skeletal muscle hypertrophic response. Also, considering the importance of vitamin D in the maintenance of muscle plasticity, regeneration and satellite cell function [138], vitamin D deficiency is expected to impair satellite cell function and consequently attenuate hypertrophic response. In line with the deleterious effects of smoking and vitamin D on skeletal muscle structure and function, an attenuated hypertrophic response is plausible with smoking or vitamin D deficiency. Also, taking into account the similarity between smoking- and vitamin D deficiency-induced skeletal muscle dysfunction, a combination of both factors could produce a synergistic deleterious effect on the hypertrophic response.

1.9 Smoking mouse model

The use of mouse models to study human health and disease has provided useful insights in physiology and pathophysiology and helped developing treatment strategies for various diseases. The extent of similarity between human and mouse genomes validates the study of mouse biology in a bid to better understand and characterize human biology [139]. For example, in the musculoskeletal system, it has been reported that the mouse soleus muscle, a predominantly oxidative muscle, expresses the closest molecular resemblance to several skeletal muscles in humans and is therefore suitable for the study of skeletal muscle physiology and mitochondrial function [140,141]. However, the inherent biological, biomechanical and temporal differences between species must be properly recognized and carefully considered in the interpretation and translation of the results to humans [141].

All experiments in this project were done using an established smoking mouse model. The model consists of mice placed in soft restrains attached to exposure towers where they are exposed to controlled quantities of cigarette smoke through a nose-only exposure system (InExpose System, SCIREQ, Montreal, Canada). The smoke-exposure system consists of a computer-generated puff which leads to the release of smoke from a lit cigarette through a pump system for 10 seconds followed by 50 seconds of room air. Mice are only exposed by the nose, another clear advantage over models where the whole body is continuously exposed to cigarette smoke [4,142]. Each cigarette generates 9 – 10 puffs. This method of active smoking in mice closely resembles the typical human smoking situation in which puffs of mainstream smoke are inhaled, and the levels of smoke and particulate matter are comparable to other studies in literature [87,143–145]. In our studies, the mice were exposed nose-only to six 3R4F research cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky) in soft restraints twice daily, 5 days a week for 14 to 18 weeks. Control mice were exposed to room air in soft restrains with the same frequency and for the same duration.

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CHAPTER 2

Rationale and Objectives

2 Rationale and Objectives

2.1 Rationale

The adverse effects of cigarette smoking on the lungs have been extensively studied and documented. However, in the last decades there has been much interest in the extrapulmonary impact of cigarette smoking. In addition to the indirect effects of cigarette smoke on muscles as a result of systemic inflammation and lung emphysema, recent evidence shows direct deleterious effects of smoking on limb and respiratory skeletal muscles. This evidence has been further supported by the observation of skeletal muscle dysfunction in smokers even before any respiratory symptoms were observed. However, the effects and mechanisms of smoking-induced skeletal muscle dysfunction are not fully understood. Also, mindful of the influence of physical activity and diet on skeletal muscle structure and function, it is important to properly control for physical activity and diet in the study of smoking-induced skeletal muscle dysfunction to ensure that the dysfunction observed is indeed due to smoking. This has not always been the case in previous studies.

Skeletal muscles have high plasticity, and, unlike the lungs, they may well recover from dysfunction induced by chronic cigarette smoking. In fact, the reversal of skeletal muscle atrophy after prolonged inactivity by exercising is well known and demonstrates their recovery capacity. However, this recovery capacity upon cessation of cigarette smoking and the mechanisms involved have been poorly studied. The long-term benefits of smoking cessation on skeletal muscles such as increased lean and fat mass, improved exercise tolerance, increased skeletal muscle mass and enhanced energy production and consumption have been propounded in long-term smoking cessation studies. Though the structural improvements in skeletal muscles may take long to develop and may be a consequence of gains in body weight, metabolic improvements such as improved mitochondrial function and enhanced protein synthesis may be already present within days to a few weeks after smoking cessation. This has not been investigated previously.

The skeletal muscle hypertrophic response is a normal response of the muscle to resistance training and sustained overload stimuli. It is generally characterized by increased muscle mass

and fiber cross-sectional area, increased protein synthesis and increased angiogenesis. However, vitamin D deficiency and noxious constituents of cigarette smoke are known to inhibit these processes and instead lead to muscle atrophy and increased catabolism. In addition, it is suggested that local hypoxia and systemic inflammation caused by cigarette smoking would also alter the muscle regeneration process and its response to hypertrophic stimuli. Also, in line with the role of vitamin D in skeletal muscle growth and regeneration, its deficiency may attenuate the skeletal muscle hypertrophic response. This suggests that cigarette smoking, vitamin D deficiency or both conditions may dampen the muscle hypertrophic response. This has hitherto not been investigated extensively.

With this project we intend to clarify whether 1 to 2 weeks of smoking cessation are associated with immediate improvements in skeletal muscle structure and function. If so, this could be a motivating factor for patients with muscle weakness struggling to stop smoking as they may feel the immediate beneficial effects of smoking cessation. This is mainly pertinent for patients in rehabilitation programs who are still actively smoking, or for those admitted to hospitals and suffering from acute or chronic muscle deterioration yet struggling with smoking cessation. The project will further help to determine the role of smoking and/or vitamin D deficiency on the skeletal muscle hypertrophic response, which is a normal response to muscle resistance training and overload. This is a relevant issue as it may provide useful insights on skeletal muscle response to exercise training and could be the first step towards interventions to restore or improve this response.

All studies were carried out using a C57Bl/6JolaH mouse model developed and optimized in our laboratory. In this model, mice were nose-only exposed to cigarette smoke.

2.2 Objectives and hypothesis

1. To assess whether short-term smoking cessation would improve the structure and mitochondrial function of limb and respiratory skeletal muscles. We hypothesized that short-term smoking cessation leads to improvements in the structure, mitochondrial function and capillarization of limb and respiratory muscle.

To assess the possible immediate benefits smoking cessation on skeletal muscles, we investigated if 1- to 2-weeks smoking cessation would improve respiratory and limb muscle structure, capillarization, contractile properties and mitochondrial function in a smoking mouse model.

2. To assess the effects of smoking on limb muscle hypertrophic response. We hypothesized that cigarette smoking would impair the hypertrophic response in skeletal muscles and even more so in combination with vitamin D deficiency.

3. To determine the impact of vitamin D deficiency on skeletal muscle regeneration and hypertrophic response during cigarette smoking. We hypothesized that vitamin D deficiency would blunt muscle hypertrophy in response to overload and even more so when combined with cigarette smoke.

   Knowing that the deleterious effects of vitamin D deficiency or smoking on skeletal muscle regeneration process share similarities, we explored the impact of vitamin D deficiency alone or smoking alone or a combination of both on skeletal muscle regeneration and hypertrophic response in mouse model of overload-induced hypertrophy.

CHAPTER 3

Two weeks smoking cessation reverses cigarette smoke-induced skeletal muscle atrophy and mitochondrial dysfunction in mice

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Nicotine & Tobacco Research, 2020 (doi:10.1093/ntr/ntaa016)

Advance Access publication January 22, 2020

This article has an online supplement that is included at the end of this chapter.

All data, text and figures are identical to the published article except for the effect sizes in percentage which have been added as well as minor additions in the text added for clarity.

3 Two weeks smoking cessation reverses cigarette smoke-induced skeletal muscle atrophy and mitochondrial dysfunction in mice

3.1 Abstract

Introduction

Apart from its adverse effects on the respiratory system, cigarette smoking also induces skeletal muscle atrophy and dysfunction. Whether short-term smoking cessation can restore muscle mass and function is unknown. We therefore studied the impact of 1- and 2-weeks smoking cessation on skeletal muscles in a mouse model.

Methods

Male mice were divided into 4 groups: Air-exposed (14 weeks); cigarette smoke (CS)-exposed (14 weeks); CS-exposed (13 weeks) followed by 1-week cessation; CS-exposed (12 weeks) followed by 2 weeks cessation to examine exercise capacity, physical activity levels, body composition, muscle function, capillarization, mitochondrial function and protein expression in the soleus, plantaris and diaphragm muscles.

Results

CS-induced loss of body and muscle mass was significantly improved within 1 week of cessation due to increased lean and fat mass. Mitochondrial respiration and protein levels of the respiratory complexes in the soleus were lower in CS-exposed mice, but similar to control values after 2 weeks of cessation. Exposing isolated soleus muscles to CS extracts reduced mitochondrial respiration that was reversed after removing the extract. While physical activity was reduced in all groups, exercise capacity, limb muscle force, fatigue resistance, fiber size and capillarization and diaphragm cytoplasmic HIF-1α were unaltered by CS-exposure. However, CS-induced diaphragm atrophy and increased capillary density was not seen after 2 weeks of smoking cessation.

Conclusion

In male mice, two weeks smoking cessation reversed smoking-induced mitochondrial dysfunction, limb muscle mass loss and diaphragm muscle atrophy, highlighting immediate benefits of cessation on skeletal muscles.

Key words: CS-exposure, Smoking cessation, muscle mass, mitochondrial function

3.2 Implications

Our study demonstrates that CS-induced skeletal muscle mitochondrial dysfunction and atrophy are significantly improved by 2 weeks cessation in male mice. We show for the first time that smoking cessation as short as 1 to 2 weeks is associated with immediate beneficial effects on skeletal muscle structure and function with the diaphragm being particularly sensitive to CS-exposure and cessation. This could help motivate smokers to quit smoking as early as possible. The knowledge that smoking cessation has potential positive extrapulmonary effects is particularly relevant for patients referred to rehabilitation programs and those admitted to hospitals suffering from acute or chronic muscle deterioration yet struggling with smoking cessation.

3.3 Introduction

Cigarette smoke (CS) is a complex mix of toxic substances exerting deleterious effects on different organs. While the adverse effects of CS on the respiratory system are well established, its extrapulmonary toxicity has only been highlighted during the last decades. In particular, the deleterious effects of CS on skeletal muscle function has emerged as an important extrapulmonary consequence of smoking [1,2].

Importantly, the observation that skeletal muscle dysfunction was present in smokers and CS-exposed mice prior to the development of any respiratory symptoms indicates that some compounds in CS could directly affect skeletal muscle function. Young smokers without respiratory symptoms have weaker and less fatigue resistant muscles compared to non-smokers [1,2]. Although part of these effects could have been related to decreased physical activity as reported in many smokers [3,4], CS-exposed animal models confirmed the loss of muscle force and resistance to fatigue prior to the development of any respiratory symptoms [5–8]. These models also revealed that both the respiratory muscles (in particular the diaphragm) and the limb muscles were affected by CS [5–8]. Muscle fiber atrophy with loss of muscle mass due to decreased protein synthesis, increased proteolysis, and enhanced protein oxidation in the muscle as reported in smokers and animal models might have contributed to the CS-induced muscle weakness [6,7,9–11]. In addition, impaired oxygen supply and blood flow to the muscle as a consequence of smoking may induce the expression of Hypoxia-inducible factor-1α (HIF-1α) that plays a crucial role in mitochondrial biogenesis, enhanced vasodilatory capacity and capillary proliferation [12]. However, these adaptations may be impaired by noxious substances in cigarette smoke [13]. Skeletal muscles require an adequate supply of blood by the capillary network for the delivery of nutrients, hormones and oxygen and removal of waste products, heat and metabolites [14]. Various substances in CS may interfere with muscle capillarization and blood supply which may culminate in impaired muscle homeostasis and deleterious structural changes in the long run [7,15,16]. Moreover, reduced oxygen delivery to the mitochondria as a result of CS-induced intermittent tissue hypoxia, or the inability of the mitochondria to use oxygen coupled to the direct effect of CS on the mitochondrial respiratory chain enzyme complexes [17,18] could also contribute to the loss of muscle function seen with CS [6,11,19]. Due to its role in breathing, the

diaphragm muscle is constitutively active even when the limb muscles are at rest and requires a constant supply of oxygen and nutrients. This implies that the noxious substances in CS may be constantly transported to the diaphragm making it more susceptible to CS-exposure. In fact, a recent study in mice reported CS-induced diaphragm muscle contractile dysfunction prior to the development of limb muscle alterations and emphysema [6].

Whether skeletal muscle impairment due to CS is reversible has been addressed in long-term smoking cessation studies. For instance, 16 months of smoking cessation was associated with an increase in fat and muscle mass in post-menopausal women [20], something also seen in other studies on smoking cessation [20,21]. Furthermore, the absence of fiber atrophy in muscles of former smokers and non-smokers pairs of monozygotic twins supports the notion that smoking cessation can lead to restoration of muscle mass [3]. Likewise, the observation that both the lean body mass and the force generating capacity of skeletal muscles in former smokers are not different from never smoking controls [22] seems to indicate that normalization of muscle function after cessation of smoking is associated with the recovery of muscle mass. A recent study in mice also confirmed that soleus mass can be normalized after 2 months of smoking cessation but gastrocnemius was still lower [23].

Studies in human lymphocytes from healthy smokers have shown that smoking cessation for as little as 24 hours to 7 days resulted in restored mitochondrial complex III and IV activities and increased maximal mitochondrial respiration [24]. These data suggest that smoking cessation may have immediate beneficial effects on mitochondrial function in blood cells. Whether this also occurs in skeletal muscles has never been explored so far. Therefore, the objective of the study was to comprehensively assess the impact of 1- or 2-weeks smoking cessation on limb and respiratory muscle function, structure, and capillarization in CS-exposed mice. We assessed maximal exercise capacity, physical activity levels, body composition, muscle force and fatigue as well as muscle histology, mitochondrial function and capillarization characteristics. We hypothesized that short-term smoking cessation restores body mass and composition, mitochondrial function and improves limb and respiratory muscle structure and capillarization.

3.4 Materials and methods

All experimental procedures were conducted according to the European, national, and institutional guidelines for animal welfare and approved by the ethical committee for animal experiments of the University of Leuven, Belgium (authorization number: P010/2014 and P050/2016).

3.4.1 Animals and study design

Eight-week-old male C57Bl/6JolaH mice (n = 88) were randomly divided into 4 groups: one exposed to CS for 14 weeks (CS); a group exposed to CS for 13 weeks and 1 week cessation (CS1W); a group exposed to CS for 12 weeks and 2 weeks cessation (CS2W) and a control group (Con). Since the amount of muscle tissue per mouse was not sufficient to perform all analyses in each muscle, the animals were further divided into two series. The first series (n = 52 [13 per group]) was used to determine muscle mass, body mass, mitochondrial function, neutrophilic inflammation, muscle force and fatigue, Dual-energy X-ray Absorptiometry (DEXA) scans, whole body strength, muscle structure and maximal exercise capacity measurements. The second series (n = 36 [9 mice per group]) were used to assess spontaneous physical activity, body mass, muscle structure and capillarization.

All mice were housed in individually ventilated cages with food and water provided *ad libitum*. Mice were acclimatized during one week to CS by gradually increasing the number of cigarettes per day while the control mice were exposed to room air in soft restrains for the same duration (Figure S1). The mice were nose-only exposed (InExpose System, SCIREQ, Montreal, Canada) to either six 3R4F research cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky) or room air (Con) in soft restrains twice daily, 5 days a week (Figure S1), as previously described [25]. The average duration of each smoking session was one hour (~ 10 minutes per cigarette). Total particle density was measured daily (Microdust, Casella CEL, Bedford, UK), and carbon monoxide (CO) levels in CS 3 times per week (EasyLog EL-USB-CO, Lascar electronics, UK), while body mass and food intake were measured weekly.

The average total particulate matter in CS was 188 ± 29 mg/m^3 and CO levels were 871 ± 49 ppm/cigarette. This exposure elevates arterial and venous carboxyhemoglobin (COHb) levels to

35% immediately after exposure, 6% within 1 hour of stopping and back to control levels (1.5%) within 2 hours. The carboxyhemoglobin levels were obtained from a pilot study in which COHb levels were measured over time in whole venous and arterial blood collected immediately after smoking, 1 hour later and 2 hours later from mice exposed to 6 cigarettes.

3.4.2 Exercise capacity and physical activity levels

3.4.2.1 Whole-body function

Maximal exercise Capacity and whole-body strength were measured at baseline (before the mice were exposed to CS or room air in soft restrains) and after 14 weeks. The exercise test consisted of a 5-minute warm up on a treadmill (0% incline, 3 m/min), and the speed was increased by 1 m/min every minute until exhaustion. Maximal exercise capacity was defined as the maximum speed [26]. Latency-to-fall time was measured after placing the mice on a grid that was subsequently inverted and served as a surrogate marker for maximal muscle strength [26].

3.4.2.2 Physical activity levels

Spontaneous physical activity was assessed at baseline, at the initiation of smoking, at 11-12 weeks and after smoking cessation, as previously described [27]. Each mouse was first acclimatized to an individual cage for 24 hours and then physical activity was recorded for the next 24 hours using a basic IP camera (Foscam C1; Foscam, Shenzhen, China). After recording, the mouse was returned to its original cage. The videos for the light and dark cycles were combined and analyzed using a customized program (Matlab Release 2017a; The MathWorks, Natick, MA) and data were reported as cumulative distance covered over time.

3.4.3 Measurements at sacrifice

Mice were anesthetized with a mixture of Ketamine (100 mg/kg, Ketalar®, Pfizer, Belgium), Xylazine (10 mg/kg, Rompun®, Bayer, Belgium) and Acepromazine (3 mg/kg, Placivet®, Kela, Belgium) administered intra peritoneally at a dose of 6 µL/g body mass.

3.4.3.1 Body composition

Fat, lean and bone mass were assessed using a DEXA scanner (Lunar Corp., Madison, WI) as described previously [26].

3.4.3.2 In situ muscle force and fatigue resistance

The left plantaris with its nerve and blood supply left intact was prepared to elicit contractions via stimulation of the tibial nerve at optimal length, to measure maximal twitch (at 1 Hz) and tetanic (at 200 Hz, train duration: 250 ms) tension normalized to muscle cross-sectional area. Fatigue resistance was assessed during repetitive isometric contractions at 30 Hz, 330ms/s, 4 min preceded by a series of 100 Hz, 100 ms/2s contractions for 1 min that does induce little or any fatigue at all to activate muscle metabolism and blood flow as described previously [28] . The fatigue index (FI) was calculated as follows: for FI100, force of the last contraction was divided by the highest contraction in this series while for FI30, force 2 min after the strongest contraction was divided by the strongest contraction in this series.

3.4.3.3 Lung cell counts

The mice were tracheotomized and the lungs were lavaged 4 times with Dulbecco's phosphate buffered saline (PBS). Total cell counts were done on pooled bronchoalveolar lavage (BAL) fractions using a Bürker hemocytometer with trypan blue. The remaining BAL fluid was centrifuged (1000 g for 10 min at 4°C) and the cell pellets dissolved in PBS were stained with Diff-Quick® (Medical Diagnostics, Düdingen, Germany) to determine differential cell counts on 300 cells per mouse to assess lung inflammation.

3.4.4 Muscle tissue assessments

3.4.4.1 Muscle mass

The gastrocnemius, plantaris and soleus muscles were removed and weighed before further analysis.

3.4.4.2 Mitochondrial function

Saponin-permeabilized fibers from the soleus muscle were used to assess mitochondrial respiratory function by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) as described previously [29,34]. In a parallel experiment, mitochondrial function was assessed in freshly extracted soleus muscles from control mice exposed to 5% and 20% cigarette smoke extracts for 30 minutes after which the extracts were removed, and mitochondrial function re-assessed after washing.

3.4.4.3 Muscle histology

Immunohistochemistry for myosin heavy chain types was done on 10 µm thick serial sections of the plantaris, soleus and diaphragm muscles to determine fiber size and fiber type composition as described previously [30] (Figure S2B). Serial sections were stained for succinate dehydrogenase (SDH) activity to measure oxidative capacity as described previously [31] (Figure S2E). Serial sections were stained with lectin for capillaries as described previously [32] (Figure S2A). Capillary domains analysis was performed as described previously [33](Figure S2C - D). This analysis provides information on fiber cross-sectional area (FCSA), fiber roundness (calculated as: $perimeter^2/[4\pi \cdot FCSA]$), capillary domain area, capillary-to-fiber ratio, local capillary-to-fiber ratio [(LCFR) - the sum of the fractions of the capillary domains overlapping a given fiber], capillary density, capillary fiber density (LCFR/FCSA) and heterogeneity in capillary spacing [logarithmic standard deviation of the domain areas (Log_DSD)].

3.4.4.4 Western immunoblotting

Forty mg of diaphragm muscle was homogenized in ice cold CER1 lysis buffer containing protease inhibitor followed by protein extraction using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific; 78833) according to the manufacturer's instructions. Equal amounts of protein from the cytoplasmic fraction (30 µg) were separated under reducing conditions using 8% SDS-PAGE (Biorad mini-PROTEAN) and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked in 5% non-fat dry milk in TBS-0.1%Tween and then incubated overnight at 4°C with anti-HIF-1α (NB400-479, Novus Biologicals) or anti-β-tubulin (T5201, Sigma) in 1:1,000 dilutions. After incubation with the appropriate HRP-conjugated secondary antibody, specific proteins were detected using an enhanced chemiluminescence system (Sigma). The resulting bands were quantified, and the results expressed as ratios normalized to β-tubulin.

3.4.4.5 Assessment of subunits of mitochondrial complexes I to IV and ATP synthase by western blotting

Protein levels of key subunits of mitochondrial complexes I-IV and ATP synthase were assessed by western blotting as described before [34]. Briefly, proteins of the soleus muscle were extracted in protein extraction buffer containing 25 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 1% sodium

deoxycholate and 0.1% SDS. Ten to 15 µg of total (undenatured) protein lysate was loaded on 8-16% gradient Criterion TGX SDS-PAGE gels and proteins were transferred onto nitrocellulose membranes and blocked for one hour with 5% non-fat dried milk powder, and subsequently incubated for 2 hours with an antibody cocktail against complex I subunit (NDUFB8), complex II subunit 30 kDa, complex III subunit core 2, complex IV subunit 1, and ATP synthase α-subunit (MS604, Abcam, Cambridge, UK) at 1:1000 dilution. Membranes were washed with TBS-Tween and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (DAKO, Heverlee, Belgium; 1:5000).

3.4.5 Statistical analysis

The sample size was calculated based on maximal tetanic force data from previous experiments (25% difference) considering a 10% mortality rate in order to reach a significance level (α) of 0.05 and a power of 0.8. A Shapiro-Wilk test for normality was performed in each group prior to further analysis. Comparison between groups was performed using one-way ANOVA with Tukey's multiple comparison post-hoc test for normally distributed data or Kruskal-Wallis test with Dunn's multiple comparison post-hoc analysis for non-normally distributed data. Capillarization parameters were analyzed using two-way ANOVA with smoking and fiber type as the two variables, followed by Sidak's multiple comparison post-hoc analysis. Mixed model analysis followed by Sidak's post-hoc test was performed when the data had random missing values. Significance level was set at $p<0.05$. Values are presented as mean ± SD.

3.5 Results

3.5.1 Lung assessments

CS-exposure increased total cell count in BAL (+74%, P<0.0001; [Figure S3A]), as well as macrophage (P<0.0001; [data not shown]), neutrophil (P<0.0001; [Figure S3B]) and lymphocyte count (P<0.0001; [data not shown]) which were similar to control levels within 2 weeks of smoking cessation.

3.5.2 Body mass, body composition and food intake

3.5.2.1 Body mass

Body mass declined significantly in all animals at the onset of CS or room air exposure and remained lower in the CS-exposed mice. This was statistically significant from 10 weeks onwards (-15 %, $p < 0.01$ vs. control) [Figure S4A]. Cessation of CS-exposure for 1 week ($p = 0.03$ vs. smoking) and 2 weeks ($p < 0.0001$ vs smoking) improved body mass (Figure S4B).

3.5.2.2 Body composition

Fat mass was 26% lower after CS exposure ($p < 0.001$ vs. Con) and remained so after cessation ($p = 0.01$ vs. control) [Figure S4C]. Lean mass reduced by 9% in the CS animals and was only 4.6% and 0.5% lower after 1- and 2-weeks smoking cessation as compared to control animals, indicative of rapid improvements (Figure S4D). However, these improvements were not statistically significant.

3.5.2.3 Food intake

Food intake was reduced briefly in all mice in the first week of CS or room air exposure (Figure S5). There was a significant increase in food intake after 1- and 2-weeks smoking cessation (+16 %, $p < 0.05$ vs. CS).

3.5.3 Physical fitness and Physical activity

3.5.3.1 Maximal Exercise Capacity, Whole-Body Strength and Spontaneous physical activity

For all these parameters, the baseline is the same and refers to measurements done before the mice were exposed to cigarette smoke or air. Compared to baseline values, maximal running speed was not different in the control group (-5.5%, p=0.18) but decreased significantly by 10% in CS (p=0.02), 9% in CS1W (p=0.05) and 9% in CS2W (p=0.01) groups at sacrifice with no effect

smoking cessation. Whole body strength showed a 65-80% decrease in latency-to-fall time in all groups ($p<0.01$ vs baseline) and no significant effect of CS-exposure or cessation. Spontaneous physical activity decreased significantly by 40 – 45% in all groups compared to baseline from the start of the experiments independently of smoking and remained as such during the entire study period (Table S1).

3.5.4 Muscle assessments

3.5.4.1 Muscle mass

Compared to the Control group, the masses of the gastrocnemius and soleus muscles were 18% and 14% lower respectively in the CS group and similar to control levels in the CS1W and CS2W groups. Though the mass of the plantaris was 16% lower in the CS mice, it only became similar to control levels in the CS2W mice while it was still significantly lower in the CS1W mice compared to controls (Table 3.1).

Table 3.1: *Muscle mass of the right calf complex*

Mass (mg)				
Muscle	**Con**	**CS**	**CS1W**	**CS2W**
Plantaris	20.5 ± 2.9	16.9 ± 1.7**	18.2 ± 2.7*	19.7 ± 2.0#
Soleus	10.8 ± 1.0	9.1 ± 0.7**	10.0 ± 1.3	10.6 ± 1.2##
Gastrocnemius	147 ± 12	126 ± 11**	137 ± 14	146 ± 16##

Con = air-exposed for 14 weeks; CS = CS-exposed for 14 weeks; CS1W = CS-exposed for 13 weeks and stop for 1 week; CS2W = CS-exposed for 12 weeks and stop for 2 weeks (n = 13/group). Values expressed as mean ± SD.

**P<0.05, **P<0.001 vs control; #p<0.05, ##p<0.007 vs smoking.*

3.5.4.2 In situ contractile properties

Despite the CS-induced decrease in muscle mass, CS-exposure did not significantly alter absolute (N) or specific (N·cm-2) tetanic (Figure S6A) and twitch (Figure S6B) plantaris muscle forces. The

100-Hz/100 ms/2s series of contractions induced little if any fatigue, and the percentage decrement in force during this series of contractions did not differ significantly between groups (Figure S6C). The following more strenuous protocol (30 Hz, 330 ms on 670 ms off for 4 min) induced fatigue that did not differ significantly between groups (Figure S6D).

3.5.4.3 Muscle histology

3.5.4.3.1 Fiber type composition, cross-sectional area (CSA)

In the soleus and plantaris muscles, the fiber CSA (Figure S7A - B) did not differ significantly between groups. There were no significant differences in fiber type composition between groups in any of the muscles (Figure S7C - D). In the diaphragm, the fiber CSA was smaller in the CS than Con mice (-16 %, P<0.001 *vs.* Con) and similar to control levels in CS2W mice (Figure S8A). The percentage of non-contractile material did not differ between groups in any of the muscles.

3.5.4.3.2 Capillarization and SDH activity

There were no significant differences in any of the capillary parameters between groups in the soleus and plantaris muscles (p>0.05; [data not shown]). By contrast, diaphragm capillary density [+19 %] (Figure S8B) and capillary fiber density of all fiber types [+19 %] (Figure S8C) were significantly increased in CS mice and similar to control levels in CS2W mice. This was not due to angiogenesis, as there were no significant differences between groups in the diaphragm for LCFR (Figure S8D). Two weeks cessation also led to significant improvements in capillary spacing (Figure S8E, +65%, P<0.05 vs control and smoking). The SDH activity of fibers was not significantly different between groups in any of the muscles. The diaphragm muscle had a higher capillary density than the plantaris and soleus muscles (Figure S8F, +41 %, P<0.001).

3.5.4.4 Muscle mitochondrial function and protein levels of key markers of mitochondrial complexes

Leak respiration, maximal electron transport capacity and succinate/rotenone (complex II)-linked respiration were 20.2 % lower in CS-exposed animals compared to controls (Figure 3.1A-C; p < 0.01). Mitochondrial respiration under all substrate conditions were similar to control levels after 2 weeks of smoking cessation with intermediate values after 1 week of cessation (Figure 3.1A – C). Total protein levels of mitochondrial complex I subunit NDUFB8, complex III subunit UQCRC2,

and complex IV subunit MT-CO1 showed ~ 20 % lower values in smoking animals and were similar to controls after smoking cessation (Figure 3.1D-E). Acute exposure of the soleus to CS extracts also led to reduced respiration (-27 %, p = 0.0012) that was readily reversed after removal of the extracts (Figure 3.1F).

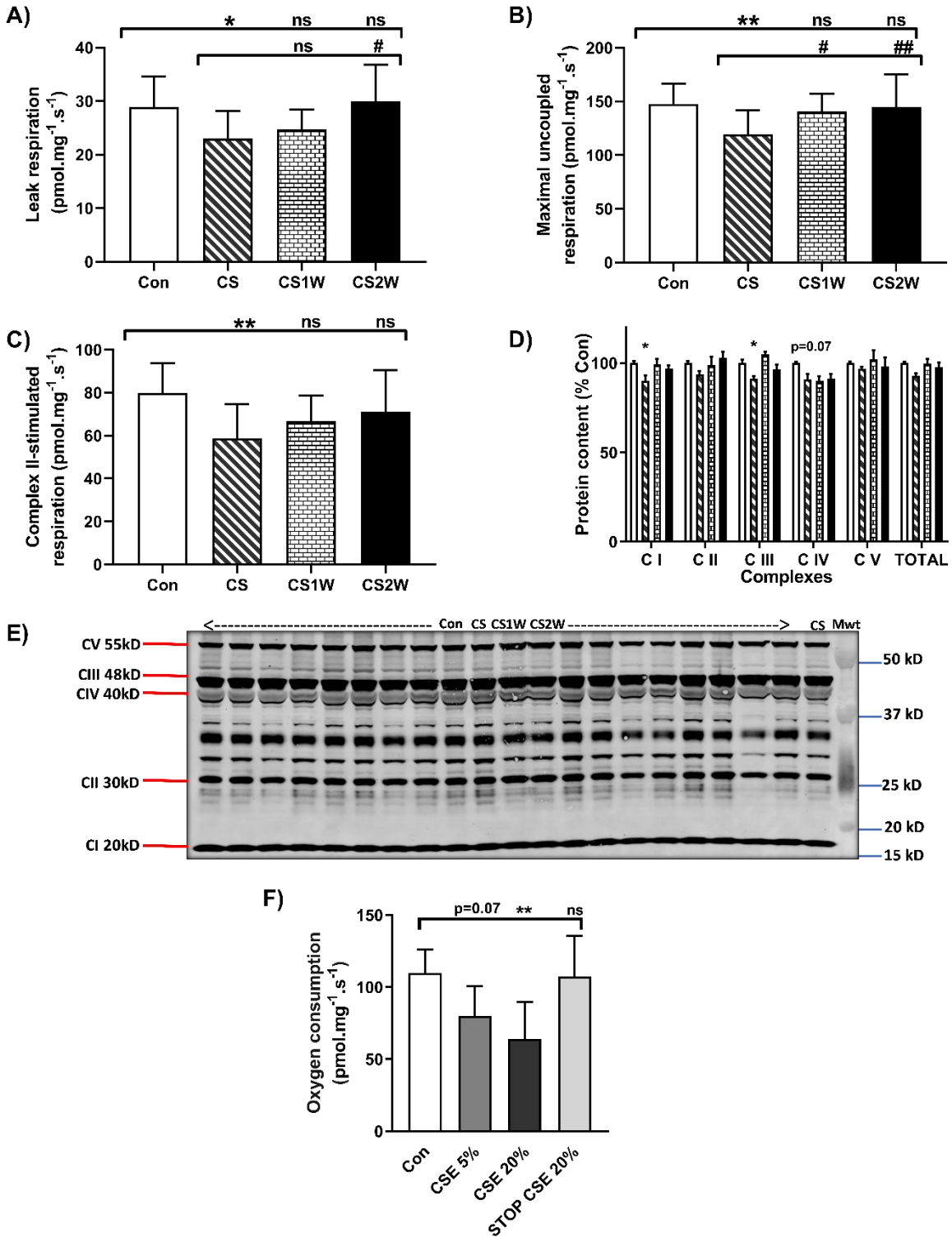

Figure 3.1: *Mitochondrial function in soleus muscle: Leak respiration (A), maximal uncoupled respiration (B), succinate/rotenone (complex II)-stimulated respiration (C) and protein levels of*

*key subunits of the mitochondrial complex I to IV (NDUFB8 (CI), complex II subunit, UQCRC2 (CIII), MTCO1 (CIV)), and ATP synthase α-subunit (CV) in Con (open bars), CS (hatched bars), CS1W (brick bars) and CS2W (solid black bars) [D] – [p = 0.07 for the comparison between CS group and Con group for mitochondrial complex IV]. Typical western blot with bands corresponding to the mitochondrial complex protein levels (E) and maximal oxygen consumption after acute exposure of the soleus to 5% and 20% CS extracts (CSE) and after a 30-minute wash out period (F). **p<0.001, *p<0.05 vs control; ##p<0.001, #p<0.05 vs smoking; ns = non-significant. Values are presented as mean and SD. Con = air-exposed for 14 weeks; CS = CS-exposed for 14 weeks; CS1W = CS-exposed for 13 weeks and stop for 1 week; CS2W = CS-exposed for 12 weeks and stop for 2 weeks (n = 13/group).*

3.5.4.5 HIF-1α protein levels

Though there were no alterations with CS-exposure, smoking cessation for 2 weeks significantly increased cytoplasmic HIF-1α protein content in the diaphragm ($p<0.05$ for CS2W *vs* CS1W; $p=0.052$ for CS2W *vs* CS) [Figure S9 A-B].

3.6 Discussion

This study shows for the first time that in male mice, the diaphragm is more susceptible to smoking than the limb muscles, as indicated by a significant smoking induced fiber atrophy in the diaphragm only. However, even the limb muscles showed a lower muscle mass and smoking-induced impairment in maximal mitochondrial respiration. Even though some emphysema could have developed after 3 months of CS exposure, all these detrimental effects of smoking, including lung inflammation, were similar to control levels after 2 weeks of smoking cessation. If the reversibility of many of the smoking-induced defects we see here are as rapid in human smokers, this information may encourage smokers to quit smoking.

In line with previous studies, CS-exposure led to neutrophilic lung inflammation [26,35], reduced body mass [36–38] and decreased gastrocnemius, plantaris and soleus muscle mass [23,26,30,38]. Studies in humans [21,39–41] and mice [23,42,43] have shown that long-term smoking cessation is accompanied by increased fat and lean body mass [20,21,44,45]. Our study shows a progressive increase in fat and lean mass after 1 to 2 weeks of CS-cessation. Potential contributors to CS-cessation-induced weight gain include amongst others, increased food intake, increased lipoprotein lipase activity and decreased resting metabolic rate [44], but our data show that increased food intake after cessation is the most likely explanation.

Despite the rapid increase in body mass during smoking cessation (7 and 12 % after 1 and 2 weeks respectively), there were no significant changes in the fiber CSA in the plantaris and soleus muscles, nor were there significant changes in force generating capacity. This is in accord with previous studies whereby 12 – 18 weeks of CS exposure in mice did not alter fiber CSA and force in the soleus and extensor digitorum longus (EDL) muscles [26,35]. The fatigue resistance in the plantaris was neither altered by CS-exposure nor smoking cessation, and such absence of changes in fatigue resistance correspond with the absence of significant changes in fiber type composition, similar to the absence of significant changes in *in vitro* contractile properties, fatigue resistance, and fiber type composition in mice exposed to CS for 12 weeks [35]. Others also have reported no significant smoking-induced changes in fiber CSA and fiber type proportions in mice [6,26,30,35] and humans [1,2,46]. Unlike the limb muscles, the diaphragm muscle showed significant reductions in the fiber CSA of all fiber types with CS-exposure. Interestingly, smoking cessation

for as short as 2 weeks led to a rapid increase in fiber CSA to control levels. Literature on CS-induced diaphragm muscle fiber atrophy are controversial. Some studies report reduced force and increased atrophic signaling [6] or altered fiber proportions [5] but no changes in fiber CSA, while others report reduced mass [47] or fiber atrophy [48]. These discrepancies may result from differences in dose and duration of CS-exposure or mouse strain used. Whatever the cause of the discrepancies between studies, our study is the first to demonstrate immediate beneficial effects of smoking cessation on diaphragm muscle structure. Taken together, and in line with previous studies [6], these results show that the diaphragm muscle is more sensitive to CS-induced changes in muscle structure than limb muscles and that it responds rapidly and positively to smoking cessation.

Despite the effects of CS-exposure in the diaphragm, the cytoplasmic protein content HIF-1α was not significantly altered. This could indicate that CS-induced transient intermittent hypoxia did not stimulate HIF-1α signaling or substances in CS may have inhibited its activation. Surprisingly, HIF-1α protein content increased after 2 weeks of cessation. However, the absence of downstream effects such as angiogenesis could show that the accumulated HIF-1α in CS2W mice is not translocated to the nucleus and therefore not activated.

The main determinant of the capillary supply to a fiber is muscle fiber size, while fiber type and oxidative capacity play a minor role at best [49,50]. Our data show that CS-exposure and consequently CS-cessation had no significant effects on the capillarization in the plantaris and soleus muscles. This observation is in discrepancy with a recent study showing capillary regression in the soleus after 8 weeks of CS-exposure [7]. However, the data obtained from the EDL (a predominantly glycolytic muscle similar to the plantaris) in the aforementioned study [7] and another [16] report no effect and thus concur with our study. In the diaphragm, we did see an increase, rather than a decrease in capillary density (CD) and capillary fiber density (CFD) after smoking. While at first glance this may suggest angiogenesis, the absence of an increase in LCFR shows that this is not the case, and the increase in capillary density must be due to the decrease in fiber CSA. Further support comes from the observation that the normal values of capillary density after smoking cessation were associated with an increase in fiber CSA, again without a significant change in the LCFR. We have no explanation for the more homogeneous distribution

of capillaries after smoking cessation than even control muscles, but it would result in improved muscle oxygenation. Perhaps it is a sort of adaptation to an impaired respiratory chain function in a bid to restore homeostasis. Further research is required to confirm these findings.

Studies in skeletal muscles of humans have reported CS-induced impairment of the electron transport system, lower maximal ATP production rate and lower oxygen consumption rates in limb muscle mitochondria [3,18,51]. Studies in mice are contentious, with some [16,52] but not all [6] reporting limb muscle mitochondrial dysfunction after CS-exposure. Here we show that the respiration of the mitochondria was indeed impaired during CS-exposure and restored with smoking cessation. To further investigate the potential direct effects of CS on skeletal muscle mitochondria, we exposed the soleus muscle from control mice to CS extracts. This resulted in reduced respiration that was readily reversed after removal of the extracts. This indicates that substances in CS acutely impair mitochondrial function. Although the smoke extracts contained a large variety of substances that could potentially explain the acute impairment of mitochondrial respiration, it fits the notion that carbon monoxide in cigarette smoke can directly inhibit complex IV activity and mitochondrial respiration [18]. In addition to potential effects of CO, we also observed a significant lower protein expression levels of subunits of mitochondrial complex I, III and IV that were similar to control levels after 1 and 2 weeks of smoking cessation.

Though maximal exercise capacity was lower in all CS-exposed mice at sacrifice compared to baseline, CS-exposure or CS-cessation had no effect on whole body strength and spontaneous physical activity. However, the physical activity levels of all mice dropped significantly as from the start of CS/room air exposure (-50%) probably as a result of external stress. This shows that the effects observed in our study were not due to alterations in physical activity (disuse or overuse) and supports the concept that CS has direct effects on skeletal muscles. These results are in line with a previous study showing no CS-induced alterations in whole body strength and maximal exercise capacity after 24 weeks of CS-exposure [26].

Though we looked very comprehensively at many parameters, our study does not present in depth assessment of specific mechanisms. Also due to the plethora of analyses, the number of mice for some of the analyses may have been rather low but no type II error was detected. In

addition, since most of the CS-induced effects on skeletal muscles observed in our study (decreased muscle mass, reduced protein content and function of mitochondrial enzymes and diaphragm atrophy) are in line with previous studies with similar sample sizes, this confirms that the probability of getting false negatives in our study is low. The absence of female mice in this study also limits the scope of interpolation and extrapolation of these data. We present here an extensive assessment of the short-term effects of smoking cessation on skeletal muscles in male mice and whether similar data would be observed in female mice needs to be addressed. Further research is needed to clarify the mechanisms of changes in muscle mass and capillarization, effects of intermittent hypoxia and physical activity alterations.

In conclusion, our study shows that smoking cessation for as short as 2 weeks exerted immediate benefit on the diaphragm and limb muscles with reversal of muscle mass loss, especially lean mass, and improvement of mitochondrial function at least in male mice. Obviously, these data could help motivating and stimulating stopping smoking as they highlight a real benefit of short-term smoking cessation on skeletal muscle and body mass.

3.7 Funding

This study was financed by the Education, Audiovisual and Culture Executive Agency of the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate program.

3.8 Declaration of interests

All authors have no conflicts of interest to disclose

3.9 Acknowledgements

The authors thank Simone Denis for her help with the assessment of the subunits of the mitochondrial complexes and ATP synthase by Western blotting.

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3.11 Online data supplement

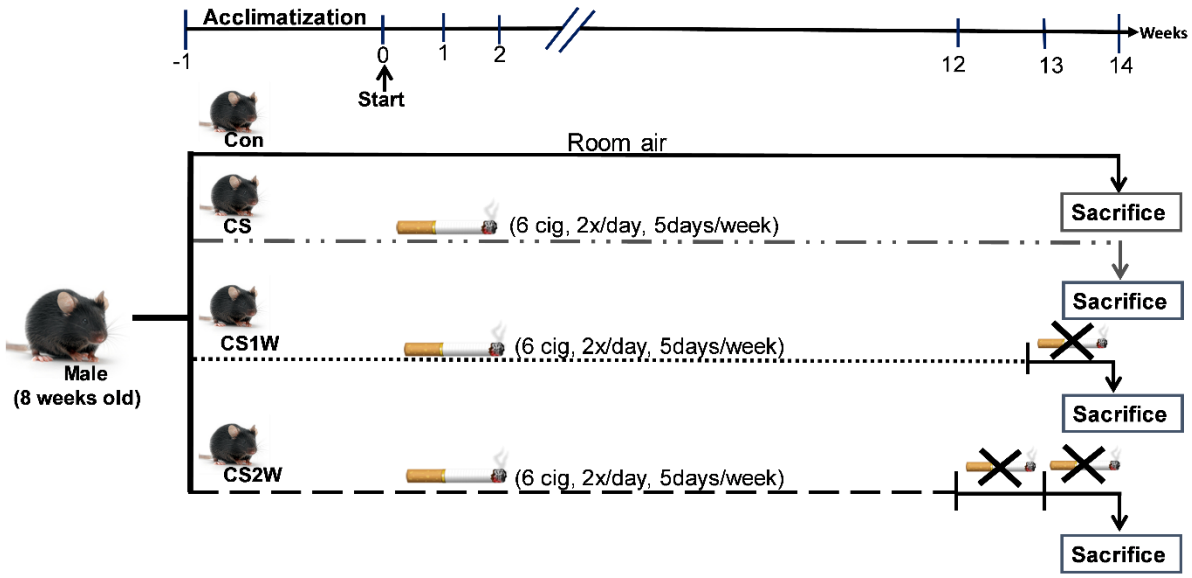

Figure S1: *Study design. Con = air-exposed for 14 weeks; CS = CS-exposed for 14 weeks; CS1W = CS-exposed for 13 weeks and stop for 1 week; CS2W = CS-exposed for 12 weeks and stop for 2 weeks; Cig = cigarette.*

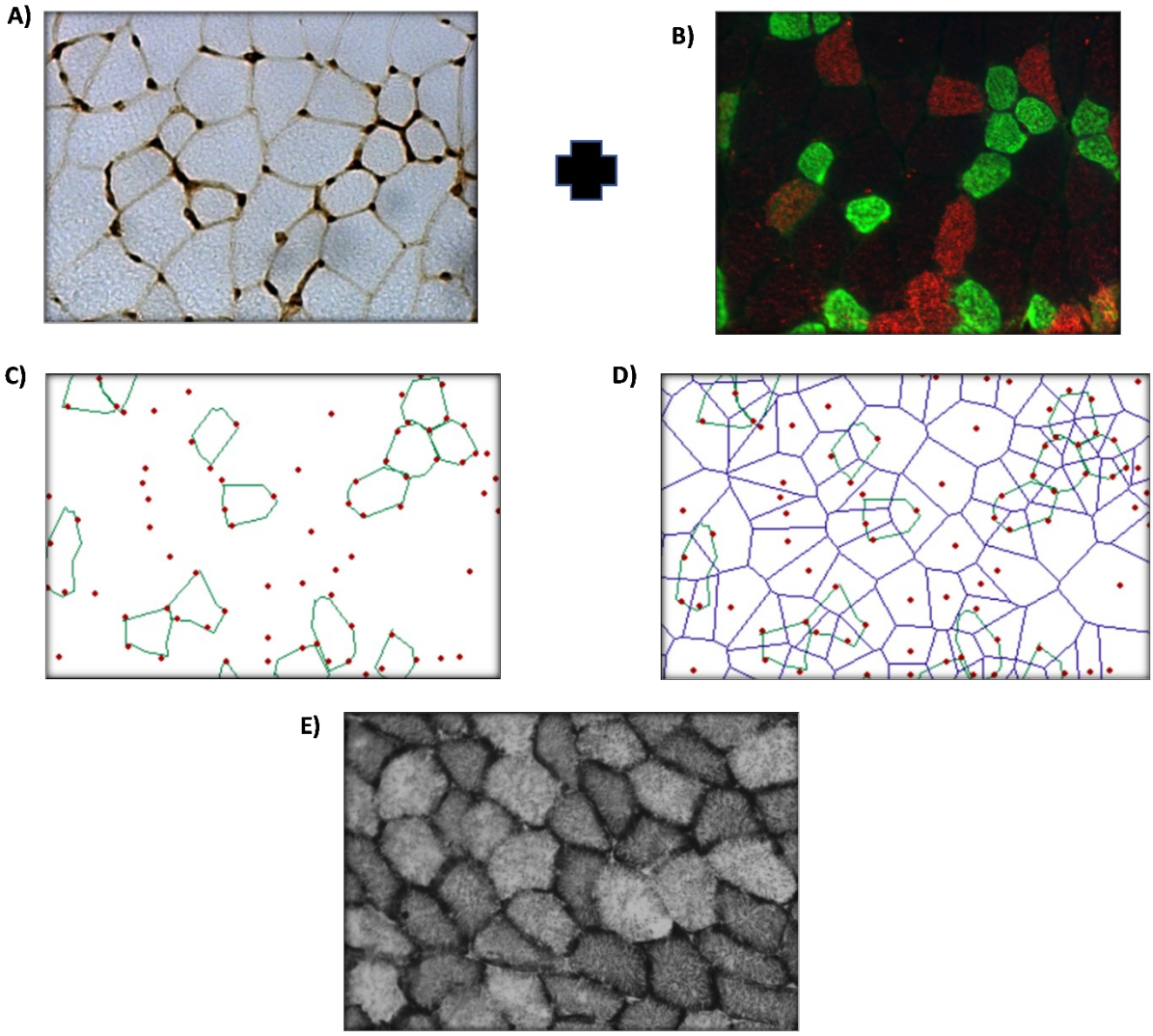

Figure S2: *Fiber cross sectional area (FCSA), capillarization and SDH activity measurement. (A) Lectin stained 10 µm section of the plantaris showing capillaries (black dots) around fibers; (B) MHC-Stained serial section for identification of fibers and fiber types (Green = IIa, Red = IIx, unstained = IIb). The black plus sign between A and B indicates that both images are used simultaneously to produce C and D in the digitizing tablet. (C) Trace of type IIa fibers (green outlines) with capillaries (red dots) using digitizing tablet which provides FCSA data; (D) The overlap of automatically calculated capillary domains (blue outlines) and type IIa fibers to determine capillarization parameters. (E) Typical example of 10 µm section of the soleus stained for SDH activity. The darker stained fibers have more SDH activity.*

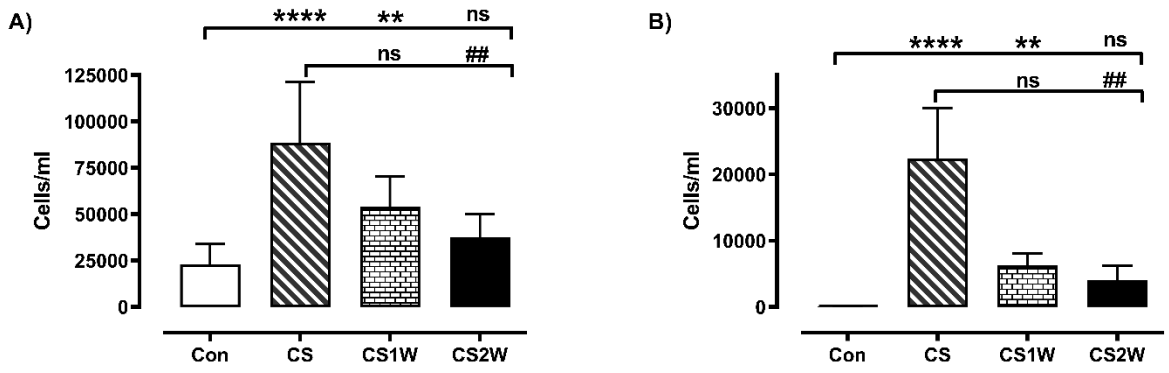

Figure S3: *Effects of CS-exposure and CS-cessation on lung inflammation (n = 13 mice per group measured). CS caused neutrophilic lung inflammation which was similar to controls after two weeks of smoking cessation Total cell count [A] and Neutrophil count [B] in BAL. ****p<0.00 01, **p<0.001, *p<0.05 vs control; ##p<0.001 vs smoking ns: not significant. Values are presented as mean and SD.*

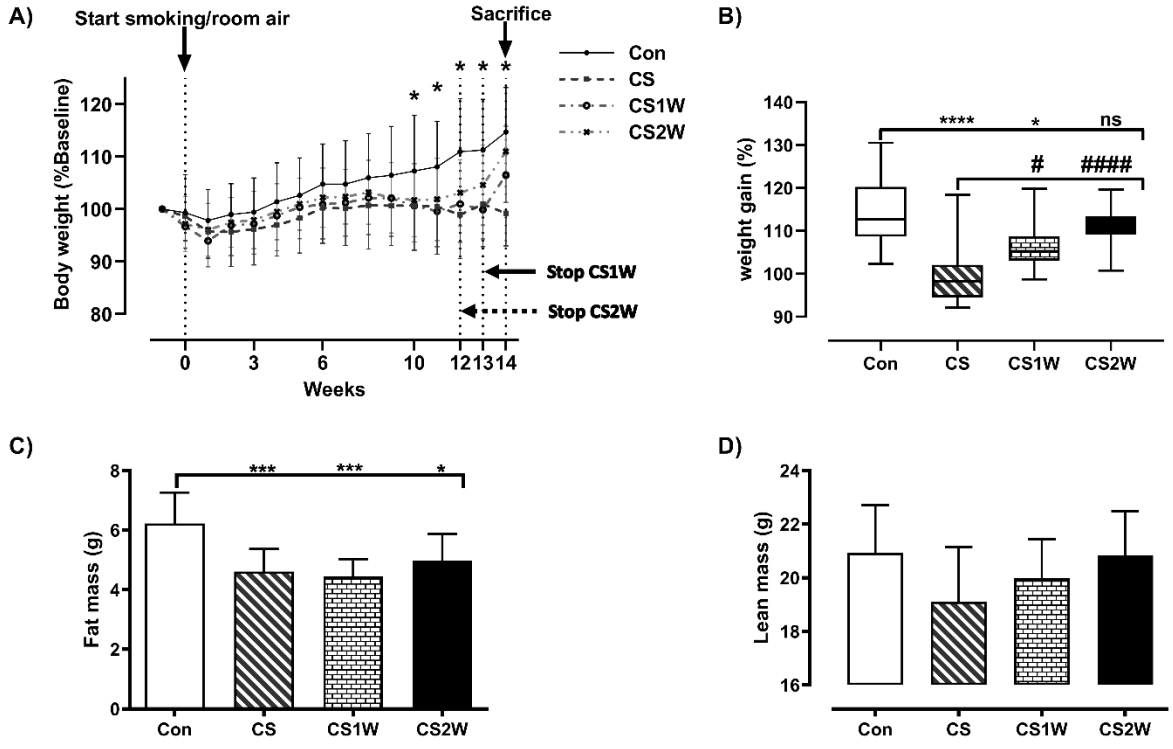

Figure S4: *A) Body mass changes (n=19/group) [Full and dashed arrows indicate smoking cessation for CS1W and CS2W respectively. Asterisks indicate statistically significant differences between all CS-exposed animals and the air-exposed group]; B) weight gain (%) from the end of CS or room air exposure in CS1W, CS2W, CS and Con showing clear improvements in the cessation groups (n=19/group); C) fat mass and; D) lean mass changes (n=11/group) showing non-statistically significant improvement after cessation. Con (closed circles and open bars), CS (closed squares and hatched bars), CS1W (open circles and brick bars) and CS2W (crosses and solid black bars).* ***Con*** *= air-exposed for 14 weeks;* ***CS*** *= CS-exposed for 14 weeks;* ***CS1W*** *= CS-exposed for 13 weeks and stop for 1 week;* ***CS2W*** *= CS-exposed for 12 weeks and stop for 2 weeks. Values are presented as mean and standard deviation (A, C and D) or median and interquartile range (B).* ****p<0.0001, *p<0.05 vs Con; ####p<0.0001, #p<0.05 vs CS, ns: not significant.*

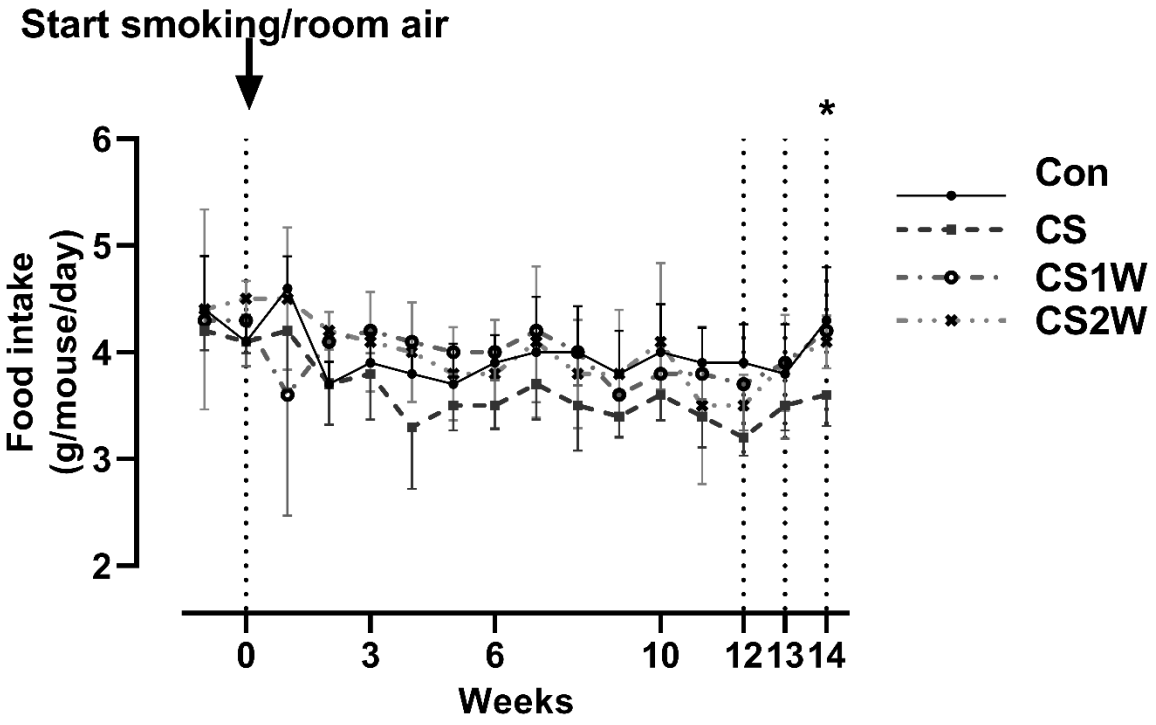

Figure S5: *Average weekly food intake during protocol. CS1W and CS2W mice ate significantly more after smoking cessation. Con (closed circles), CS (closed squares), CS1W (open circles) and CS2W (crosses). Data are shown as mean ± SD. *p<0.05 for CS vs others.*

Table S1: *Maximal Exercise Capacity, Whole-Body Strength and Spontaneous physical activity*

	Maximal exercise capacity (Maximum running speed) [m/min]					
	Con	**CS**	**CS1W**	**CS2W**	**ANOVA**	**Post Hoc**
Baseline	19.9 ± 2.6	22.8 ± 2.5	21.8 ± 2.3	21.2 ± 1.8	*p = 0.004*	CS > Con
Terminal	18.8 ± 2.8	20.6 ± 2.2	19.9 ± 1.7	19.3 ± 1.9	*p = 0.28*	ns
p-Value (Bl vs Ter)	*0.18*	*0.02*	*0.05*	*0.01*		
	Whole body strength (Latency-to-fall time [min])					
Baseline	4.4 ± 3.7	5.4 ± 3.6	6.5 ± 5.6	2.8 ± 1.2	*p = 0.30*	ns
Terminal	1.5 ± 1.5	1.9 ± 1.2	1.0 ± 0.3	1.0 ± 0.6	*p = 0.15*	ns
p-Value (Bl vs Ter)	*0.002*	*0.002*	*0.018*	*0.001*		
	Spontaneous physical activity (night activity [m/h])					
Baseline	38.0 ± 10.3	42.2 ± 10.8	40.1 ± 11.0	44.8 ± 10.0	*p = 0.50*	ns
Start smoking or air	16.9 ± 4.8	18.2 ± 3.9	18.0 ± 3.9	21.8 ± 3.8	*p = 0.09*	ns
p-Value (Bl vs start)	*0.18*	0.06	*0.006*	*0.07*		
11 - 12 weeks	14.9 ± 6.9	14.4 ± 3.3	17.6 ± 6.0	14.7 ± 5.1	*p = 0.78*	ns
p-Value (Bl vs 11-12)	*0.009*	*0.049*	*0.003*	*0.03*		
Week 13	11.8 ± 4.3	11.6 ± 4.4	12.7 ± 6.0	14.0 ± 4.2	*p = 0.74*	ns
p-Value (Bl vs Wk 13)	*0.0004*	*0.01*	*0.0009*	*0.03*		
Week 14 (Terminal)	14.7 ± 5.6	12.6 ± 3.0	16.9 ± 4.3	16.5 ± 4.9	*p = 0.22*	ns
p-Value (Bl vs Wk 14)	*0.04*	*0.047*	*0.0009*	*0.02*		

Con = air-exposed for 14 weeks; CS = CS-exposed for 14 weeks; CS1W = CS-exposed for 13 weeks and stop for 1 week; CS2W = CS-exposed for 12 weeks and stop for 2 weeks (n = 13/group). Values expressed as mean ± SD. Maximal exercise capacity at baseline (BL) and at sacrifice (Ter)[n = 13 mice/ group]. Whole body strength at sacrifice compared to baseline (n = 13 mice/ group). Spontaneous physical activity expressed as distance in meters covered per hour obtained from night activity at baseline, start of CS or room air exposure, at 11-12 weeks of exposure and at week 13 and 14 of the study representing 1 or 2 weeks smoking cessation (n = 9 mice/ group). There was no effect of CS-exposure or cessation on functional physical activity measurements. Baseline is the same for all these parameters and refers to measurements done before the mice were exposed to cigarette smoke or air.

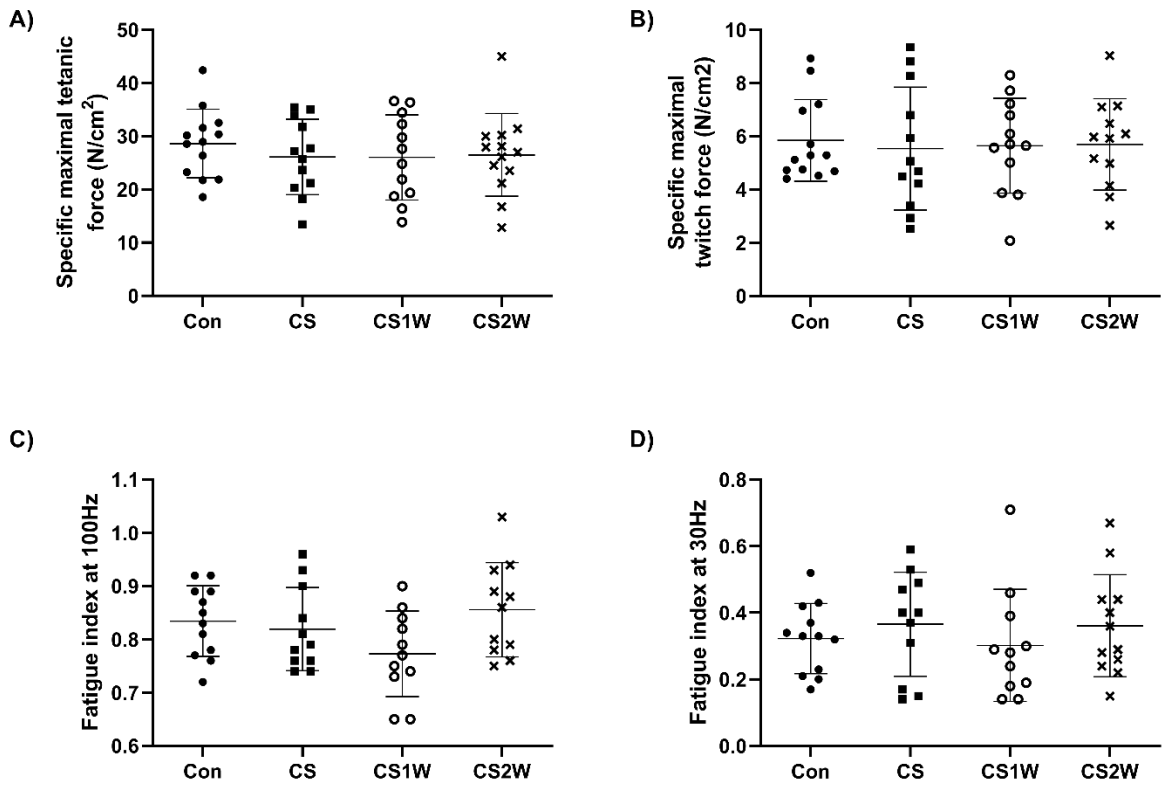

Figure S6: *In situ contractile properties of the plantaris muscle. Specific tetanic (A) and twitch (B) force. Fatigue index at high (FI100) [C] and low (FI30) [D] stimulation frequency. Con (closed*

circles), CS (closed squares), CS1W (open circles) and CS2W (crosses). Values are presented as mean and SD.

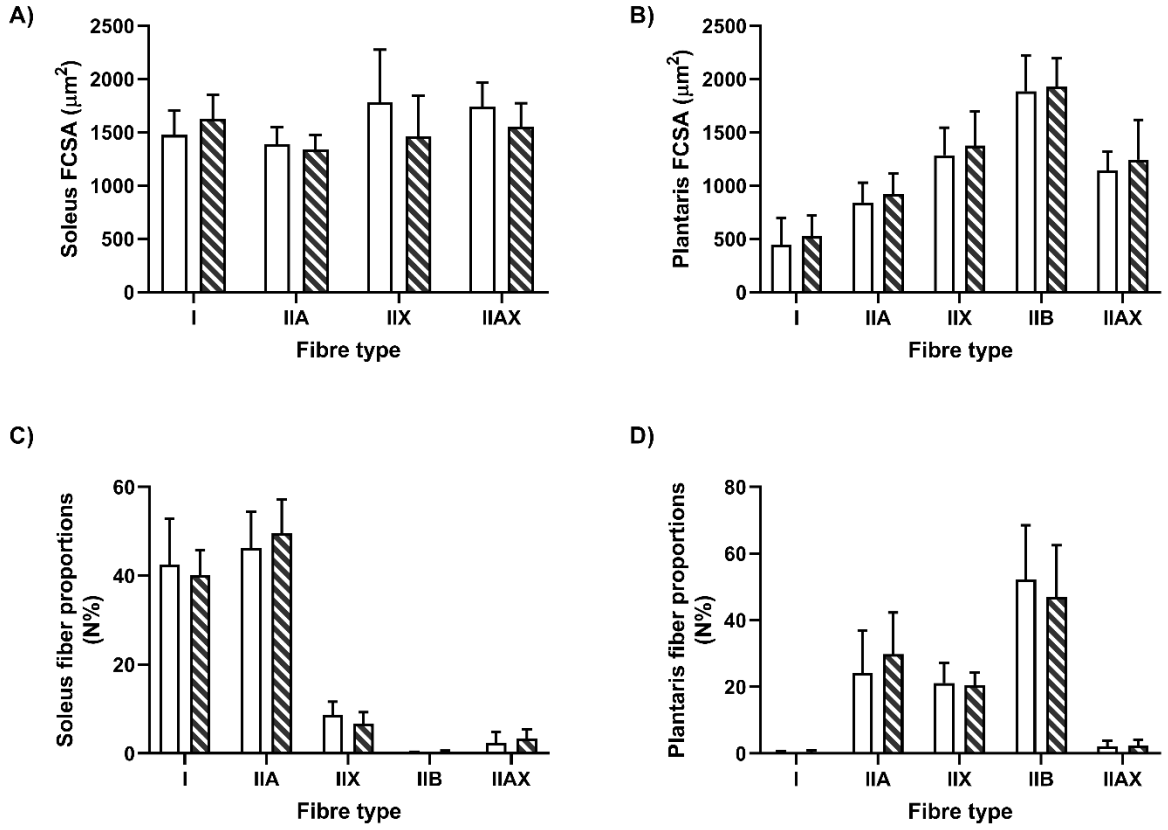

Figure S7: *Fiber dimensions (FCSA, [A – B]) and proportions (C – D) in the soleus and plantaris muscles in Con (open bars) and CS (hatched bars). Values are presented as mean and SD.*

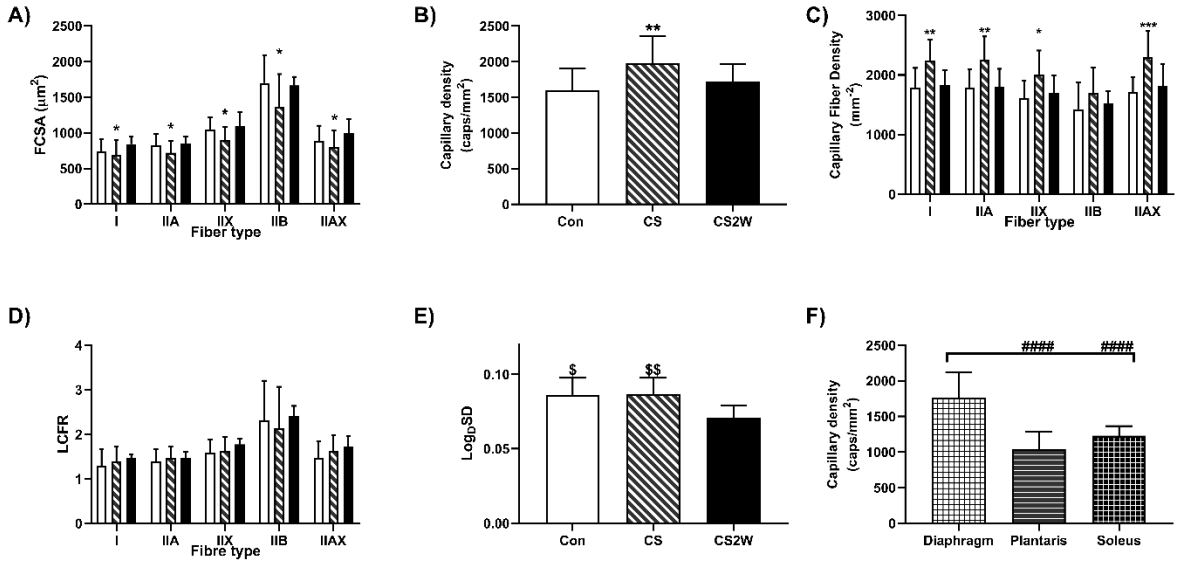

Figure S8: *Diaphragm fiber cross-sectional area (FCSA) (A), capillary density (B) and capillary fiber density in the different fiber types (C) are altered by CS-exposure and similar to control with cessation, though LCFR remained unchanged in CS and CS2W (D), indicative of no angiogenesis. Improved diaphragm capillary distribution in CS2W (E). Higher capillary density of the diaphragm compared to the plantaris and soleus muscles (F). Con (open bars), CS (hatched bars) and CS2W (solid black bars) (n = 13 mice/group). ***p<0.001; **p<0.01; *p<0.05 vs control and smoking, ####p<0.0001 vs diaphragm, $$p=0.008; $p<0.05 vs CS2W). Log_DSD = logarithmic standard deviation of the domain areas Values presented as mean and SD.*

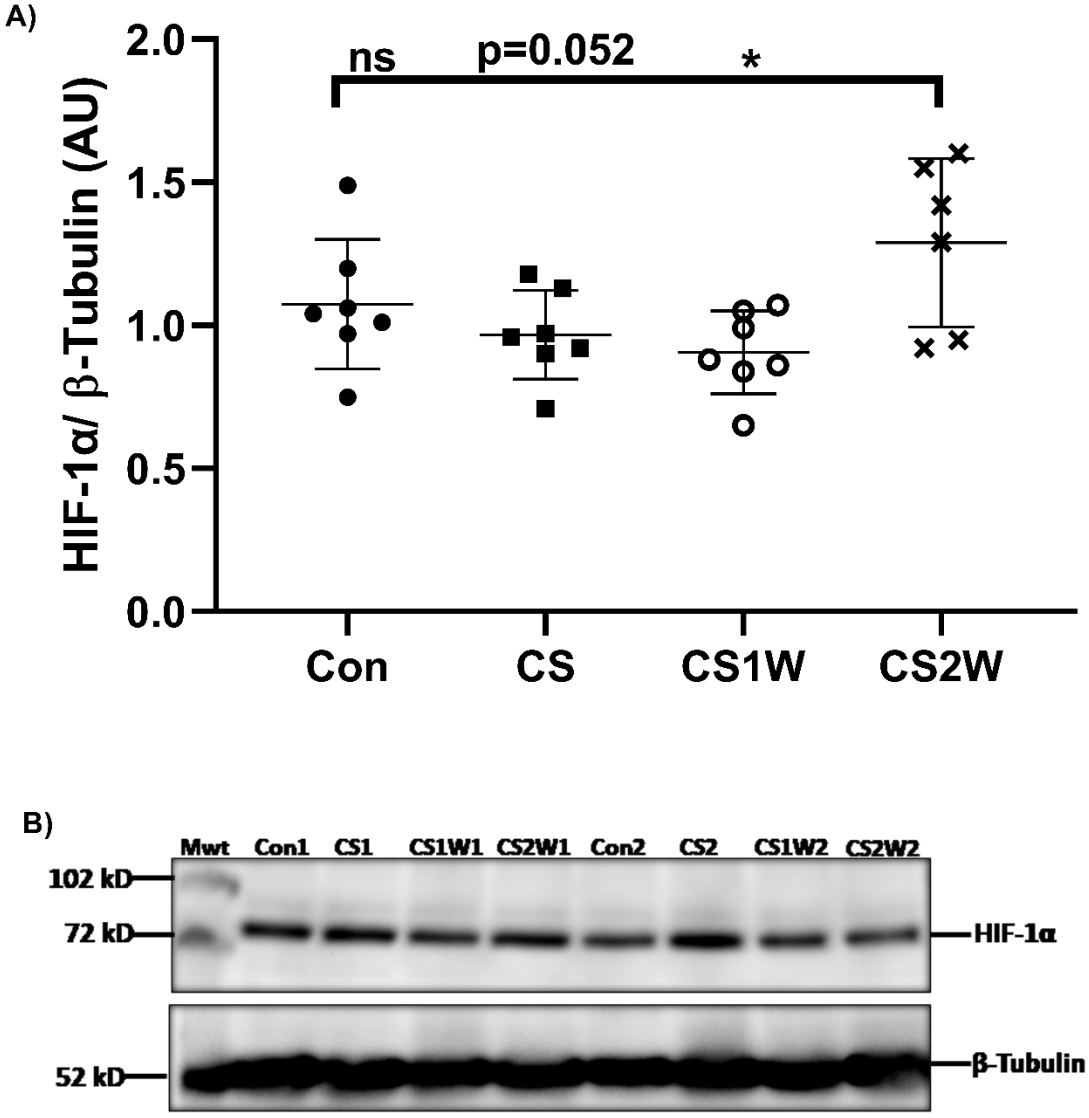

Figure S9: *HIF-1α western blots. Cytoplasmic protein levels of HIF-1α normalized by β-tubulin in arbitrary units (AU) increased significantly in CS2W (n = 7 mice/group) [A]. Typical western blot with bands corresponding to HIF-1α and β-tubulin cytoplasmic protein levels (B). Each point represents an individual value, horizontal bars represent mean values while vertical lines represent standard deviation. *P<0.05 vs CS2W, ns: not significant.*

CHAPTER 4

Smoking combined with vitamin D deficiency attenuates the skeletal muscle hypertrophic response in mice

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Manuscript under review, Journal of Applied Physiology, 2020

4 Smoking combined with vitamin D deficiency attenuates the skeletal muscle hypertrophic response in mice

4.1 Abstract

Introduction

Vitamin D deficiency, which is highly prevalent in the general population, exerts similar deleterious effects on skeletal muscles to those induced by cigarette smoking. We examined whether cigarette smoke (CS) exposure and/or vitamin D deficiency would impair the skeletal muscle hypertrophic response to overload.

Methods

Male C57Bl/6JolaH mice on a normal or vitamin D-deficient diet were exposed to CS or room air for 18 weeks. Six weeks after initiation of smoke or air exposure, sham surgery, or denervation of the agonists of the left plantaris muscle was performed. The right leg served as internal control. Twelve weeks later, the hypertrophic response was assessed.

Results

CS exposure instigated loss of body and muscle mass and increased lung inflammatory cell infiltration, independently of diet. Maximal exercise capacity, whole body strength, in situ plantaris muscle force and key markers of hypertrophic signaling (Akt, 4EBP1 and FoxO1) were not significantly affected by smoking or diet. The increase in plantaris muscle mass and fiber cross-sectional area in response to overload was attenuated in vitamin D-deficient CS-exposed mice (smoking x diet interaction, $p = 0.03$). In situ fatigue resistance was elevated in hypertrophied plantaris, irrespective of vitamin D deficiency and/or CS exposure.

Conclusion

In conclusion, our data show that CS exposure or vitamin D deficiency alone did not attenuate the hypertrophic response of overloaded plantaris muscles, but this hypertrophic response was weakened when both conditions were combined. This data should be considered when strategies to improve muscle mass are applied to smoking and vitamin D-deficient individuals.

Keywords: overload, muscle fatigue resistance, muscle force, muscle fiber size, maximal exercise capacity, hypertrophy

4.2 Introduction

For several decades, cigarette smoking has remained a major avoidable public health hazard as about 20% of adults worldwide are active cigarette smokers [1]. Cigarette smoke (CS) contains more than 5000 known toxic constituents and several other unidentified components, and is one of the greatest sources of human exposure to poisonous chemicals [2,3]. CS does not only affect pulmonary function, but also has extra-pulmonary deleterious effects such as CS-induced skeletal muscle dysfunction [4–8]. In humans and animal models, chronic cigarette smoking has been associated with skeletal muscle weakness and atrophy, impaired mitochondrial function, reduced vasodilation, decreased perfusion and diminished fatigue resistance [4,9–11].

Skeletal muscle hypertrophy is defined as an increase in muscle mass due to an increase in fiber size [12,13]. It occurs mainly in response to resistance exercise training in humans [14] and in animal models by exercise training, overloading a muscle via denervation or elimination of synergist muscles [14–17]. Skeletal muscle mass and fiber cross-sectional areas are maintained by a tight balance between protein synthesis and degradation, both of which depend on physical activity, diet, disease or injury and hormonal balance, amongst others [18]. CS through its negative effects on muscle protein turnover [11,19] together with its potential to induce systemic inflammation and local muscle hypoxia may potentially impair the skeletal muscle hypertrophic response. This has, however, not yet been investigated.

Beside the many functions of vitamin D in metabolism, recent studies in humans and mice have indicated that chronic vitamin D deficiency, which is highly prevalent in the general population, leads to muscle atrophy (particularly in type II fibers), lower strength, lower mitochondrial function and decreased vitamin D receptor expression [20–23]. In addition, vitamin D deficiency may impair muscle regeneration [24] and therefore perhaps also the muscle hypertrophic response to overload. Cigarette smoking has been shown to augment the risk for vitamin D deficiency by impairing the enzymes involved in the generation of vitamin D, increasing the activity of enzymatic markers of liver damage and other mechanisms [25–27]. Given that both vitamin D deficiency and cigarette smoking have been associated with muscle wasting and mitochondrial dysfunction, it is perhaps no surprise that our recent study reported that vitamin D deficiency aggravated the muscle wasting induced by cigarette smoking in a mouse model [21]. However,

whether vitamin D deficiency alone or in combination with cigarette smoking impairs the skeletal muscle hypertrophic response has hitherto not been explored. Better understanding of the interaction may be relevant to human exercise training studies where poor response to exercise training in approximately 30% of patients with COPD remains a poorly understood problem.

The objective of this study was to assess whether cigarette smoking, vitamin D deficiency or their combination affect the muscle hypertrophic response to overload in smoking mice made vitamin D-deficient by diet. We hypothesized that 1) chronic cigarette smoke exposure or vitamin D deficiency blunts the hypertrophic response of skeletal muscle to overload and 2) this effect is larger when cigarette smoke exposure is combined with vitamin D deficiency. To induce muscle hypertrophy, we used the compensatory overload model in which the plantaris muscle is overloaded through denervation of the gastrocnemius and soleus muscles in one hindlimb.

4.3 Materials and methods

4.3.1 Study design

Seventy-two 3-week-old male C57Bl/6JolaH mice were randomly divided into two groups of 36 mice: one group received a standard diet (w/w: 1% calcium and 0.7% phosphorus), while the other received a vitamin D depleted diet (<200 IU/kg body mass vitamin D) with 20% lactose, 2% calcium and 1.25% phosphorus to preserve serum calcium and phosphorus homeostasis [21,28,29]. All mice were housed in an ultraviolet-light-free environment to prevent *de novo* synthesis of vitamin D in the skin. At the age of 8 weeks, mice were subdivided into 4 groups of 18 mice each: Normal Vitamin D control (NAir); Normal Vitamin D smoking (NSmo); Vitamin D-deficient control (DAir) and Vitamin D-deficient smoking (DSmo) groups. After acclimatization by progressive exposure to Cigarette smoke (CS) or room air in soft restrains, the CS-exposed mice were exposed via a nose-only exposure system (InExpose System, SCIREQ, Montreal, Canada) to six 3R4F research cigarettes with filter (Kentucky Tobacco Research and Development Center, University of Kentucky) twice daily, five days a week [11,30] for 18 weeks. The air-exposed animals were exposed to room air in soft restrains for the same length of time. After 6 weeks of exposure to either room air or CS, the left plantaris muscle was overloaded to induce compensatory hypertrophy (Fig. 4.1). Total particle density in CS was measured daily using a particle density meter (Microdust, Casella CEL, Bedford, UK). The average level of total particulate density in CS was 188 ± 29 mg/m^3 during each smoking session. Body mass and food intake were measured weekly using a laboratory balance (KERN Precision balance). Food intake was not measured during the weeks of surgery and recovery.

All experiments were performed in line with the institutional, national, and European guidelines for animal welfare, and were approved by the ethical committee for animal experimentation of the KU- Leuven (authorization number: P050/2016).

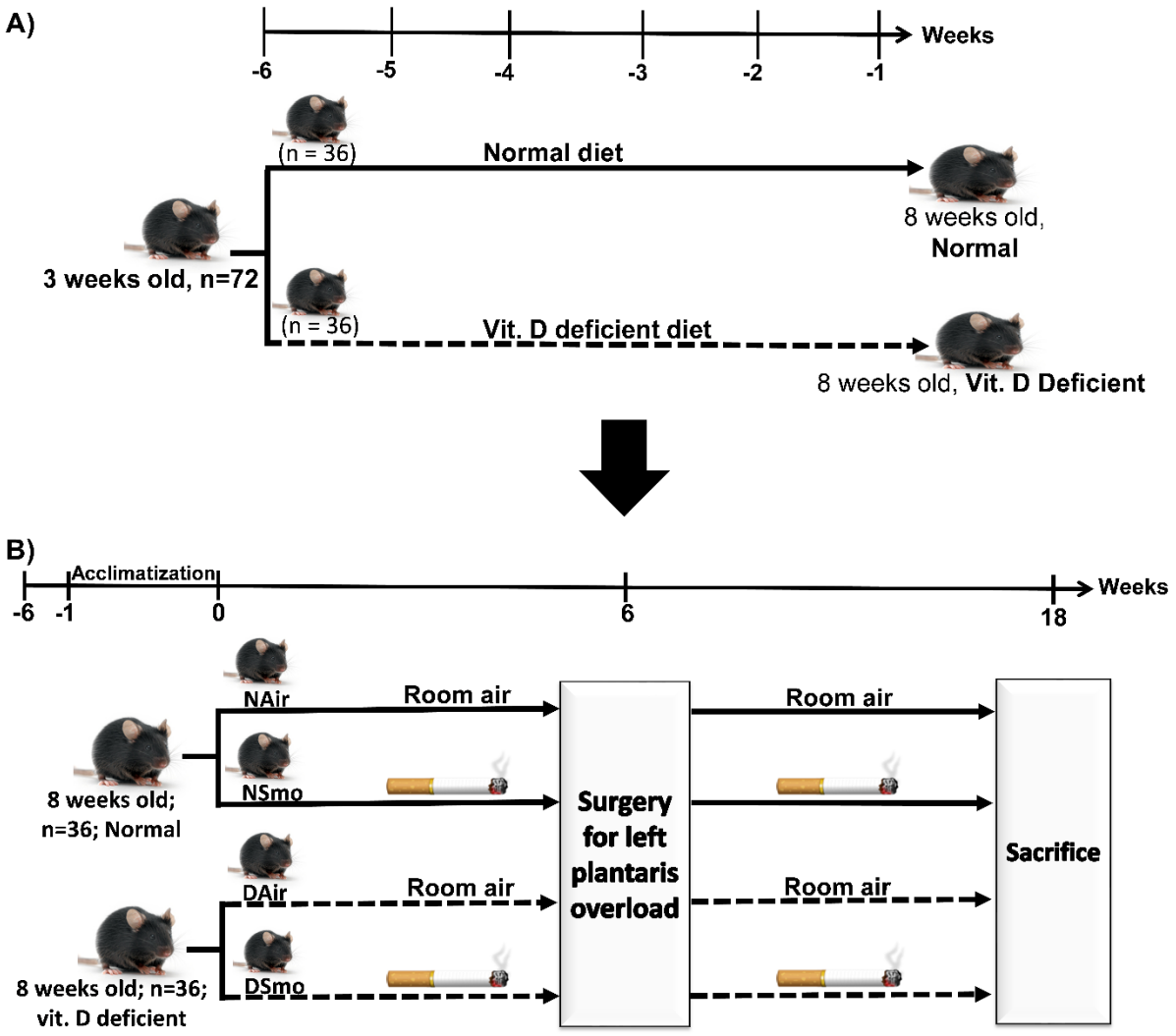

Figure 4.1: *Study design. (A) Seventy-two 3-week-old male C57Bl/6JolaH mice were randomly assigned to 2 equal groups and fed for 5 weeks with a normal diet or vitamin D-deficient diet. (B) At the age of 8 weeks the mice were subdivided into 4 equal groups (n=18/36) and acclimatized to the setup for one week to be exposed to cigarette smoke (CS) or room air. Surgery to overload the left plantaris muscle was performed 6 weeks after starting exposure to CS or room air. Analysis was performed 18 weeks after the start of exposure. More details are presented in the 'study design' section of the data supplement. NAir = Normal diet-air exposed; NSmo = Normal diet-smoke-exposed; DAir = Vitamin D-deficient diet-air exposed; DSmo = Vitamin D-deficient diet-smoke-exposed. (vit. D = Vitamin D).*

4.3.2 Induction of hypertrophy in the left plantaris muscle

The overload of the left plantaris muscle started after 6 weeks of cigarette smoke/room air exposure. Mice were anesthetized with a mixture of Ketamine (100 mg/kg, Ketalar®, Pfizer, Belgium), Xylazine (10 mg/kg, Rompun®, Bayer, Belgium) and Acepromazine (3 mg/kg, Placivet®, Kela, Belgium) administered intra-peritoneally at a volume of 150 µL/25g. After cessation of nociceptive responses, an incision was made in the popliteal area of the left hind limb to expose the tibial nerve with blunt dissection. The branches of the tibial nerve that innervate the soleus and gastrocnemius muscles were cut and small segments removed to prevent re-innervation. This strategy imposes an overload and subsequent compensatory hypertrophy of the plantaris muscle [31,32]. A sham operation was performed on the right hind limb that served as an internal control. Post-operative care included intraperitoneal injections of buprenorphine (0.2 mg/kg body weight) once a day for 3 days. After this 3-day recovery, mice were again exposed to either room air or CS.

4.3.3 Physical fitness

Maximal Exercise Capacity and whole-body strength were measured before the start of CS or room air exposure (baseline), before surgery (week 6) and after 18 weeks. The test to measure maximal exercise capacity consisted of a 5-minute warm up on a treadmill (0% incline, 3 m/min), followed by 1 m/min increments in speed every minute until exhaustion. Maximal exercise capacity was defined as the maximum speed attained by each animal. Whole body strength was measured by placing the mouse on a grid that was subsequently inverted and the latency-to-fall time was recorded. This served as a proxy for maximal muscle strength.

4.3.4 *In situ* contractile properties

The force generating capacity and fatigue resistance were determined *in situ* in the overloaded and sham plantaris muscle, as described before [11,31]. Mice were anaesthetized with a mixture of ketamine (100 mg/kg, Ketalar®, Pfizer, Belgium), Xylazine (10 mg/kg, Rompun®, Bayer, Belgium) and acepromazine (3 mg/kg, Placivet®, Kela, Belgium) administered intra-peritoneally at a volume of 150 µL/25g. The plantaris muscle was prepared free, keeping its blood and nerve supply intact, and the distal tendon was attached to a FORT-100 force transducer (World

Precision Instruments, Sarasota, Florida). The tibial nerve was cut proximally, and the distal end placed over stimulating electrodes to elicit contractions by supramaximal stimulation. The force was digitally recorded. Optimal muscle length was set with repeated twitch contractions 30 s apart and defined as the length at which maximal active twitch force was developed. The maximal twitch (at 1 Hz) and tetanic (at 200 Hz, train duration: 250 ms) tensions were recorded.

Subsequently, two sequential fatigue resistance tests were performed. The first one, which activates muscle metabolism and blood flow [11,31], consisted of a 1-min test with 100-Hz contractions with a duty cycle of 100 ms on / 1900 ms off for 1 min (FI100). The second fatigue test that consisted of repetitive isometric contractions at 30 Hz, 330 ms on / 670 ms off for 4 minutes (FI30). Fatigue indices were computed as follows: for FI100, force of the last contraction was divided by the highest contraction in this cycle, while for FI30, force 2 minutes after the strongest contraction was divided by the strongest contraction in this cycle. After the fatigue test, the optimal length was measured, the plantaris muscle excised and weighed. Forces were reported in mN and as specific force (force normalized to the anatomical cross-sectional area of the muscle). Anatomical cross-sectional area was calculated thus:

Anatomical CSA = ((muscle mass (g)) / [(optimal length (cm)) x (1.056 g/cm^3)]) [equation 1]

where 1.056 represents the muscle density

4.3.5 Inflammatory cell counts in the bronchoalveolar lavage fluid

To assess lung inflammation, mice were tracheotomized, and the lungs were lavaged 4 times using Dulbecco's phosphate-buffered saline to obtain bronchoalveolar lavage fluid. Total cell counts were performed with pooled fractions using a hemocytometer (Bürker bright-line, Optic Labor) with trypan blue staining. The rest of the fluid was centrifuged at 1000 g for 10 minutes at 4°C and the resulting cell pellets were stained with Diff-Quick (Medical Diagnostics, Düdingen, Germany) and 3×100 cells were counted to obtain differential cell counts (neutrophils, macrophages and lymphocytes).

4.3.6 Serum measurements

Serum from venous blood collected from the vena cava was used to measure 25-OH Vitamin-D3-D6 levels using liquid chromatography-tandem mass spectrometry (LC–MS/MS) (LC, Shimadzu and MS, Qtrap 5500, Sciex) as described previously [30].

4.3.7 Muscle histology

Left and right plantaris muscles were frozen using isopentane cooled in liquid nitrogen and stored at -80^{o}C. Immunohistochemical staining for myosin heavy chain isoforms was performed on 5 µm thick serial sections to measure the fiber cross sectional areas (FCSA) and fiber type distribution [21]. The pooled FCSA was calculated as total FCSA normalized for fiber type distribution. Areal fiber proportions were calculated as (e.g., for type IIA):

Areal fiber proportion (IIA) = 100% x ((CSA IIA x %IIA) / [(CSA IIA x %IIA) + (CSA IIX x %IIX) + (CSA IIB x %IIB)]) [equation 2]

4.3.8 Western immunoblotting of key markers of hypertrophic signaling

The hypertrophied left plantaris muscles were homogenized in Tris-HCl buffer (5 mM EDTA and 5 mM Tris·HCl, pH 7.5) 1:10 (wt/vol) containing a protease inhibitor cocktail (Roche, Complete) as described previously [33]. Equal concentrations of protein (15-30 µg) were separated under reducing conditions using 12% SDS-PAGE (Biorad mini-PROTEAN) and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked in 5% non-fat dry milk or 5% bovine serum albumin in TBS-0.1% Tween20 and then incubated overnight at 4°C with primary antibody. The following primary antibodies from Cell Signaling Technologies (Leiden, The Netherlands) were used: anti-phospho-Akt (#4060S, 1:2000), anti-Akt (#9272S, 1:2000), anti-phospho-4EBP1 (#2855S, 1:1000), anti-4EBP1 (#9644S, 1:1000), anti-phospho FoxO1 (#9461S, 1:1000) and anti-FoxO1 (#2880S, 1:1000). After incubation with the HRP-conjugated secondary antibody (P0217, Sigma), protein bands were detected using an enhanced chemiluminescence system (Sigma-Aldrich, Belgium and Thermo Fisher Scientific, USA), analyzed using the software package (Bio 1D) of the blot imaging system (Photo print, Vilber, France) and phosphorylation status (activity) was determined as the ratio of phosphorylated to total protein.

4.3.9 Statistical analysis

The data were analyzed using GraphPad prism for windows, version 8.2.1 (GraphPad Software, California) and SPSS statistics version 26.0 (IBM corporation, New York). A Shapiro-Wilk test was used to test whether the data in each group were normally distributed. Comparison of whole-body mass, whole-body strength, maximal exercise capacity, protein levels and lung inflammation between groups was done using a two-way analysis of variance (ANOVA) with CS exposure and vitamin D deficiency as independent factors. Muscle mass and histology data were analyzed using a three-way ANOVA with CS exposure, vitamin D deficiency and hypertrophy/atrophy as independent factors. Tukey's post-hoc tests were used for multiple comparisons. Mixed model analysis followed by Tukey's post-hoc test was done when the data had random missing values. Multi-factor ANOVA was used when the comparison between groups involved more than 3 variables as was the case with FCSA and proportions. Significance level was set at $p<0.05$. Values are presented as mean ± standard deviation (SD).

4.4 Results

4.4.1 Vitamin D levels in serum

Serum levels of 25(OH)D in the mice on vitamin D-deficient diet were below the level of detection (< 2.1 µg/L) and lower than in the mice on normal diet (19.4 ± 2.8 µg/L). Exposure to CS did not significantly alter the serum vitamin D concentration.

4.4.2 Lung inflammatory cell count

Exposure to CS caused significant lung inflammation as neutrophil, macrophage, and total cell count (all p<0.0001) were all higher (+56 %) in the bronchoalveolar lavage fluid after smoking, irrespective of diet (Fig. 4.2A–C).

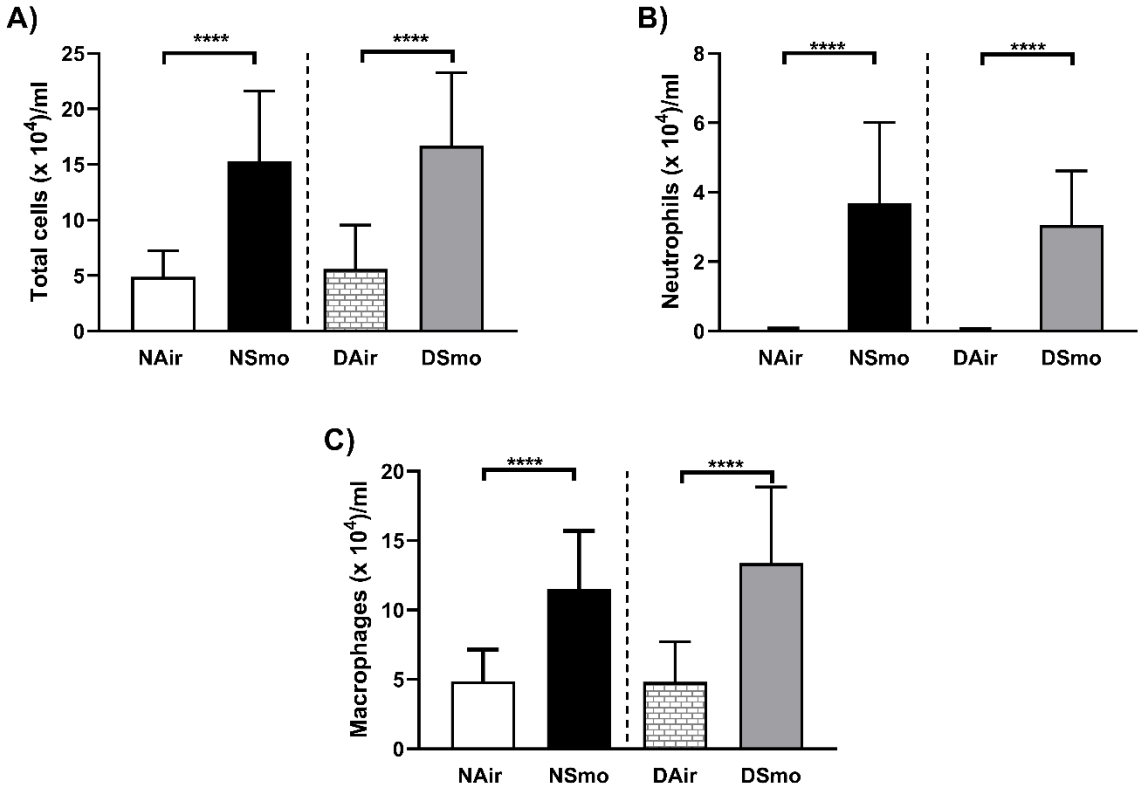

Figure 4.2: *Lung inflammatory cell count. Total cell count (A), number of neutrophils (B) and macrophages (C) were significantly higher in the bronchoalveolar lavage fluid after smoking, irrespective of diet. NAir = Normal diet-air exposed (open bars, n=15); NSmo = Normal diet-smoke-exposed (black bars, n=16); DAir = Vitamin D-deficient diet-air exposed (brick bars, n=16); DSmo*

*= Vitamin D-deficient diet-smoke-exposed (grey bars, n=11) mice. ****: p < 0.0001. Data are presented as mean ± SD.*

4.4.3 Body mass and food intake

Body mass increased over time but significantly less in CS-exposed animals from the 11th week on, irrespective of diet (Fig. 4.3A).

Food intake was similar in all groups for the first 2 weeks. From week 3 on, mice on a vitamin D-deficient diet ate less than their counterparts on a normal diet (-15%, $p < 0.05$). Also, CS-exposed mice generally ate less than their air-exposed counterparts from week 5 on (-10 %, $p < 0.05$). There were no interaction effects (Figure 4.3B).

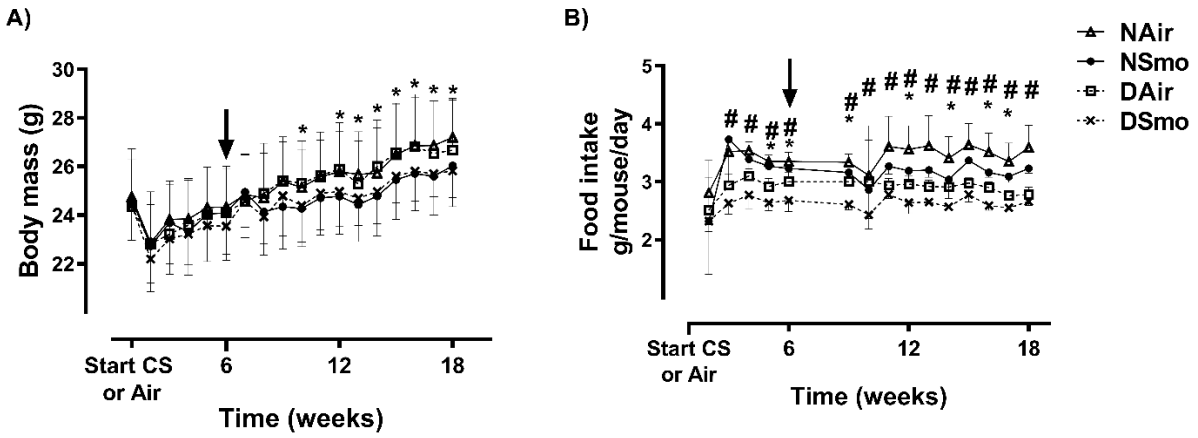

Figure 4.3: *Body mass and food intake during cigarette smoke or air exposure. A: Body weight gain was lower in the cigarette smoke (CS)-exposed mice and food intake (B) in vitamin D-deficient mice was less from week 2. NAir = Normal diet-air-exposed (open triangles, n=15); NSmo = Normal diet-smoke-exposed (closed circles, n=16); DAir = Vitamin D-deficient diet-air-exposed (open squares, n=16); DSmo = Vitamin D-deficient diet-smoke-exposed (crosses, n=11) mice. Black arrows indicate overload surgery. *: p < 0.05 vs. smoking, #: p < 0.05 vs. vitamin D deficiency. Values are presented as mean ± SD.*

4.4.4 Physical fitness

CS exposure and/or vitamin D deficiency did not significantly affect maximal exercise capacity at any time point (Fig. 4.4A). There were also no differences in whole-body strength between groups at any time point (Fig. 4.4B).

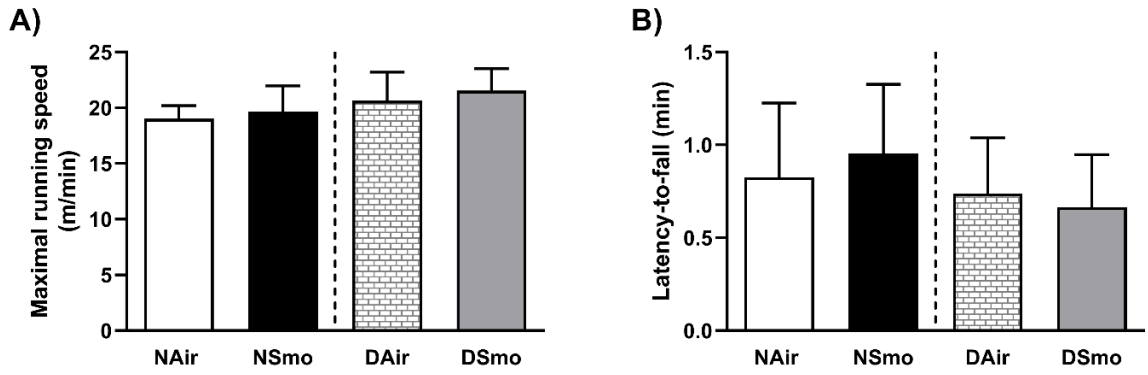

Figure 4.4: *Physical fitness at sacrifice. Maximal exercise capacity (A) and Whole-Body Strength (latency-to-fall time) (B) at sacrifice in all groups. There was no significant difference in maximal exercise capacity or whole-body strength between groups. NAir = Normal diet-air exposed (open bars); NSmo = Normal diet-smoke-exposed (black bars); DAir = Vitamin D-deficient diet-air exposed (brick bars); DSmo = Vitamin D-deficient diet-smoke-exposed (grey bars) mice. (n = 11/group). Values are presented as mean ± SD.*

4.4.5 Skeletal muscle structure and contractile function

4.4.5.1 Muscle mass

There was no significant effect of either smoking or vitamin D deficiency on the mass of the control and overloaded plantaris muscle (Fig. 4.5A). As expected, the mass of the overloaded left plantaris muscles was higher (+ 20 – 25 %) than that of the contralateral muscle ($p < 0.0001$). This response was not significantly affected by CS exposure or vitamin D deficiency (Fig. 4.5A).

The soleus and gastrocnemius (Fig. 4.5B and C) mass in the smoking mice was lower than that of non-smoking mice, irrespective of diet (-10 %, $p = 0.012$). The mass of the denervated soleus and gastrocnemius (Fig. 4.5B and C) muscles were lower than the contralateral muscles (-25 % and -60 % respectively, $p < 0.0001$), irrespective of group (Fig. 4.5B). The vitamin D x atrophy

interaction (p = 0.008) for the gastrocnemius muscle was reflected by a larger decrease in muscle mass in vitamin D-deficient mice, irrespective of smoking status (Fig. 4.5C).

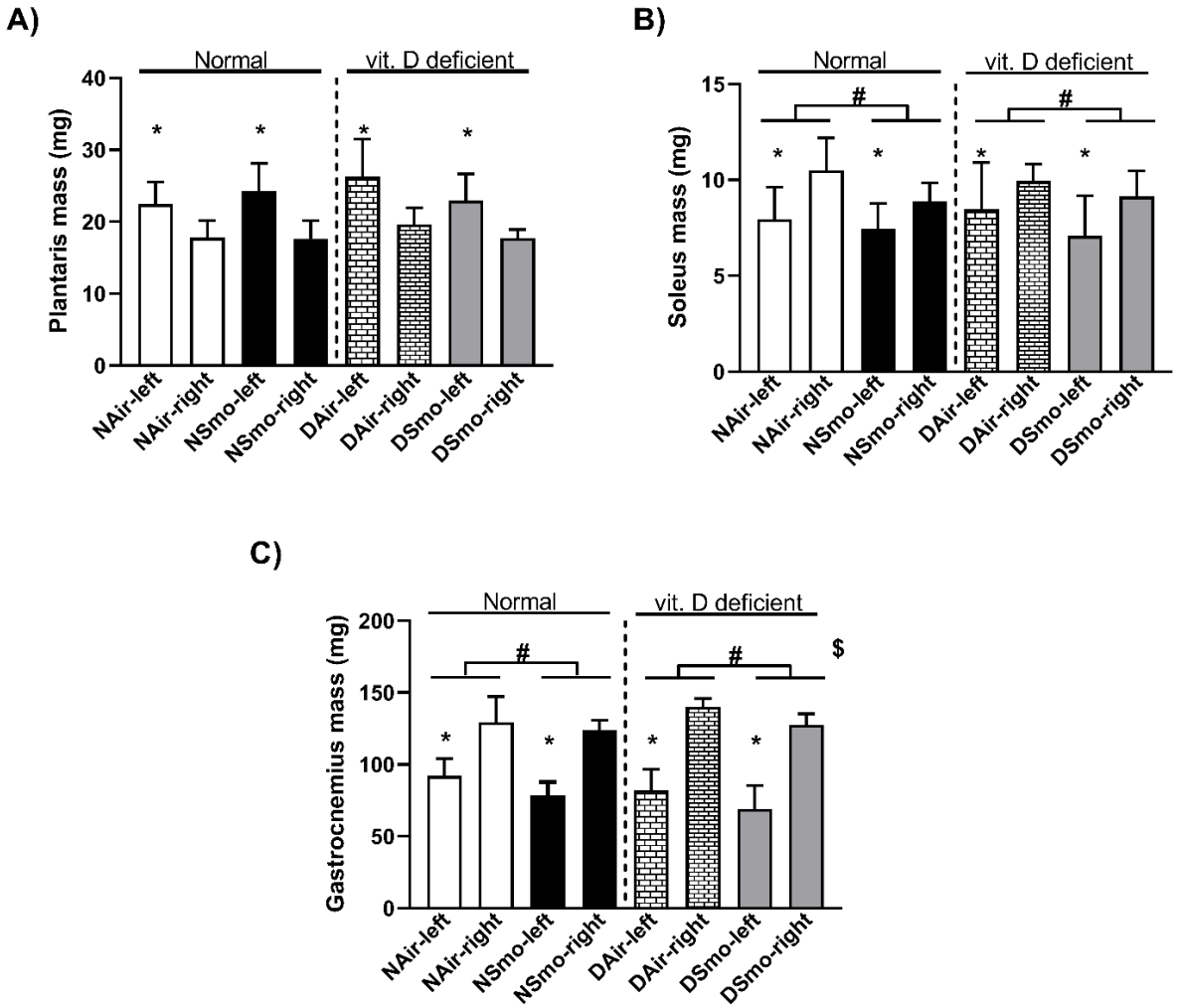

Figure 4.5: *Effects of cigarette smoke exposure, vitamin D deficiency and overload/denervation on plantaris, soleus and gastrocnemius muscle mass. Mass of the overloaded (left) and sham (right) plantaris (A); denervated and sham soleus (B) and denervated and sham gastrocnemius (C). Plantaris muscle overload led to a significant increase in mass, while denervation of the soleus and gastrocnemius caused significant decrease in mass. NAir = Normal diet-air-exposed (open bars); NSmo = Normal diet-smoke-exposed (black bars); DAir = Vitamin D-deficient diet-air-exposed (brick bars); DSmo = Vitamin D-deficient diet-smoke-exposed (grey bars) mice. (n = 11/group);* ***** *= Significant hypertrophic/atrophic response (p<0.05);* **#***: p<0.05 for air vs CS;* **$** *=*

more atrophy in the gastrocnemius muscles of vitamin D-deficient mice. Values are presented as mean ± SD.

4.4.5.2 Plantaris muscle force and fatigue index

Despite plantaris hypertrophy, no significant differences were observed in absolute (N) or specific (N/cm^2) twitch (Fig. 4.6A) and tetanic (Fig. 4.6B) forces compared to the contralateral muscle. However, when we compared force measurements from the sham and hypertrophied muscles irrespective of vitamin D or smoking status, we found that the overloaded muscles produced higher absolute twitch (+22 %, p = 0.043) and tetanic (+23 %, p = 0.038) forces than the sham muscles, though the specific forces were not higher (data not shown). The hypertrophied plantaris muscles had a higher fatigue index (FI100 and FI30) than the contralateral muscle (+20 %, p = 0.010 for FI100 and +21 %, p = 0.009 for FI30, Fig. 4.6C and D) with no significant effect of smoking and/or vitamin D deficiency.

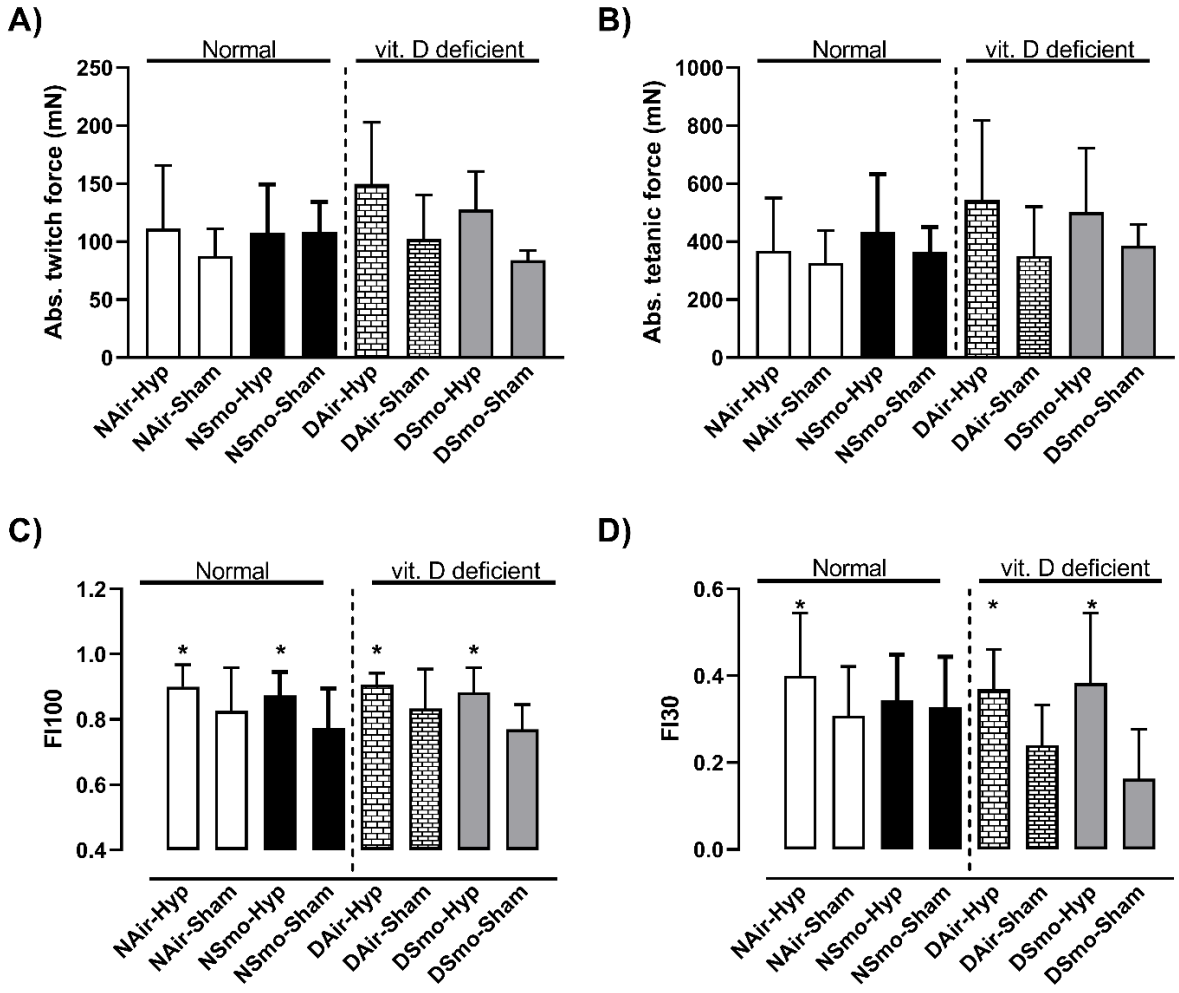

Figure 4.6: *Effects of cigarette smoke exposure and vitamin D deficiency on contractile properties. In situ left hypertrophied (Hyp) and right (Sham) plantaris muscle absolute twitch [1Hz] force (A), absolute tetanic [200 Hz] force (B), fatigue index at 100 Hz stimulation (C) and fatigue index at 30 Hz stimulation (D). Hypertrophied plantaris muscles had a higher fatigue resistance but not force, irrespective of diet and/or smoking. NAir = Normal diet-air-exposed (open bars); NSmo = Normal diet-smoke-exposed (black bars); DAir = Vitamin D-deficient diet-air-exposed (brick bars); DSmo = Vitamin D-deficient diet-smoke-exposed (grey bars) mice. (n = 6/group); * = p < 0.05 vs. contralateral control. Values are mean ± SD.*

4.4.5.3 Plantaris muscle fiber cross-sectional area and fiber type composition

4.4.5.3.1 Fiber cross-sectional area (FCSA)

A typical example of a plantaris muscle cross section stained for different myosin heavy chain isoforms used to determine fiber types, areas and proportions is shown in figure 4.7 (A and B). Due to the low presence or complete absence of type I muscle fibers in the plantaris, they were not considered in all histological analyses.

The fibers of overloaded plantaris muscles were larger (+34 %, p = 0.0003 [IIA]; +33 %, p = 0.0002 [IIX]; +34 %, p = 0.0004 [IIB]) compared to the contralateral muscle, when analyzed per type (Fig. 4.7C – E) or pooled (Fig. 4.7F, +30 %, p = 0.0016), irrespective of vitamin D deficiency or smoking. However, the increase in FCSA (hypertrophic response) in the Vitamin D-deficient smoking mice was smaller (interaction effect between smoking and vitamin D deficiency; p = 0.03), suggestive of a blunted hypertrophic response in the vitamin D-deficient, CS-exposed mice.

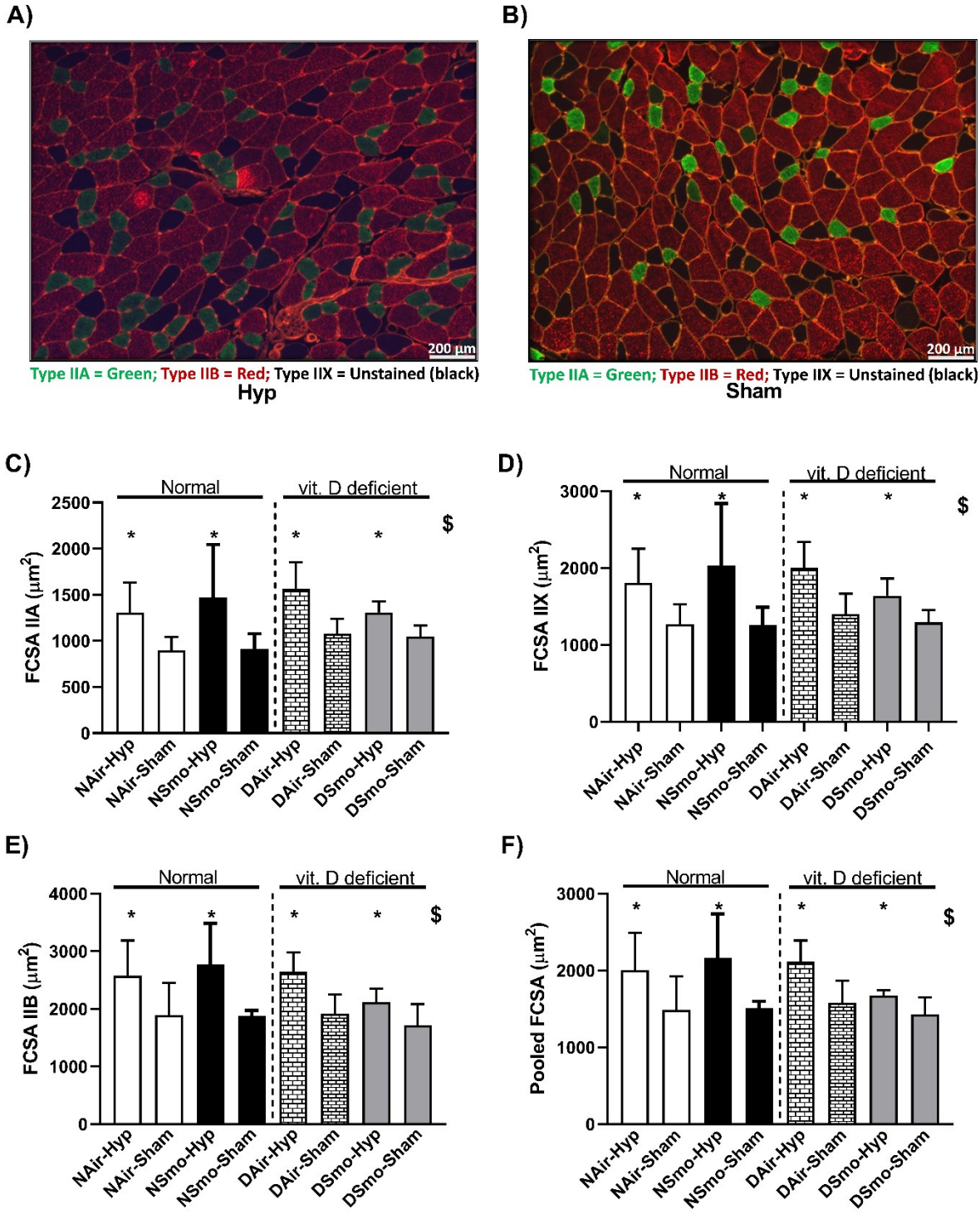

Figure 4.7: *Effects of cigarette smoke exposure, vitamin D deficiency and overload on plantaris muscle fiber cross-sectional area (FCSA). Representative example of left hypertrophied (Hyp) [A] and right (Sham) [B] plantaris muscle cross-sections stained for the different myosin heavy chain*

isoforms. (C) IIA, (D) IIX, (E) IIB and (F) Pooled fiber cross-sectional areas (FCSA) in hypertrophied (Hyp) and sham plantaris in NAir = Normal diet-air-exposed (open bars); NSmo = Normal diet-smoke-exposed (black bars); DAir = Vitamin D-deficient diet-air-exposed (brick bars); DSmo = Vitamin D-deficient diet-smoke-exposed (grey bars) mice. Hypertrophy led to an increase in FCSA, irrespective of diet or smoking. (n = 6/group); ***** *= Significant effect of hypertrophy (p < 0.05 vs contralateral control);* ***$*** *= Blunted hypertrophic response in vitamin D-deficient CS-exposed mice (p < 0.05). Values are as mean ± SD.*

4.4.5.3.2 Fiber type distribution and areal proportions

There were no significant main effects of CS exposure, hypertrophy, or vitamin D deficiency on the proportion of type IIA and IIX fibers. However, there were significant interactions between vitamin D deficiency and hypertrophy (p = 0.021), and between CS exposure and hypertrophy (p = 0.038) for the proportion of IIX fibers (Fig. 4.8A), which implies that the combination of vitamin D deficiency and CS exposure led to a higher percentage of IIX fibers in the overloaded muscles, but not in the other groups. The numerical (Fig. 4.8B) and areal (Fig. 4.8C) proportions of type IIB fibers was lower in the hypertrophied muscles, irrespective of CS-exposure and/or vitamin D deficiency (p = 0.04).

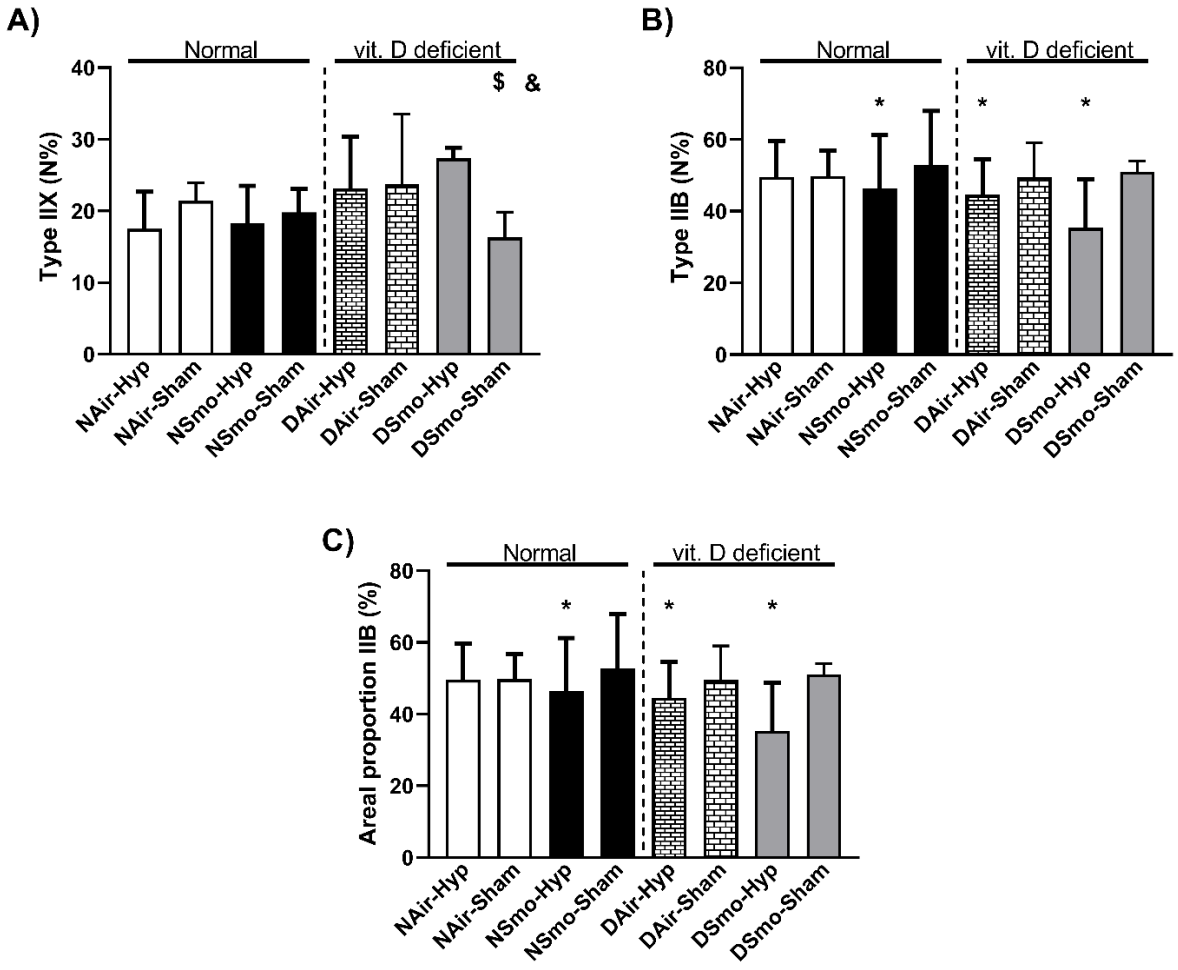

Figure 4.8: *Fiber proportions and contribution to total area. Numerical proportions of type IIX (A), IIB (B) and areal proportions of IIB (C) fibers in the hypertrophied (Hyp) and sham plantaris muscle in NAir = Normal diet-air-exposed (open bars); NSmo = Normal diet-smoke-exposed (black bars); DAir = Vitamin D-deficient diet-air-exposed (brick bars); DSmo = Vitamin D-deficient diet-smoke-exposed (grey bars) mice. The hypertrophied plantaris muscle had a lower numerical and areal proportion of type IIB fibers, while type IIX fiber proportions only showed interactions (vitamin D deficiency x hypertrophy; CS-exposure x hypertrophy) with no main effects of CS-exposure, hypertrophy, or vitamin D deficiency. (n = 6/group);* ***** *= Significant effect of hypertrophy (p < 0.05 vs contralateral control);* ***$*** *= Larger overload effect compared to CS-exposure (p = 0.038);* ***&*** *= Larger overload effect compared to vitamin D deficiency. The interactions suggest that combined treatment caused higher %IIX in overloaded muscles. Values are as mean ± SD.*

4.4.6 Protein synthesis markers

There were no significant main effects of, or interactions between, CS exposure and Vitamin D status on the total protein concentration and activity of Akt (Fig. 4.9B), 4EBP1 (Fig. 4.9C) and FoxO1 (Fig. 4.9D) in the hypertrophied plantaris muscle. Typical examples of western blots for Akt, phospho-Akt, 4EBP1, phospho-4EBP1, FoxO1 and phospho-FoxO1 can be seen in Fig. 4.9A.

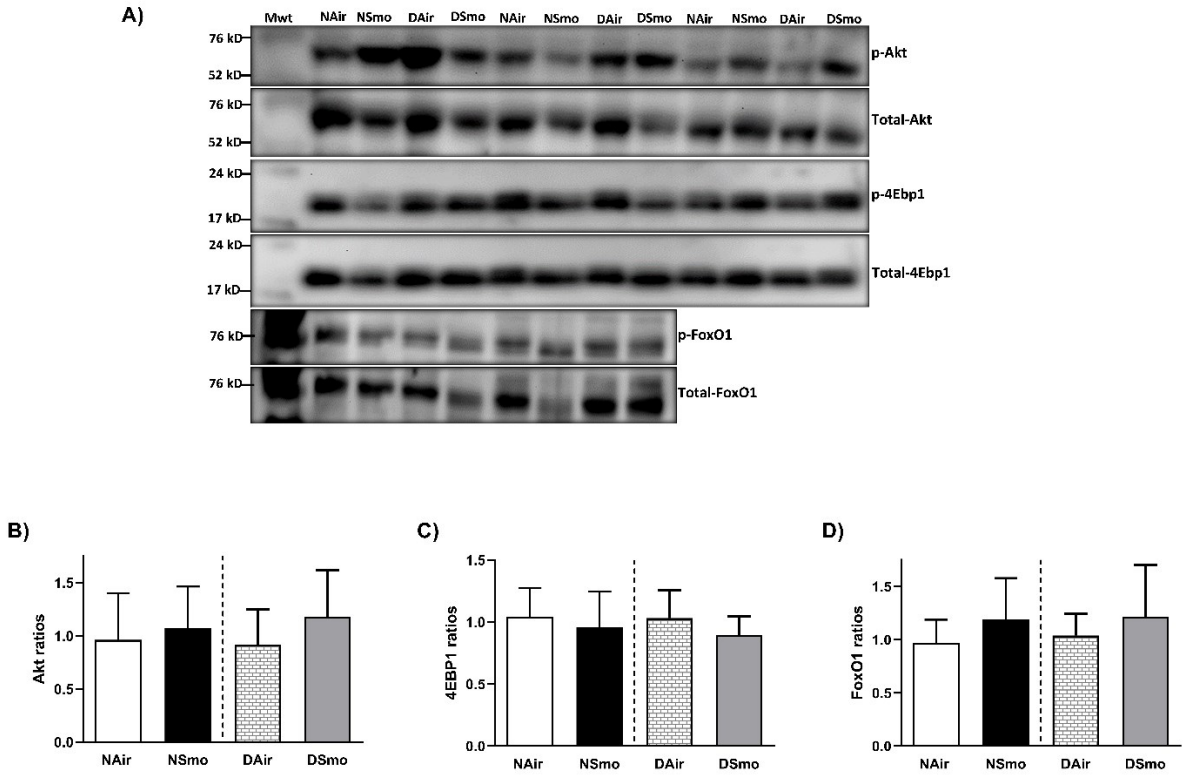

Figure 4.9: *Protein synthesis markers in left plantaris. A: Representative examples of western immunoblots from hypertrophied plantaris muscles for phospho-Akt, total-Akt, phospho-4EBP1, total-4EBP1, phospho-FoxO1 and total-FoxO1. Phospho-Akt/total-Akt ratios (B), phospho-4EBP1/total-4EBP1 ratios (C), phospho-FoxO1/total-FoxO1 ratios (D) in. NAir = Normal diet-air exposed (open bars); NSmo = Normal diet-smoke-exposed (black bars); DAir = Vitamin D-deficient diet air-exposed (brick bars); DSmo = Vitamin D-deficient diet smoke-exposed (grey bars) mice (n = 6/group). There were no significant effects of cigarette smoking or vitamin D status. Values are presented as mean ± SD.*

4.5 Discussion

This study examined for the first time whether cigarette smoking and/or vitamin D deficiency affect the hypertrophic response of skeletal muscle to overload in mice. Our data reveal that CS exposure or vitamin D alone induced a similar hypertrophy response in the overloaded plantaris, but this response was reduced when both conditions were combined, as shown by the attenuated increase in FCSA in the hypertrophied plantaris muscles in vitamin D-deficient CS-exposed mice. This suggests that current smokers who also suffer from vitamin D deficiency, may benefit less from regular resistance training to increase muscle mass.

In agreement with previous studies, CS exposure led to significantly lower body mass [5,7,11,34,35], loss of soleus and gastrocnemius muscle mass [8,21,34] and lung inflammatory cell infiltration [35,36], irrespective of diet. We confirmed that maximal exercise capacity and whole-body strength remained unaffected after 14-24 weeks of CS exposure [11,36].

Corresponding with previous studies, mice on the vitamin D-deficient diet became severely deficient as compared to those on a normal diet [30]. Even though CS exposure is a known risk factor for vitamin D-deficiency [25], it did not alter the levels of vitamin D. Although the mice on a vitamin D-deficient diet generally ate less, their body mass gain was not lower than their counterparts on a normal diet. This suggests that the energy efficiency was elevated, or physical activity levels reduced in mice on a vitamin D-deficient diet. However, the maximal exercise capacity, whole-body strength and skeletal muscle fatigue resistance were not diminished by smoking or vitamin D deficiency, all indicative that the lowered physical activity was not pronounced and thus unlikely to have an impact on skeletal muscle structure and function. In line with this, it has been shown that vitamin D-deficient mice showed no alterations in physical fitness even after 24 weeks except when specific muscles were immobilized [37].

In agreement with previous studies reporting muscle wasting due to smoking [36,38], we also observed a lower muscle mass in smoking mice. Similarly, in our study, there was no significant effect of the Vitamin D status on muscle mass in line with previous studies in vitamin D deficient rats where calcium and phosphorus serum levels were maintained within the normal range [39,40]. Another factor that may underlie the muscle wasting in these conditions is disuse as suggested

by the reduced exercise capacity in vitamin D deficiency [41], although the mice in our and another study [37] showed no indication of reduced exercise capacity.

As expected [17,42], overload of the left plantaris muscle in our study led to hypertrophy as demonstrated by the increase in plantaris mass and FCSA. While CS-exposure and/or vitamin D deficiency on their own did not attenuate the compensatory increase in plantaris mass, combination of both reduced the increase in FCSA of the overloaded plantaris muscle, irrespective of fiber type. This is a novel finding that may explain why some individuals fail to respond to stimuli such as exercise training meant to increase muscle size.

Despite the overload-induced increase in plantaris muscle mass and FCSA, there were no significant changes in plantaris force-generating capacity, irrespective of CS-exposure and/or vitamin D deficiency. This is in conformity with data reported in an overload model of rats on a normal diet [43] but in contradiction with a functional overload model in mice on a normal diet [16]. The discrepancies may be due to a difference in the age at the time of analysis (5 months at the end versus 6.5 months in our study), the duration of overload stimulus (4 weeks vs 12 weeks in our study), the way overload was induced (ablation of soleus and gastrocnemius vs their denervation) and the difference in mouse strain used.

Although there were no alterations in twitch and tetanic force, the hypertrophied plantaris muscles of the mice in our study were more resistant to fatigue, irrespective of CS exposure and/or vitamin D deficiency. This improved fatigue resistance may be the result of a decreased proportion of type IIB fibers as observed here and by others [44,45], as the ATP cost for isometric tension is higher for type IIB than any other fiber type [46]. Thus, although the hypertrophic response was blunted in the presence of both vitamin D deficiency and smoking, this combination did not attenuate the improvement in fatigue resistance during hypertrophy.

To investigate whether the molecular or biochemical changes during overload were affected by CS exposure and/or vitamin D deficiency, key markers of hypertrophic signaling pathways were measured in the hypertrophied plantaris muscles. No differences in protein levels of positive regulators (Akt and 4EBP1) [44] and negative regulators (FoxO1) [47] of muscle protein homeostasis were observed between the different groups indicating no effect of CS and/or vitamin D

deficiency on these hypertrophic markers. A study by Huey *et al.* showed that the hypertrophic response after functional overload in mice is already significant 7 days after surgery [16] and it is accompanied by an increase in hypertrophic markers such as IGF-I and Akt. However, this increase was transient and had disappeared after 14 days, where phosphorylated Akt even decreased below baseline levels at 30 days [16]. The lack of differences in the levels of hypertrophic markers in our study is in agreement with the time course of these events and is likely reflecting that a new steady state has long been reached. Indeed, we have previously observed in rats that the development of hypertrophy tapers off after 4 weeks and has reached a new steady state after 6 weeks of overload [48]. It is possible that the hypertrophic response was somewhat delayed during smoking or vitamin D deficiency as a consequence of an attenuated activation of these signaling pathways. Such a situation is reminiscent to the delayed response to electrical stimulation in muscles [49] and the delayed hypertrophy after reloading of disused muscle [50] from old rodents. It therefore remains to be seen whether the development of hypertrophy is delayed in Vitamin D deficiency and/or smoking.

To conclude, even though smoking and Vitamin D deficiency individually did not alter the hypertrophic response of skeletal muscle to overload, the combination of smoking and Vitamin D deficiency did blunt the development of overload-induced hypertrophy. These findings should be kept in mind especially when attempting to improve muscle mass in individuals who are smoking and vitamin D deficient.

4.6 Data availability statement

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

4.7 Competing interests

None to declare

4.8 Author contributions

Conception and design – H.D., G.G.-R., T.T., T.A., and W.J.; Data acquisition and analysis – T.A., J.S., K.M., G.G.-R, R.W., J.B., and H.D.; Interpretation of data, drafting of manuscript and final approval - T.A., J.S., J.B., K.M., R.W., W.J., T.T., G.G.-R., and H.D.

4.9 Funding

This study was funded by the Directorate-General for Education and Culture of the European Commission through MOVE-AGE, an Erasmus Mundus Joint Doctorate program (2011-2015).

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CHAPTER 5

General Discussion

5 General discussion

5.1 Summary of the findings

The studies in this PhD thesis were designed to gain more insights into cigarette smoke (CS)-induced skeletal muscle dysfunction, highlight the potential benefits of smoking cessation on skeletal muscle structure and function and evaluate the effects of smoking and/or vitamin D deficiency on the hypertrophic response in a smoking mouse model. In the first study (chapter 3) we showed that CS-induced neutrophilic lung inflammation, muscle mass loss, mitochondrial dysfunction and impaired capillarization were back to control levels within 1 – 2 weeks of smoking cessation. This study also revealed that compared to limb muscles, the diaphragm is more susceptible to CS exposure and recovers rapidly after smoking cessation. The results of the second study (chapter 4) showed that though vitamin D deficiency or CS exposure on their own did not blunt the hypertrophic response, the combination of both attenuated the development of overload-induced skeletal muscle hypertrophy.

5.2 Skeletal muscle dysfunction in CS-exposed mice

Skeletal muscle dysfunction has been described in cigarette smokers and CS-exposed animal models, and it involves both limb and respiratory muscles [1–4]. Several consequences of smoking on skeletal muscles have been described including reduction in muscle mass, mitochondrial dysfunction, fibre atrophy and capillary rarefaction.

In line with some previous studies in mice [5–7], our results show that 12 – 18 weeks of CS exposure result in loss of whole body fat, lean mass and skeletal muscle wasting as indicated by the significant reductions (~20%) in soleus, plantaris and gastrocnemius muscle mass. Data on the effects of chronic CS exposure on skeletal muscle mass is controversial. Some studies report a reduction in skeletal muscle mass in CS-exposed mice [5–8] and humans [4,9–11], while others indicate little or no effects in mice [12,13] and humans [14,15]. These divergences may be due to differences in age, sex, diet, physical activity, smoking status, and mouse strain. Whatever the cause of the discrepancies, the fact that we observed significant reductions in whole body fat and lean mass in CS-exposed mice as compared to age, physical activity and diet-matched controls indicates that chronic smoking does lead to muscle wasting. This is further supported by our observation

that CS did not cause any decrement in physical activity and physical fitness (Table S1 [Chapter 3]; Figure 4.4 [Chapter 4]). Several studies have shown that components of CS and CS-induced cytokines could cause muscle wasting by inhibiting protein synthesis and activating proteolysis [5,16–18]. For example, an increase in the expression of muscle specific enhancers of ubiquitin-mediated proteolysis such as muscle atrophy F-box (MAFBx) protein and Muscle Ring Finger-1 (MuRF1) protein has been observed in the limb muscles of CS-exposed mice and in the vastus lateralis muscle of smokers [16,17]. Also, a decrease in quadriceps muscle protein synthesis as assessed by incorporation of labelled leucine has been observed in smokers, which was coupled with increased expression of myostatin that inhibits Akt, a positive regulator of protein synthesis [17]. A decrease in Akt expression has also been observed in the muscles of CS-exposed mice [5]. An increase in the levels of proinflammatory cytokines such as TNF-α in mice [16] and IL-6 in humans [17,19] due to smoking is also known to enhance proteolysis and inhibit protein synthesis. The CS-induced muscle wasting we observed is thus most likely the result of a multifactorial process.

Despite the CS-induced loss of limb muscle mass, CS exposure did not lead to fibre atrophy or altered fibre type composition in the plantaris and soleus muscles, as shown in chapter 3. This is in accordance with previous studies in mice exposed to cigarette smoke for 12 – 18 weeks [6,12]. The lack of changes in force and fatigue resistance in CS-exposed mice in our studies are in line with the absence of fibre atrophy and maintenance of fibre type composition which are known to affect force-generating capacity and fatigue resistance, respectively. As opposed to limb muscles, the diaphragm muscle showed significant atrophy of all fibre types after 14 weeks of CS exposure. Though the literature on diaphragm muscle atrophy are inconclusive with some [20,21] but not all [3,13] reporting CS-induced atrophy, in agreement with previous studies [13], our results show that CS-induced structural and morphological changes in the diaphragm that precede those in the peripheral muscles and occurs in the face of unchanged levels of physical activity. This may indicate that the diaphragm function is compromised earlier during smoking than peripheral muscle function. Though our data may not provide full evidence to support this claim, our conclusion is in line with some studies in the literature [13]. The rapid reversal of CS induced changes in the diaphragm after smoking cessation also support this claim. Thanks to its participation in every breathing cycle even at rest, the diaphragm muscle is much more active

than the limb muscles which are used intermittently during movement. In order to keep up with the high functional demands, the diaphragm requires a dense capillary network to supply all the nutrients and oxygen necessary. Our results showed that the diaphragm muscle had a higher capillary density than the limb muscles. Therefore, due to the higher levels of blood flow through the diaphragm as compared to the limb muscles, they may be more readily exposed to the deleterious components of CS through the bloodstream.

Due to their central role in movement and posture, skeletal muscles are regularly active and require a rapid and stable source of ATP that is primarily provided by mitochondria. Oxidative skeletal muscles have more mitochondria than predominantly glycolytic muscles [22]. The soleus muscle was used in our study to evaluate the alterations in muscle mitochondrial respiration and protein content with CS exposure. This muscle which presents a predominantly oxidative phenotype [23] and is rich in mitochondria, has been reported to have the closest molecular similitude to many human skeletal muscles [24]. In accordance with previous studies in mice [25] and in smokers [26], our results show that chronic cigarette smoking impairs skeletal muscle mitochondrial respiration and leads to lower protein levels of subunits of complex I, III and IV. Studies in human limb muscles have also reported decreased oxygenation, reduced ATP synthesis and impaired electron transport in the mitochondria resulting from cigarette smoking [26–28]. Studies in mice are controversial, with some reporting CS-induced limb muscle mitochondrial dysfunction [16,25], while others indicate no alterations [13]. These discrepancies are likely related to the protocol of CS exposure (duration, amount of cigarette per session, number of sessions per day), type of muscle analysed (oxidative or glycolytic/respiratory or limb) and mouse strain used (C57BL/6 or A/JolaHSD or BALB/C) as well as gender. To further assess the direct effect of CS on skeletal muscle mitochondrial function, we exposed soleus muscles from air-exposed mice to CS extracts, and this resulted in reduced mitochondrial respiration that was readily reversed after removal of the CS extract. In accordance with previous studies in humans [27,28], these data suggest that carbon monoxide and other substances in CS can directly impair the function of respiratory chain complexes. In summary, our data show that chronic exposure to CS impairs mitochondrial respiration and leads to reduced expression of mitochondrial enzyme complexes which also contributes to the process muscle wasting.

Skeletal muscles require an adequate supply of oxygen for the aerobic generation of ATP and this is provided by the exchange of oxygen between the blood and muscle cells through the capillary network. It is important to note that the main contributing factor to skeletal muscle capillarization is muscle fibre size, while oxidative capacity and fibre type are much less relevant [29,30]. The results in chapter 3 show that CS exposure had no effects on capillarization in the limb muscles. This is in concordance with data from the extensor digitorum longus which has similar properties to the plantaris muscle [16]. Considering that there were no changes in fibre dimensions and proportions in the limb muscles of CS-exposed mice, and the fact that capillarity depends on the size, and to a smaller extent on the fibre type composition, it is not surprising that CS exposure did not alter capillarization in the limb muscles of the mice in our study. Studies on the effects of CS-exposure on respiratory muscle capillarization are lacking. Our results show for the first time that CS exposure in mice leads to an increase in capillary density (CD) and capillary fibre density (CFD) in the diaphragm muscle, which, in the absence of alterations in average number of capillaries per fibre (capillary-to-fibre ratio) and no change in local capillary-to-fibre ratio indicates no angiogenesis and no capillary loss. Since CS exposure reduced the size of all fibre types in the diaphragm, we conclude that the increase in CD and CFD resulted from diaphragm fibre atrophy and not from angiogenesis. In summary, our data in chapter 3 showed that while 14 weeks of CS exposure did not alter limb muscle capillarization, the diaphragm muscle displayed increased CD and CFD which were a consequence of fibre atrophy and not angiogenesis.

A clinical rationale for studying CS-induced skeletal muscle dysfunction is that CS directly impairs skeletal muscle metabolism. This over time can lead to structural alterations that may impair exercise capacity and physical activity which, in turn will contribute to the increased morbidity and mortality seen in smokers. Due to the influence of several inherent genetic factors and external factors such as diet, weather, physical activity status and lifestyle, the translation of alterations in muscle fiber CSA to changes in muscle and body mass to skeletal muscle function and then to exercise capacity and whole-body strength is not always straightforward. Though each of the events in the sequence is related, like other organ systems, skeletal muscles possess several mechanisms to combat any homeostatic disturbances. CS-induced skeletal muscle

dysfunction is a multifactorial process, and CS-induced deleterious changes in, for instance fiber size, may not necessarily translate into changes in muscle mass.

Though our data from both studies show that chronic CS exposure causes significant loss of whole-body lean and fat mass, the effect on skeletal muscles is muscle specific. Different muscles have different fiber type composition tailored to their function. In chapter 3, our data show that 12 - 14 weeks of CS exposure lead to loss of limb muscle mass with no change in fiber CSA, while the diaphragm showed a decrease in fiber CSA. Such an apparent discrepancy between changes in fiber CSA and muscle mass are not unique to our study and have been observed by others in mice [31]and humans [32,33]. Perhaps such discrepancies are related to differences in the amount of connective tissue and/or fat infiltration and changes in muscle architecture. Whatever the cause, we have no clear explanation for these discrepancies.

We agree that it is somewhat surprising that despite the loss in limb muscle mass, there was no significant reduction in muscle force generating capacity, which did fit the absence of changes in fiber proportions and CSA. Again, such discrepancies may be attributable to differences in muscle architecture, where atrophy results in a reduction in pennation angle, causing the muscle fibers to be more aligned with the line of pull (a diagrammatic presentation of this phenomenon can be found in [34]). Similar results were obtained in chapter 4.

Measurement of mitochondrial function and enzyme content in the soleus muscle showed that chronic CS exposure leads to muscle mitochondrial dysfunction. This shows that even when CS-induced phenotypic alterations are not obvious, mitochondrial dysfunction may be present, which may culminate in structural and functional changes in the long run. The time spent smoking and intervals between smoking sessions are important considerations since effects of CS are usually acute, intermittent and time dependent. The focus of chapter 3 was to demonstrate improvement in skeletal muscle metabolism and structure with smoking cessation. It was shown that all the alterations with CS exposure were reversed within 2 weeks of smoking cessation in mice.

Although CS did not result in a reduction in muscle fatigue resistance, it should be noted that, while the test we used was related to single fiber and motor unit oxidative capacity, this may not

be so in whole muscle (for a review see [35]). It may well be that the microcirculation is a more important determinant of fatigue resistance than oxidative capacity, as reflected by a reduced fatigue resistance when capillaries were randomly blocked with microspheres [36]. In line with this, in our study, smoking did neither reduce muscle fatigue resistance, nor muscle capillarization.

Chapter 5 shows that CS exposure led to a loss of muscle mass in the soleus and gastrocnemius muscles but not in the plantaris muscles. This confirms that the effects of CS exposure differ from one muscle to the other. It was in the plantaris muscle that we measured the force generating capacity and fatigue resistance. Although CS exposure did not alter muscle force production, overload led to an increase in mass, CSA, and a proportional increase in force. Similar to the results in chapter 3, and in line with the absence of significant alterations in fiber type composition, CS exposure did not decrease plantaris muscle fatigue resistance, even when combined with vitamin D deficiency. The hypertrophic response of the left plantaris and denervation of its synergists may have caused some imbalance which could have imposed a little extra effort on the contralateral control plantaris which may have overshadowed the effects of smoking.

Even though CS exposure alone or vitamin D deficiency alone did not alter muscle fiber CSA, we saw that a combination of both factors attenuated the overload-induced increase in fiber CSA. This indicates that while CS alone may take a longer time to produce phenotypic alterations, combined with other common negative regulators of muscle mass and function, such as vitamin D deficiency, the process may well be accelerated.

5.3 Short-term smoking cessation restores muscle structure and mitochondrial function

In chapter 3, we show for the first time in mice that smoking cessation for as short as 1 to 2 weeks restores body and muscle mass, re-establishes mitochondrial function and ATP production, decreases neutrophilic lung inflammation to control levels and improves body composition. Our data also indicate that the diaphragm muscle is more susceptible to CS but recovers within 2 weeks of smoking cessation.

Data on the immediate effects of smoking cessation on body and muscle mass are scarce. The majority of previous smoking cessation studies evaluating muscle effects in mice and humans

involved cessation periods of at least 2 months [5,37]. While long term smoking cessation in mice [5,38] and humans [39,40] leads to increased body weight, the results in chapter 3 actually demonstrate that these effects are already evident within one to two weeks of smoking cessation in mice. The increase in body weight after long-term smoking cessation in humans results from increased fat mass, lean mass and, to a lesser extent, bone mass [39,41–43]. In line with these observations, body composition data from the mice in our study show a progressive increase in lean and fat mass after 1 to 2 weeks of cessation with a tendency for lean mass to recover faster. This indicates that the rapid recovery of soleus, gastrocnemius and plantaris muscle mass observed after 1 and 2 weeks of smoking cessation results from an increase in fat and to a greater extent lean mass, indicating rapid muscle recovery. These observations agree with data in mice whereby 60 days of cessation restored soleus mass and abolished gastrocnemius muscle atrophic signaling [5]. Our data show that these effects are already present within 7 - 14 days of smoking cessation emphasizing that smoking cessation is associated with rapid beneficial effects on skeletal muscle.

Several factors are known to contribute to body and muscle weight gain after smoking cessation. These include increased food intake, increased appetite, decreased physical activity, decreased resting metabolic rate, and increased lipoprotein lipase activity [37,44–46]. Since all groups of mice in our study had similar levels of physical activity and physical fitness at all time points, a decrease in physical activity was not the cause of muscle and body weight gain. However, our results show that CS exposure slightly reduced food intake, which was restored after smoking cessation for 1 to 2 weeks. We can therefore infer that the sharp increase in body and muscle mass after cessation is, at least in part, due to increased food intake after cessation.

Studies on the effects of smoking cessation on diaphragm muscle structure are lacking. Our study is the first to demonstrate immediate beneficial effects of CS cessation on diaphragm muscle structure. Though the diaphragm muscle was more affected by CS exposure than the limb muscles, it however recovered rapidly within 2 weeks of smoking cessation. Indeed, smoking cessation for as short as 2 weeks led to a rapid increase in FCSA to control levels and a more homogeneous distribution of capillaries in the diaphragm. These results thus show that the diaphragm muscle is more sensitive to CS-induced changes in muscle structure than limb muscles but responds rapidly and positively to CS cessation.

Our results show, for the first time, that smoking cessation for 1 to 2 weeks rapidly restores muscle mitochondrial function and increases protein expression levels of subunits of mitochondrial complex I, III, and IV to control levels. Previous studies have shown that histological measurements of succinate dehydrogenase activity correlates well with the maximum cellular oxygen uptake and mitochondrial content [29,30]. Our results show that CS exposure does not alter the oxidative capacity of the soleus and diaphragm muscle. This indicates that the changes seen with CS exposure are not due to a reduction in mitochondrial content but could be a result of decreased synthesis or reversible inhibition of some mitochondrial enzyme complexes or both and/or the interaction of substances in cigarette smoke, such as carbon monoxide and cyanide, with the complexes of the respiratory chain. The latter is illustrated by the acute reduction in mitochondrial function when soleus muscle was exposed to a cigarette smoke extract and recovery of respiration after removal of the extract.

Taken together, these data indicate that immediate reversibility of smoking-related alterations in skeletal muscle structure and function is possible within 14 days of smoking cessation, at least in mice.

5.4 Overload induced hypertrophy mouse model

In chapter 4, the compensatory overload model was used to investigate the effects of smoking and/or vitamin D deficiency on the skeletal muscle hypertrophic response to overload. This model is expected to produce a hypertrophy of 25 – 35 % in rats and mice as indicated by increases in mass and fibre cross-sectional area within 6 weeks [47,48]. This was also the case in our study where an average of 25% hypertrophy was seen in the plantaris muscle after 12 weeks of overload stimulus. Though this model produces a chronic hypertrophic stimulus that is dissimilar to conventional resistance training bouts in humans, it has been suggested to be an adequate proxy to study the hypertrophic response since the resultant hypertrophy is structurally and physiologically similar [49].

5.5 Vitamin D deficiency, CS exposure and the skeletal muscle hypertrophic response

Vitamin D deficiency is highly prevalent in the general population [50], especially during periods of less sunshine [51], and is known to cause skeletal muscle alterations that have also been reported

in chronic smokers [7,52–54]. It is, indeed, well known that vitamin D is vital for the maintenance of skeletal muscle trophic status and it also stimulates muscle cell differentiation and myogenesis and may take part in muscle regeneration and remodelling [55–60]. As such, impairment of muscle hypertrophic response is likely to occur with vitamin D deficiency. Furthermore, a recent study from our research group showed that vitamin D deficiency aggravated the muscle wasting induced by cigarette smoking in a mouse model with normal levels of calcium and phosphorus [7]. Based on these observations, we expected that CS exposure or vitamin D deficiency will each blunt the skeletal muscle hypertrophic response to overload and these effects would be exacerbated when vitamin D deficiency and CS exposure are combined. Considering that the effects of CS exposure and/or vitamin D deficiency on the skeletal muscle hypertrophic response had not been studied previously we decided to investigate this in a mouse model.

Denervation of the left soleus and gastrocnemius muscles led to a significant hypertrophic response in the overloaded plantaris muscles as shown by a significant increase in muscle mass and cross-sectional area of all fibres. Though Vitamin D deficiency or smoking on their own did not diminish the hypertrophic response, the combination blunted the development of overload-induced hypertrophy. However, this synergistic effect was not reflected in plantaris force measurements and protein levels of Akt and 4EBP1 (positive regulators) and FoxO1 (negative regulators), which showed no CS or vitamin D deficiency-induced alterations. Also, the increase in fatigue resistance in the hypertrophied muscles were not diminished by CS or vitamin D deficiency or a combination of both. The relevance of the blunted hypertrophic response caused by CS when combined with vitamin D deficiency should not be neglected, since it may explain why some individuals fail to respond to exercise training and other stimuli meant to augment and maintain muscle size. This is particularly relevant in groups such as the elderly and COPD patients for whom interventions to restore or improve the hypertrophic response and muscle regeneration is crucial.

5.6 Advantages and limitations of the smoking mouse model

Mice have been the animal model of choice in biomedical research representing more than 59% of the total animals used thanks to their genetic and physiological resemblance to humans,

reflected also by the similarity of basic protein structures and metabolic processes in mice and men [61,62]. The smoking mouse model used in our studies showcases several advantages.

Firstly, nose-only smoke exposure closely resembles the typical human situation with intermittent intake of puffs of smoke. Also, the computer-controlled puffs provide a standardized and reproducible exposure protocol enabling us to control the experiments and reduce variability.

Secondly, the animals are of similar age, exercise levels, genetic background and diet, which enhances the reproducibility, sensitivity and reliability of data produced.

Unlike in humans where only small portions of the muscle can be obtained from biopsies which may not be representative for adaptations occurring throughout the whole muscle, mice are sacrificed at the end of experiments and whole muscles extracted for analysis. Prior to sacrifice, several *in vivo* and invasive procedures, impossible to perform in humans, can be done to better understand muscle physiology *in vivo* to complement the *in-situ* findings. Also, thanks to the similarity of basic protein structures and metabolic processes in mice and men [61], the interactions between components of CS and muscle proteins are expected to be similar. Mice are, therefore, good candidates to study the mechanisms of CS-induced skeletal muscle dysfunction although their use should be carefully justified.

Finally, considering the known adverse effects of CS and the addictive properties of nicotine, it will be unethical to deliberately expose non-smoking humans to CS in order to investigate the muscle related effects. Since smoking cessation attempts are unfortunately frequently unsuccessful and are often accompanied by pharmacologic or behavioural changes with possible impact on peripheral muscle, such experiments in humans would also require exposing many subjects to the (rather invasive) study procedures to yield only minimal and probably confounded data in patients that successfully quit smoking.

The age range of the mice in our studies (2 – 6 months) is equivalent to young and mature adult humans (20-30 years) [63].

Our smoking mouse model also presents some limitations. Firstly, our studies made use of male mice only which may limit the scope of applicability of the data obtained since there are several metabolic and hormonal differences between males and females.

The essence of animal models is to provide useful data to enhance our understanding of human health and diseases. However, specific anatomical, biomechanical (for muscle) and physiological differences must be considered prior to generalization to humans. The lungs, which are the main route of entry of CS, have several anatomical differences between mice and humans: mice have less extensive airway branching and do not possess respiratory bronchioles or bronchial glands. This may reduce the area of contact between the lungs and CS and thereby reduce the diffusion of components of CS and the actual amounts of substances in CS to be transported via the circulation into the muscles. Also, due to their higher basal metabolic rates and short lifespans, mice may undergo faster clearance of CS-induced acute systemic and local inflammation than humans, which will lead to milder inflammatory effects in their muscles over time. In order to draw clinically useful conclusions, it is necessary to consider the differences in life history, physiology and morphology between mice and men.

Despite these limitations, the smoking-mouse model is widely used and considered to be an ethically and physiologically appropriate animal model to study the effects of CS on skeletal muscles and other organ systems.

5.7 Relevance of our data

Given the importance of skeletal muscles in the maintenance of balance, posture, respiration and activities of daily living, the maintenance of their structure and function is vital. Considering the high prevalence of smoking worldwide and the fact that smoking has been linked to skeletal muscle dysfunction even before the development of permanent lung damage, understanding the structural and metabolic alterations involved in this process is important. Even though the mechanisms of smoking-induced skeletal muscle dysfunction are not yet fully understood, our data add to the available evidence indicating that skeletal muscle structure and function is restored after smoking cessation. Previous studies have reported long term benefits of smoking cessation such as increased body and muscle mass. Our data show that these improvements are

already evident within 2 weeks of smoking cessation in mice, accompanied by improved muscle metabolism demonstrated by restored mitochondrial function. This is particularly important for constantly active muscles like the diaphragm which showed marked improvements after smoking cessation in our study. This may be particularly relevant in currently smoking patients who need to undergo thoracic surgery and will be mechanically ventilated, whereby smoking cessation in the run-up to surgery will lead to healthier muscles and expedite recovery after the period of mechanical ventilation. Cessation of cigarette smoking has been shown to cause considerable weight gain associated with increased appetite, which could discourage some people from ceasing to smoke. However, we show that this increase in mass involves both fat and lean mass, with lean mass recovering faster demonstrating an increase in 'healthy' muscle mass. Besides exercise training, smoking cessation is the most effective method to reverse smoking-induced skeletal muscle dysfunction and the benefits are already evident within 1 to 2 weeks after cessation. These data could provide an important stimulus to stop smoking and thereby avert the long-term detrimental effects. It is also possible that the improved mitochondrial function reported here for skeletal muscle after smoking cessation might also be observed in other vital organs, such as the heart and the brain, which are depending on mitochondria for optimal functioning.

The skeletal muscle hypertrophic response is crucial in the maintenance of muscle plasticity and homeostasis, especially in the elderly and individuals suffering from degenerative muscle diseases and COPD. The synergistic deleterious effect of the cumulation of smoking and vitamin D deficiency provides further evidence in favour of the implementation of smoking cessation and improvement of vitamin D status in a bid to maintain or improve skeletal muscle homeostasis and plasticity.

5.8 Conclusion

In this thesis, we showed that the diaphragm muscle appears to be more sensitive to CS-induced alterations than the limb muscles, while in both the diaphragm and limb muscles the CS-induced changes in muscle structure and function are reversed within 2 weeks of smoking cessation. Our data also revealed that lung inflammatory cell infiltration, limb muscle atrophy and mitochondrial dysfunction induced by CS were normalized within 2 weeks of smoking cessation. These results

demonstrate beneficial effects of short-term smoking cessation on skeletal muscle structure and function. These findings indicate that smoking cessation in the run-up to surgery, for example, may be of clinical importance to early recovery.

We also showed that vitamin D deficiency alone or cigarette smoking alone did not blunt the hypertrophic response of the overloaded plantaris but, combination of cigarette smoking with vitamin D deficiency led to synergistic deleterious effects on the skeletal muscle hypertrophic response. These findings should be considered particularly when rehabilitation programs attempting to improve skeletal muscle status are provided to patients with muscle wasting who may be struggling with smoking, whereby the restoration or improvement of skeletal muscle structure, function and the hypertrophic response is crucial and may well be helped by smoking cessation and/or treatment for vitamin D deficiency.

5.9 Future perspectives

Presently, there are still many missing links that require further research to guide rehabilitation strategies for people suffering from smoking-induced skeletal muscle dysfunction and exercise intolerance. Though we report some benefits of short-term smoking cessation on skeletal muscle and synergistic deleterious effects of smoking combined with vitamin D deficiency on the hypertrophic response, our research could be extended further to better understand the mechanisms involved and clarify some issues. Investigation of protein synthesis pathways such as IGF-1/Akt/mTOR signaling [64]and proteolytic pathways such as FoxO and NF-κB [64]signaling in the muscles after hypertrophy will provide useful mechanistic information to explain the metabolic alterations that lead to diminution of the hypertrophic response with vitamin D deficiency and smoking. Also, though the mice in our smoking cessation study ate more after smoking cessation, food intake alone may not entirely explain the weight gain observed after smoking cessation. Exploration of the aforementioned pathways could provide vital insights into the biochemical and metabolic processes involved in the rapid increases in muscle and body mass after smoking cessation. Investigation of these pathways is possible in muscle biopsies from humans and muscle tissues from animal models.

To gain more insight into the effects of smoking and/or vitamin D deficiency on the skeletal muscle hypertrophic response, measurement of satellite cell density and function as well as evaluation of expression and concentration of regulators of muscle mass such as myostatin, muscle atrophy F-box (MAFBx) and muscle ring-finger protein-1 (MuRF-1) in the hypertrophied and control muscles is necessary. Furthermore, considering the important role of the presence and adequate distribution of blood in the skeletal muscle hypertrophic response, and the potential inhibitory effects of smoking and/or vitamin D deficiency on muscle circulation, measurement of capillarization and angiogenesis will improve our understanding of the mechanisms involved in the impairment of the hypertrophic response when smoking and vitamin D deficiency are combined.

To better understand contractile changes with smoking and/or vitamin D deficiency and smoking cessation, measurement of single fibre contractile properties can be performed [65]. These measurements will enable us to detect fibre specific differences in metabolism and contractility. Due to the inherent metabolic and structural differences between type I and type II fibers, the effects of CS smoke on a muscle may vary depending on the fiber type composition. Measurements in single fibers will enable us to determine fiber-specific effects of CS, which will help to explain the differential effects of CS on limb and respiratory muscles. Also, CS- induced differences in contractile properties may not be visible in the whole muscle as a result of compensation from different fiber types, but such changes may already be detectable in single fibers, particularly in terms of maximal shortening velocity and power generating capacity that were not evaluated in the present work. Additionally, measurement of single fiber calcium sensitivity will provide more insight in CS-induced problems with submaximal activation of muscle fibers, as seen in single muscle fibers from patients with COPD [66].

A good follow up on our smoking cessation study in mice will be a similar study in age- and physical activity-matched humans to see if rapid restoration of muscle function and capillarization also occur. Also, mindful of the increasingly ageing population in the developed world and the fact that smoking is highly prevalent even among the aged [67], coupled to the deterioration of muscle function with age, the study of smoking and/or vitamin D deficiency induced skeletal muscle dysfunction, potential benefits of smoking cessation and evaluation of

the hypertrophic response is necessary in this age group. Muscle biopsies from these studies could also be used to investigate muscle protein turnover and signaling pathways.

Cigarette smoke and vitamin D deficiency are known to induce oxidative stress in skeletal muscles, and oxidative stress is an important determinant of muscle mass [1,68]. It is therefore necessary to investigate oxidative stress in the muscles together with systemic inflammation. This will enable us to see if smoking-induced skeletal muscle oxidative stress and systemic inflammation are reversed after short-term smoking cessation. Also, an increase in muscle oxidative stress could further explain the synergistic deleterious effects of smoking and vitamin D deficiency on the skeletal muscle hypertrophic response.

Finally, to better explain the synergistic attenuating effect of smoking and vitamin D deficiency on the skeletal muscle hypertrophic response, the measurement of spontaneous physical activity will help us understand the role, if any, of physical activity when these risk factors are cumulated.

5.10 References

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CHAPTER 6

Summary

6 Summary

Cigarette smoking is a prime cause of morbidity and mortality worldwide. It is a major risk factor for the development of cardiovascular diseases, various cancers, respiratory disorders, and skeletal muscle dysfunction. The adverse effects of cigarette smoking beyond the respiratory system has been an area of rising interest, particularly its impact on skeletal muscles. Considering the capital role of skeletal muscles in movement, maintenance of posture, metabolism and vital functions such as respiration, a proper understanding of the manifestations and mechanisms of smoking-induced skeletal muscle dysfunction and development of strategies to curb the ensuing morbidities and mortality is necessary. In humans and animal models the manifestations of smoking-induced skeletal muscle dysfunction include: atrophy, weakness, decreased mitochondrial function and bioenergetics, reduced force and fatigue resistance, decreased protein synthesis, increased proteolysis, capillary regression, reduced vasodilation, decreased perfusion, increased inflammation and oxidative stress.

Smoking cessation is the most efficient and cheapest way to avert these deleterious alterations in skeletal muscle structure and function as a result of smoking. In fact, long term smoking cessation has been shown to restore whole body and skeletal muscle mass, increase muscle mitochondrial function, and improve muscle plasticity in humans and animal models. However, prior to our study, evidence of short-term (1 to 2 weeks) benefits of smoking cessation were lacking. Our study in mice reveals that smoking cessation for as short as 1 to 2 weeks leads to immediate benefits on the diaphragm and limb muscles demonstrated by the reversal of muscle mass loss, recovery of whole-body fat and especially lean mass, and improvement of mitochondrial function. We also show that the constantly active diaphragm muscle is most affected by cigarette smoking and recovers rapidly after smoking cessation. Considering the fact that the devastating effects of smoking on skeletal muscles occur slowly and may only manifest later in life, these results are important as they could motivate smokers to stop smoking as soon as possible to avoid long term muscle wasting and weakness.

The maintenance and improvement of skeletal muscle mass and plasticity is highly recommended, especially for people suffering from muscle wasting such as patients with COPD,

cancer, dystrophic muscle diseases, chronic smokers and the elderly in whom smoking and vitamin D deficiency is also prevalent. Skeletal muscle hypertrophy is a normal response of skeletal muscles to resistance training and overload which lead to increased mass and fiber cross-sectional area. It is usually accompanied by beneficial adaptations, such as muscle mitochondrial biogenesis, increased protein anabolism, reduced proteolysis, enhanced vasodilatory capacity and capillary proliferation that are associated with an increased force generating capacity and fatigue resistance of the muscle. However, cigarette smoke may impair the hypertrophic response to overload. Vitamin D plays a vital role in skeletal muscle plasticity and regeneration and vitamin D deficiency, which is highly prevalent worldwide, leads to skeletal muscle dysfunction with similar manifestations to those orchestrated by cigarette smoke such as fiber atrophy, loss of muscle mass and muscle mitochondrial dysfunction. Our second study was therefore designed to understand the impact of smoking alone, vitamin D deficiency alone or a combination of these factors on the skeletal muscle hypertrophic response in mice. Our results showed that smoking or vitamin D deficiency alone did not diminish the hypertrophic response to overload in the plantaris muscles, but this hypertrophic response was attenuated in the presence of both conditions, as indicated by the attenuated fiber hypertrophy in vitamin D-deficient smoking mice. Therefore, a combination of these risk factors impairs the skeletal muscle hypertrophic response. These data are relevant for consideration in the design and implementation of strategies to improve muscle mass, especially in smokers and vitamin D deficient individuals.

Our studies report the short-term benefits of smoking cessation that include an improved mitochondrial function and limb muscle mass. This is particularly relevant as an incentive to encourage smoking cessation in smokers and individuals suffering from muscle deterioration yet struggling with smoking cessation. We also show the synergistic deleterious effect of smoking combined with vitamin D deficiency on the skeletal muscle hypertrophic response to overload. This is relevant as it may help in understanding the differences in skeletal muscle response to exercise training and could improve the interventions to restore or improve muscle mass.

7 Samenvatting

Het roken van sigaretten is wereldwijd een van de belangrijkste oorzaken van morbiditeit en mortaliteit. Het is een belangrijke risicofactor voor de ontwikkeling van hart- en vaatziekten, verschillende soorten kankers, ademhalingsstoornissen en skeletspierstoornissen. De nadelige effecten van het roken van sigaretten zijn niet enkel zichtbaar in het ademhalingssysteem maar ook op de skeletspieren. Dit gebied heeft een toenemende belangstelling gekregen, gezien de skeletspieren een cruciale rol spelen bij lichaamsbeweging, handhaving van houding, metabolisme en andere vitale functies zoals de ademhaling. Een goed begrip van de ontwikkelingen van skeletspierstoornissen en zijn achterliggende mechanismen, kan ons helpen in de ontwikkeling van strategieën om de morbiditeit en mortaliteit te verminderen. In mens- en diermodellen zijn de verschijnselen van roken geïnduceerde skeletspierstoornissen: atrofie, spierzwakte, verminderde mitochondriale functie en bio-energetica, verminderde kracht en weerstand tegen vermoeidheid, verminderde eiwitsynthese, verhoogde proteolyse, capillaire regressie, verminderde vasodilatatie, verminderde perfusie, verhoogde ontsteking en oxidatieve stress.

Stoppen met roken is de meest efficiënte en goedkoopste manier om deze schadelijke veranderingen in de structuur en het functioneren van skeletspieren te voorkomen. In feite is aangetoond dat langdurig stoppen met roken het hele lichaam en de skeletspiermassa herstelt, de mitochondriale spierfunctie verhoogt en de spierplasticiteit verbetert bij mensen en diermodellen. Voorafgaand aan onze studie ontbrak er echter bewijs van korte termijn voordelen van stoppen met roken (1 tot 2 weken). Onze studie bij muizen laat zien dat stoppen met roken gedurende slechts 1 tot 2 weken leidt tot onmiddellijke voordelen voor de middenrif- en l skeletspieren, aangetoond door de omkering van het verlies van spiermassa, het herstel van het gehele lichaamsvet maar voornamelijk de vetvrije massa en de verbetering van de mitochondriale functie. We laten ook zien dat de constant actieve middenrifspier het meest wordt aangetast door het roken van sigaretten en snel herstelt na het stoppen met roken. Gezien het feit dat de effecten van roken op skeletspieren traag optreden en pas later in het leven tot uiting komen, zijn deze resultaten belangrijk om rokers te motiveren om snel te stoppen met roken om op die manier, op latere termijn, spieratrofie en spierzwakte te voorkomen.

Het in stand houden en verbeteren van skeletspiermassa en plasticiteit wordt sterk aanbevolen, vooral voor mensen die lijden aan spieratrofie zoals in COPD, kanker, dystrofische spierziekten, chronische rokers en ouderen bij wie roken en een vitamine D-tekort is vastgesteld. Skeletspierhypertrofie is een normale reactie van skeletspieren op weerstandstraining en overbelasting, wat leidt tot een grotere spiermassa en dwarsdoorsnede van de vezels. Het gaat meestal gepaard met gunstige aanpassingen, zoals mitochondriale biogenese van de spier, verhoogde eiwitsynthese, verminderde proteolyse, verbeterde vaatverwijdingsvermogen en capillaire proliferatie die gepaard gaan met een verhoogd kracht en betere weerstand tegen vermoeidheidvan de spier. Sigarettenrook kan echter de hypertrofische reactie op overbelasting verminderen. Vitamine D speelt een vitale rol in de plasticiteit en regeneratie van skeletspieren. Een vitamine D-tekort komt wereldwijd voor en leidt tot skeletspierstoornissen die gelijken op de sigarettenrook geïnduceerde vezelatrofie, verlies van spiermassa en spier-mitochondriale dysfunctie. Onze tweede studie was daarom bedoeld om de impact van alleen roken, vitamine D-tekort of een combinatie van deze factoren op de hypertrofische respons van skeletspieren bij muizen te begrijpen. Onze resultaten toonden aan dat roken of vitamine D-tekort alleen de hypertrofische respons op overbelasting van de plantaris-spieren niet verminderde, maar deze hypertrofische respons werd verzwakt in de aanwezigheid van beide condities, zoals aangetoond in ons vitamine D-deficiënt rokend muismodel. Daarom verstoort een combinatie van deze risicofactoren de hypertrofische respons van de skeletspieren. Deze onderzoeksgegevens zijn daardoor relevant voor het ontwerpen en implementeren van strategieën om de spiermassa te verbeteren, vooral bij rokers en personen met een vitamine D-deficiëntie.

Onze studies rapporteren de korte termijn voordelen van het stoppen met roken, waaronder een verbeterde mitochondriale functie en spiermassa van de ledematen. Dit is met name relevant als stimulans om het stoppen met roken aan te moedigen bij rokers en personen die lijden aan spierverslechtering. We laten ook het synergetische schadelijke effect zien van roken in combinatie met een vitamine D-tekort op de hypertrofische reactie van skeletspieren op overbelasting. Dit is relevant omdat het kan helpen bij het begrijpen van de verschillen in skeletspierresponsen op trainingen en om mogelijke interventies te verbeteren om de spiermassa te herstellen.

8 Appositions

1. Involvement of scientists in public policy making is necessary to bridge the gap between the laboratory bench and the public.
2. Mindful of the increasingly ageing population of Europe and the benefits of physical activity and healthy muscles in the aged, the government should encourage healthy ageing by implementing simple strategies such as free gym subscriptions for individuals aged 65 years or over.
3. The recent increase in teleworking could lead to chronic physical inactivity and increased risk of obesity and cardiovascular diseases. Corporate physical activity sessions during working days and diet advice could be helpful.

9 Scientific acknowledgements, Personal Contribution and Conflicts of Interest

9.1 Scientific acknowledgements

Chapter 3: Two Weeks of Smoking Cessation Reverse Cigarette Smoke-Induced Skeletal Muscle Atrophy and Mitochondrial Dysfunction in Mice.

Ajime Tom Tanjeko, Jef Serré, Rob C.I. Wüst, Guy Anselme Mpaka Messa, Chiel Poffé, Anandini Swaminathan, Karen Maes, Wim Janssens, Thierry Troosters, Hans Degens, Ghislaine Gayan-Ramirez

Published: Nicotine & Tobacco Research, 2020 (doi:10.1093/ntr/ntaa016)

The mitochondrial respiratory function tests were done with the help of Dr Rob Wüst.

We are grateful to Simone Denis for helping with the assessment of the subunits of the mitochondrial complexes and ATP synthase by Western blotting.

All other experiments were done by Ajime Tom with the help of colleagues from BREATHE (KU Leuven) and MMU.

Data analysis, compilation of results, figures and first draft were done by Ajime Tom.

Some of the data in this manuscript were included in a poster presentation at the Physiological Society annual conference, 08 - 10 July 2019, Aberdeen, UK (PC187).

Chapter 4: Smoking combined with vitamin D deficiency attenuates the skeletal muscle hypertrophic response in mice.

Ajime Tom Tanjeko, Jef Serré, Rob C.I. Wüst, Jatin Burniston, Karen Maes; Wim Janssens; Thierry Troosters; Ghislaine Gayan-Ramirez; Hans Degens

Publication in progress, Journal of Applied Physiology, 2020

All experiments in this study were done by Ajime Tom with the help of colleagues from BREATHE (KU Leuven) and MMU.

Data analysis, compilation of results, figures and first draft were done by Ajime Tom.

We thank Prof. Jatin Burniston for providing the set up used for *in situ* skeletal muscle contractile measurements for the studies in chapters 3 and 4.

We wish to thank Prof. Wim Janssens and Dr. Karen Maes for their advice, technical support, and constructive comments.

9.2 Personal contribution

Ajime Tom participated in the design (75%) of all studies carried out in this thesis. He also executed the studies (75%), collected and analyzed the data (80%) and drafted the manuscripts (80%).

9.3 Conflicts of interest

The authors have no affiliations or financial involvement with the tobacco industry and no conflicts of interest to declare.

CHAPTER 7

Short curriculum vitae and list of publications during the PhD

Short curriculum vitae

Ajime Tom Tanjeko was born on the 19th of September 1986 in Yaoundé, Cameroon. After obtaining the GCE Advanced level in science from the Government Bilingual Practicing High School, Yaoundé, in 2006, he was admitted into the University of Buea, Cameroon, where he obtained a BSc (Hons) in Biochemistry in 2009, followed by an MSc (Hons) in Chemical Pathology in 2012. In 2013, he obtained the Erasmus Mundus scholarship from the European commission for the European Joint Master in Quality in Analytical Laboratories (University of Barcelona, Spain and University of Algarve, Portugal) which he completed with distinction in 2015. In October 2015 he was selected for the Erasmus Mundus Joint Doctorate scholarship (MOVE-AGE) in KU Leuven (Belgium) and Manchester Metropolitan University (UK). His research focused on the variations in skeletal muscle plasticity with smoking, smoking cessation, and vitamin D deficiency in mice. His work was supervised by Prof. Ghislaine Gayan-Ramirez (KU Leuven), Prof. Thierry Troosters (KU Leuven) and Prof. Hans Degens (Manchester Metropolitan University).

List of publications during the PhD

International peer-reviewed publications

1. **Tom Tanjeko Ajime**, Jef Serré, Rob C I Wüst, Guy Anselme Mpaka Messa, Chiel Poffé, Anandini Swaminathan, Karen Maes, Wim Janssens, Thierry Troosters, Hans Degens, Ghislaine Gayan-Ramirez (2020). Two Weeks of Smoking Cessation Reverse Cigarette Smoke-Induced Skeletal Muscle Atrophy and Mitochondrial Dysfunction in Mice. Nicotine & Tobacco Research. https://doi-org.kuleuven.ezproxy.kuleuven.be/10.1093/ntr/ntaa016.
2. **Tom Tanjeko Ajime**, Jef Serré, Rob C.I. Wüst, Jatin Burniston, Karen Maes; Wim Janssens; Thierry Troosters; Ghislaine Gayan-Ramirez; Hans Degens. Smoking combined with vitamin D deficiency attenuates the skeletal muscle hypertrophic response in mice. (Manuscript under review, Journal of Applied Physiology, 2020)
3. Jef Serré; **Ajime Tom Tanjeko**; Carolien Mathyssen; An-Sofie Vanherwegen; Tobias Heigl; Rob Janssen; Eric Verbeken; Karen Maes; Bart Vanaudenaerde; Wim Janssens; Ghislaine Gayan-Ramirez. Enhanced lung inflammatory response in whole-body compared to nose-only cigarette smoke-exposed mice. (Manuscript under review, Respiratory Research, 2020)
4. Sandro Satta, Alex Langford Smith, Glenn R Ferris, Jack Teasdale, **Tom Tanjeko Ajime**, Jef Serré, Georgina Hazell, Graciela Sala Newby, Jason L Johnson, Martin J Humphries, Ghislaine Gayan-Ramirez, Peter Libby, Hans Degens, Thomas Johnson, Amir Keshmiri, Yvonne Alexander, Andrew C Newby, Stephen J White. A pivotal role for Nrf2 in endothelial detachment– implications for endothelial erosion of stenotic plaques. (Manuscript under review, Cardiovascular Research 2020)
5. Serré, J., Mathyssen, C., **Ajime, T. T.**, Korf, H., Maes, K., Heulens, N., Gysemans, C., Mathieu, C., Vanaudenaerde, B., Janssens, W., & Gayan-Ramirez, G. (2019). Airway infection with Nontypeable Haemophilus influenzae is more rapidly eradicated in vitamin D deficient mice. The Journal of steroid biochemistry and molecular biology, *187*, 42–51. https://doi.org/10.1016/j.jsbmb.2018.10.021.
6. Castaldo, S. A., **Ajime, T.**, Serrão, G., Anastácio, F., Rosa, J. T., Giacomantonio, C. A., Howarth, A., Hill, R., & Madureira, P. A. (2019). Annexin A2 Regulates AKT Upon H_2O_2

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Abstract presented at international conference

Tom Tanjeko Ajime, Jef Serré, Rob C I Wüst, Guy Anselme Mpaka Messa, Chiel Poffé, Anandini Swaminathan, Karen Maes, Wim Janssens, Thierry Troosters, Hans Degens, Ghislaine Gayan-Ramirez. Short-term smoking cessation improves skeletal muscle structure and mitochondrial function. Poster presentation (PC187) at the Physiological Society annual conference, 08 - 10 July 2019, Aberdeen, UK.