

Original Paper

Cigarette Smoking Impairs the Diaphragm Muscle Structure of Patients without Respiratory Pathologies: An Autopsy Study

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Key Words

Autopsy study • Cigarette smoke • Diaphragm muscle • Histopathological analysis

Abstract

Background/Aims: Smoking is a major risk factor for several cardiovascular and pulmonary diseases, and it has also been associated with the loss of skeletal muscle mass and strength leading to sarcopenia. The aim of this study is to analyze the effects of cigarette smoking on the diaphragm muscle histopathology of postmortem samples from patients without respiratory diseases. **Methods:** Diaphragm samples were stained with hematoxylin and eosin for histopathological analysis. Picosirius stain was used to highlight the collagen fibers. **Results:** Cigarette smokers had an increase of histopathological alterations as abnormal cytoplasm, abnormal fiber size and shape, and central nucleus. Additionally, smokers had an increase of collagen fibers on diaphragm muscle. **Conclusion:** Smoking may influence in a negatively fashion the diaphragm musculature.

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Introduction

Tobacco use is a major public health problem because smoking is a major risk factor for cardiovascular disease, chronic obstructive pulmonary disease (COPD), and lung cancer [1, 2]. It has also been associated with other debilitating conditions such as sarcopenia [3-7].

Sarcopenia describes the loss of skeletal muscle mass and strength that occurs with advancing age leading to increased risk of falls and fractures, loss of independence, mobility disorders, and increased risk of death [8]. Interestingly, experimental studies showed that even respiratory muscles as the diaphragm have sarcopenia with the advancing age [9-11].

Decreased function of the diaphragm muscle, the main inspiratory muscle, may contribute significantly to the increased susceptibility to respiratory complications, such as pneumonia and respiratory infections during aging [9, 12, 13].

Although, it is suggested that oxidative stress and chronic inflammation due to cigarette smoking in aged skeletal muscle activate signaling pathways that are related to the imbalance between protein synthesis and breakdown, causing fibers abnormalities and atrophy leading to sarcopenia [6, 14, 15], there is still unclear evidences about the effects of smoking on diaphragm structure during aging.

Therefore, this study aimed to analyze the effects of cigarette smoking on the diaphragm histopathology of postmortem samples from patients without respiratory pathologies.

Materials and Methods

Study subjects

Ethical approval was obtained from the review board for human studies at the University of São Paulo Medical School (São Paulo, Brazil). The study was consistent with the Helsinki Declaration. Consent was obtained from the next of kin. Hospital charts were reviewed by a physician. Subjects with the following conditions, which may affect the muscular structure [16-18], were excluded: postmortem interval > 24h, Hepatitis B and C, HIV, heart failure, neuromuscular disease, myopathy, and respiratory pathologies (e.g. COPD). We included subjects with a smoking history of > 20 years in the smoking group (heavy smokers) [16]. Inclusion criterion for controls was never had smoked, and a lack of respiratory pathology in the history and at autopsy.

Pathological assessment and histochemical techniques

All samples were provided from the São Paulo Autopsy Service of University of São Paulo Medical School. Samples were collected at approximately 5 cm from the central tendon (midcostal region) to avoid the muscle fibers that radiate toward this tendon insert, which may influence the collagen analysis, as previous described [16, 19]. The blocks were cut with a microtome (6 μm - thick section) [16]. Transverse sections were mounted on a glass slide and stained with picosirius red for collagen fibers and hematoxylin and eosin (H&E) for histopathology [16, 19]. Histopathological analysis was conducted by two experienced morphologists blinded to all clinical data to avoid bias [20].

Quantification of collagen and abnormal myofibers

Twenty randomly selected fields were analyzed with a light microscope (Zeiss, x100 magnifications), with both standard filter (blue) and polarized filter (dark red), for each staining technique, i.e. a total of 40 scanned images per patient, using an image analysis program (Axio Vision Software, Zeiss). For volume density (Vv) of the collagen fibers (in both standard and polarized filters) and abnormal myofibers (standard filter), the photomicrographs of the diaphragm were analyzed using the Image J software (version 1.47, National Institutes of Health) by a stereological test-system with 336 points and values were expressed as a percentage [16, 21, 22]. We used a test-system with 336 points because we are analyzing small structures regarding abnormal morphology in images with $\times 100$ increase [16, 21]. Table 1 summarizes the characteristics of normal muscle fibers, as well as, abnormal muscle morphology (adapted from [16]).

Table 1. Characteristics of the diaphragm muscle in the hematoxylin and eosin stained sections

Features	Definition
Normal	
Peripheral nucleus	Nucleus situated within 2 μm of sarcolemma
Normal cytoplasm	Evenly textured cytoplasm
Normal size and shape	Homogenous size and polygonal shape
Abnormal	
Central nucleus	Minimum 2 μm of cytoplasm between nucleus and sarcolemma
Abnormal cytoplasm	Pale color fiber with granular texture (inflammatory infiltrate); vacuolated or ruptured indicating loss of cytoplasmic integrity; accumulation of lipofuscin
Abnormal size or shape	Small fiber (<1/3 diameter of three largest fibers in the field), angled fiber (resembling a "c" or "s")

The Vv was estimated for the collagen fibers, central nucleus, abnormal cytoplasm, and abnormal size and/or shape as: (Vv [structure] = PP [structure]/PT), where PP is the number of points that hit the structure, and PT is the total test-points [22]. Additionally, we analyzed the cross-sectional area (μm^2) of 10 normal muscle fibers in each H&E staining scanned images using Axio Vision software (100x magnification), i.e. a total of 200 normal muscle fibers per patient.

Statistical Analysis

Data were expressed as mean \pm standard error (SEM) for continuous variables, and absolute and relative frequencies for categorical ones. We initially conducted unpaired Student's t-test to examine whether the groups were different regarding demographics and pathological continuous variables. We used χ^2 tests or Fisher exact test when appropriate for categorical variables. To test whether the myofiber size distributions were different between the groups, the Kolmogorov-Smirnov test was used [16, 23].

The statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Prism, Inc., San Diego, CA). The alpha level was set at the 0.05 level, and all tests were two-tailed.

Results

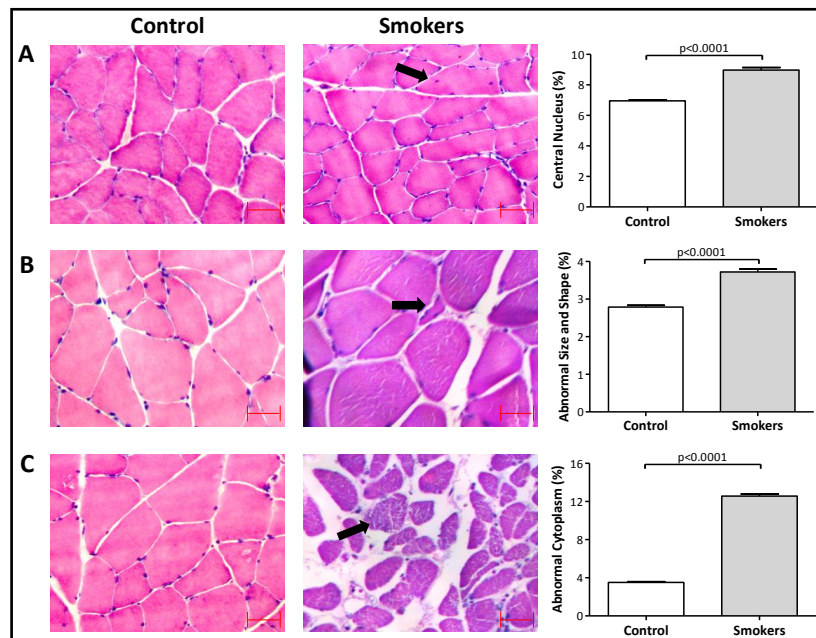
From the 39 biopsies gathered in the postmortem study undertaken, 23 met the inclusion and exclusion criteria for the Smoker and Control groups and could be age- and sex-matched (Table 2). This sample size is sufficient to answer our research question, as has already been demonstrated in the literature with COPD patients with a history of more than 20 years of smoking [16]. However, we highlight that none of the subjects included in the studied groups showed any evidence of respiratory pathology. Eleven of the 23 included patients had pulmonary edema deaths due to cardiovascular complications. Although patients had stayed in bed for at least one day before death, none of them experienced mechanical ventilation.

H&E sections revealed a variety of abnormalities that were significantly increased with smoking (Fig. 1). Smokers diaphragm had a significant higher percentage of central nucleus (8.97 ± 0.16 versus $6.95 \pm 0.05\%$) and abnormal size and shape (3.72 ± 0.07 versus $2.78 \pm 0.04\%$) with a wide range of small fibers, suggesting atrophy. However, the most predominant feature was an abnormal cytoplasm in smokers (12.56 ± 0.22 versus $3.51 \pm 0.08\%$). Common observations of abnormal cytoplasm were loss of cytoplasm integrity and inflammatory infiltrates.

Table 2. Patient characteristics. F: female. M: male. BMI: body mass index. No.: number. Data expressed as mean \pm SEM. * $p < 0.05$

Characteristics	Control	Smokers
Number of subjects	9	14
Age (years)	84.33 ± 3.05	$71.21 \pm 3.53^*$
Sex (F/M)	6/3	6/8
Weight (kg)	52.93 ± 5.65	58.07 ± 3.37
BMI (kg/m^2)	18.79 ± 1.85	20.99 ± 1.16
Sedentary lifestyle (No.)	6	10
Smoking period (years)	-	39.79 ± 4.23
No. of pack/years	-	59.71 ± 9.64
Cause of death (No.)		
Cerebrovascular disease	2	4
Chronic kidney disease	1	1
Myocardial infarction	0	1
Pulmonary edema	5	6
Liver disease	0	1
Sepsis	1	1

Fig. 1. Percentage of (A) central nucleus (black arrow); (B) abnormal size and shape (black arrow indicates a small fiber); and (C) abnormal cytoplasm (black arrow indicates the ruptured cytoplasm) in the studied groups. Representative photomicrographs for both groups in each analysis are shown (Scale bars = 50 μ m). Data are mean \pm SEM values.



In cross-sections of the diaphragm stained with picrosirius red under polarized filter (Fig. 2), collagen was in the endomysium, perimysium, epimysium, and vessel walls. The only other structures that stained positively for collagen were nerve sheaths, which were not included in the point counting. Point counting revealed a greater percentage of collagen fibers in samples from the smoking group compared with the controls (20.21 \pm 0.14 versus 15.32 \pm 0.08%, respectively).

We observed that smoker patients had a significant decrease ($p < 0.001$) in normal fiber area of the diaphragm when compared to the control group (1,147 \pm 18.26 versus 1,284 \pm 17.66 μ m², respectively). Additionally, the Kolmogorov-Smirnov test showed their cumulative distributions to be different ($p < 0.001$), with a greater proportion of smaller fibers in the smokers' samples (Fig. 3).

Discussion

Our study analyzed the effects of smoking on the diaphragm histology of human postmortem samples. Previous studies characterized the muscle fibers as homogenous fibers with polygonal shape and peripheral nucleus [16, 24]. However, Scott et al. [16] observed abnormal fibers

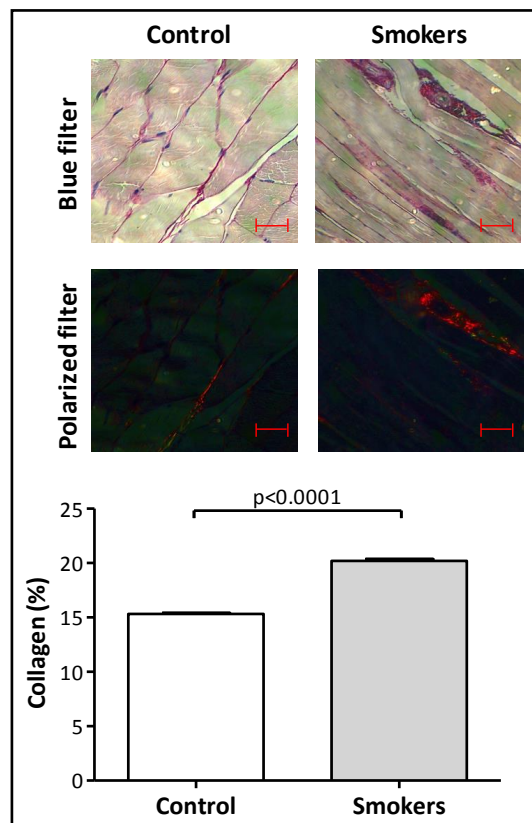


Fig. 2. Cross-sectional images of the diaphragm muscle stained with the Picrosirius technique in both blue filter (standard) and polarized filter (dark red), showing the collagen fibers in both control and smoker group (scale bars = 50 μ m). Representative graphic was obtained after stereological analysis. Data are mean \pm SEM values.

in the diaphragm of COPD patients that smoked more than 20 years. These patients had a decrease in cross-sectional area, suggesting muscle wasting, followed by migration of the peripheral nucleus to a central nucleus, increased inflammatory infiltrates which may lead to degradation of muscle fibers (ruptured cytoplasm) and increased fibrosis as previously suggested as histopathological changes of COPD due to oxidative damage and chronic inflammation [25-31]. In our study, we observed the same characteristics in non-COPD smokers when compared to control group.

Interestingly, Barreiro et al. [32] have investigated oxidative stress in skeletal muscles of human smokers and guinea pigs chronically exposed to cigarette smoke (CS). Compared with control subjects, protein oxidation and carbonylated contractile protein levels were increased in quadriceps of smokers and patients with COPD and in limb muscles of CS-exposed animals. Additionally, muscle force of quadriceps was mildly but significantly reduced in smokers compared with control subjects [32]. These results suggest that CS causes direct oxidative damage to muscle proteins, which might contribute to muscle dysfunction in smokers and in patients with COPD. In addition, Petersen et al. [6] found lower muscle protein synthesis rates and higher muscle atrophy F-box (MAFbx/atrogen-1) expression in the muscle of smokers compared with that of non-smokers and concluded that smoking impairs the muscle protein synthesis processes and increases the expression of genes associated with impaired muscle. Moreover, in chronic smoking, the number of inflammatory cells (e.g. neutrophils) increase, as well inflammatory mediators (e.g. tumour necrosis factor alpha, interleukins, and nuclear factor kappa B) [33-37]. We highlight in our results a great number of inflammatory infiltrates surrounding, mainly, fibers with abnormal cytoplasm. So, we suggest that the increase of inflammatory cells and its mediators combined with oxidative damage may contribute to diaphragm muscle histopathology in smokers, independent of respiratory diseases (e.g. COPD), which may explain our results [6, 14, 32-38].

Finally, smoker patients had a significant increase of collagen accumulation when compared to controls, indicating fibrosis. Diaphragm injury is characterized by structural abnormalities and fibrosis leading to loss of force-generation with a more prolonged time course of recovery, corresponding to the need to regenerate or repair the injury [39-41]. If exertional muscle injury is chronically repeated, regeneration may be compromised resulting in the pathological, long-term accumulation of collagen [40, 42]. An association between collagen accumulation and injury of the human diaphragm has also been reported in the histopathology of both COPD and sudden infant death syndrome [16, 43].

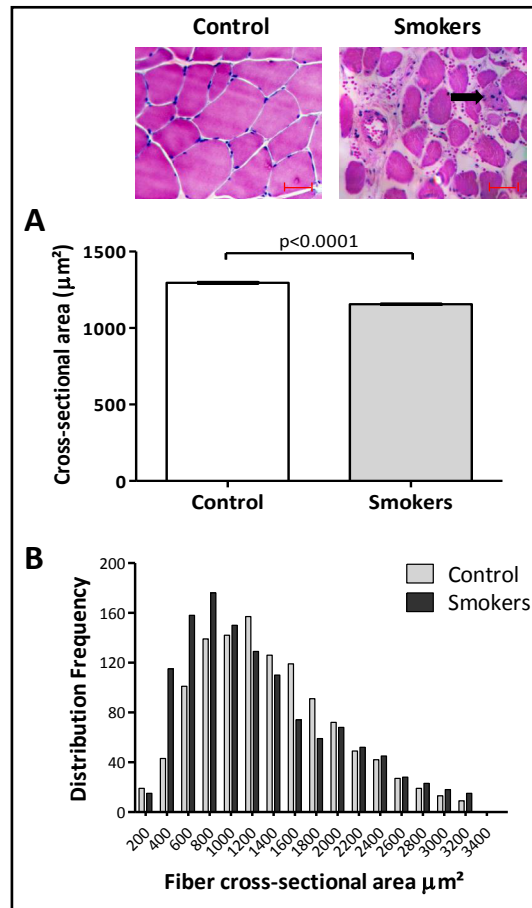


Fig. 3. Cross-sectional area. (A) Total cross-sectional area (μm^2) between the groups. Data are mean \pm SEM values. (B) Cumulative distribution of myofiber cross-sectional areas in smoking patients and control subjects. Representative photomicrographs are shown above (scale bars = 50 μm). Note the increased proportion of small fibers in the smoking group as well as an inflammatory infiltrate (black arrow).

However, our results should be examined considering the study limitations. We did not follow participants during life, and clinical variables were evaluated postmortem through Hospital charts. To increase the reliability of these data, we included only participants who had contact with the next of kin and excluded individuals when the informant provided conflicting information when compared to Hospital records during the clinical interview. In addition, several conditions could potentially affect the musculature, for instance, the aging process [9-11, 44]. Most of our patients had more than 70 years and a prevalence of death from pulmonary edema. According to previous clinical and experimental studies, the diaphragm strength decreases with aging which is dependent of histological and neurotrophic alterations [9-11, 44]. However, despite the age and cause of death we observed significant alterations between the groups. On the other hand, we might have over-represented individuals with pulmonary edema death due to cardiovascular diseases, and further studies should analyze the diaphragm of smokers across the aging process considering the cause of death. Although we assessed the muscle histopathology with only H&E and picosirius staining techniques, these methods were previous used to evaluate the diaphragm histology [16, 39]. Nevertheless, we encourage future studies to add molecular and immunohistochemical analyzes to verify the modulatory effects of smoking on the diaphragm muscle. Regarding collagen analysis, using a polarized filter, we can see in both groups the presence of type I (red), intermediate (yellow/orange), and type III (green) collagen fibers [45]. However, we analyzed the total collagen deposit despite the type of fiber. We encourage studies to elucidate the presence of each type of collagen fibers, as well as enzymes responsible for muscle repair and remodeling in response to injury (e.g. metalloproteinases) caused by different intensities of smoking (light, moderate, and heavy).

Although our study has limitations, we also have some advantages. We presented clinical and histopathological data from heavy smokers who had not showed any evidence of respiratory pathology. Previous study included only COPD patients with a heavy intensity of smoking [16]. However, respiratory diseases as COPD are responsible for structural changes in the diaphragm muscle [16, 25-31]. Additionally, we did not included patients with HIV and Hepatitis as viral diseases, mainly HIV, may negatively affect the musculature [18]. Regarding the postmortem period, our methodology was in accordance with previous studies which demonstrated that store the sample less than 24 hours of postmortem interval is essential to maintain the muscular integrity [17, 19]. In addition, our histopathological assessment consisted from predeterminate muscular abnormalities, which allows for the accuracy of our findings in H&E sections [16, 28, 29]. Moreover, we investigated the collagen deposit under polarized filter, which provides a more accurate analysis of collagen content in the diaphragm, which has not been fully explored in previous studies [16, 39].

Conclusion

In conclusion, we described the association of smoking and diaphragm injury in a sample of Brazilian individuals. Accordingly, further studies are needed regarding the physiological and molecular mechanisms involving diaphragm injury and smoking for a better understanding of our results.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

- 1 Sherman CB: Health effects of cigarette smoking. *Clin Chest Med* 1991;12:643-658.
- 2 Zaher C, Halbert R, Dubois R, George D, Nonikov D: Smoking-related diseases: the importance of COPD. *Int J Tuberc Lung Dis* 2004;8:1423-1428.
- 3 Nelson HD, Nevitt MC, Scott JC, Stone KL, Cummings SR, Cummings SR: Smoking, alcohol, and neuromuscular and physical function of older women. *JAMA* 1994;272:1825-1831.
- 4 Castillo EM, Goodman-Gruen D, Krititz-Silverstein D, Morton DJ, Wingard DL, Barrett-Connor E: Sarcopenia in elderly men and women: the Rancho Bernardo study. *Am J Prev Med* 2003;25:226-231.
- 5 Szulc P, Duboeuf F, Marchand F, Delmas PD: Hormonal and lifestyle determinants of appendicular skeletal muscle mass in men: the MINOS study. *Am J Clin Nutr* 2004;80:496-503.
- 6 Petersen AMW, Magkos F, Atherton P, Selby A, Smith K, Rennie MJ, Pedersen BK, Mittendorfer B: Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. *Am J Physiol Endocrinol Metab* 2007;293:E843-E848.
- 7 Steffl M, Bohannon RW, Petr M, Kohlikova E, Holmerova I: Relation between cigarette smoking and sarcopenia: meta-analysis. *Physiol Res* 2015;64:419-426.
- 8 Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, et al.: Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* 2010;39:412-423.
- 9 Greising SM, Mantilla CB, Gorman BA, Ermilov LG, Sieck GC: Diaphragm muscle sarcopenia in aging mice. *Exp Gerontol* 2013;48:881-887.
- 10 Greising SM, Mantilla CB, Medina-Martínez JS, Stowe JM, Sieck GC: Functional impact of diaphragm muscle sarcopenia in both male and female mice. *Am J Physiol Lung Cell Mol Physiol* 2015;309:L46-L52.
- 11 Greising SM, Ermilov LG, Sieck GC, Mantilla CB: Ageing and neurotrophic signalling effects on diaphragm neuromuscular function. *J Physiol* 2015;593:431-440.
- 12 Houston MS, Silverstein MD, Suman V: Risk factors for 30-day mortality in elderly patients with lower respiratory tract infection: community-based study. *Arch Intern Med* 1997;157:2190-2195.
- 13 El-Solh AA, Sikka P, Ramadan F, Davies J: Etiology of severe pneumonia in the very elderly. *Am J Respir Crit Care Med* 2001;163:645-651.
- 14 Rom O, Kaisari S, Aizenbud D, Reznick AZ: Sarcopenia and smoking: a possible cellular model of cigarette smoke effects on muscle protein breakdown. *Ann NY Acad Sci* 2012;1259:47-53.
- 15 Rom O, Kaisari S, Aizenbud D, Reznick AZ: The effects of acetaldehyde and acrolein on muscle catabolism in C2 myotubes. *Free Radic Biol Med* 2013;65:190-200.
- 16 Scott A, Wang X, Reid WD: Increased injury and intramuscular collagen of the diaphragm in COPD: autopsy observations. *Eur Respir J* 2006;27:51-59.
- 17 Van Ee CA, Chasse AL, Myers BS: Quantifying skeletal muscle properties in cadaveric test specimens: effects of mechanical loading, postmortem time, and freezer storage. *J Biomech Eng* 2000;122:9-14.
- 18 Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S: Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. *Proc Natl Acad Sci U S A* 1998;95:14938-14943.
- 19 Nucci RAB, Jacob-Filho W, Busse AL, Maiffrino LBM, de Souza RR: Anatomopathological Assessment of the Diaphragm in Formalin-Fixed, Paraffin-Embedded Sections. *J Morphol Sci* 2018;35:173-176.
- 20 MacCoun R, Perlmutter S: Blind analysis: hide results to seek the truth. *Nature News* 2015;526:187.
- 21 Hsia CC, Hyde DM, Ochs M, Weibel ER: An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. *Am J Respir Crit Care Med* 2010;181:394-418.

- 22 Marchon C, de Marco Ornelas E, da Silva Viegas KA, Lacchini S, de Souza RR, Fonseca FLA, Maifrino LBM: Effects of moderate exercise on the biochemical, physiological, morphological and functional parameters of the aorta in the presence of estrogen deprivation and dyslipidemia: an experimental model. *Cell Physiol Biochem* 2015;35:397-405.
- 23 Conover WJ, Iman RL: Rank transformations as a bridge between parametric and nonparametric statistics. *Am Stat* 1981;35:124-129.
- 24 Brooke MH, Kaiser KK: Muscle fiber types: how many and what kind?. *Arch Neurol* 1970;23:369-379.
- 25 Ottenheijm CA, Heunks LM, Sieck GC, Zhan WZ, Jansen SM, Degens H, de Boo T, Dekhuijzen PN: Diaphragm dysfunction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2005;172:200-205.
- 26 Ottenheijm CA, Heunks LM, Li YP, Jin B, Minnaard R, Van Hees HW, Dekhuijzen PR: Activation of the ubiquitin-proteasome pathway in the diaphragm in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006;174:997-1002.
- 27 Wijnhoven JH, Janssen AJM, Van Kuppevelt TH, Rodenburg RJT, Dekhuijzen PNR: Metabolic capacity of the diaphragm in patients with COPD. *Respir Med* 2006;100:1064-1071.
- 28 Ottenheijm CA, Heunks LM, Dekhuijzen PR: Diaphragm muscle fiber dysfunction in chronic obstructive pulmonary disease: toward a pathophysiological concept. *Am J Respir Crit Care Med* 2007;175:1233-1240.
- 29 Ottenheijm CA, Heunks LM, Dekhuijzen RP: Diaphragm adaptations in patients with COPD. *Resp Res* 2008;9:12.
- 30 Marin-Corral J, Minguella J, Ramírez-Sarmiento AL, Hussain SNA, Gea J, Barreiro E: Oxidised proteins and superoxide anion production in the diaphragm of severe COPD patients. *Eur Respir J* 2009;33:1309-1319.
- 31 Testelmans D, Crul T, Maes K, Agten A, Crombach M, Decramer M, Gayan-Ramirez G: Atrophy and hypertrophy signalling in the diaphragm of patients with COPD. *Eur Respir J* 2010;35:549-556.
- 32 Barreiro E, Peinado VI, Galdiz JB, Ferrer E, Marin-Corral J, Sánchez F, Gea J, Barberà JA, ENIGMA in COPD Project: Cigarette smoke-induced oxidative stress: a role in chronic obstructive pulmonary disease skeletal muscle dysfunction. *Am J Respir Crit Care Med* 2010;182:477-488.
- 33 Anto RJ, Mukhopadhyay A, Shishodia S, Gairola CG, Aggarwal BB: Cigarette smoke condensate activates nuclear transcription factor- κ B through phosphorylation and degradation of I κ B α : correlation with induction of cyclooxygenase-2. *Carcinogenesis* 2002;23:1511-1518.
- 34 Van der Vaart H, Postma DS, Timens W, Ten Hacken NHT: Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 2004;59:713-721.
- 35 Meng SJ, Yu LJ: Oxidative stress, molecular inflammation and sarcopenia. *Int J Mol Sci* 2010;11:1509-1526.
- 36 Liu Q, Xu WG, Luo Y, Han FF, Yao XH, Yang TY, Zhang Y, pi WF, Guo XJ: Cigarette smoke-induced skeletal muscle atrophy is associated with up-regulation of USP-19 via p38 and ERK MAPKs. *J Cell Biochem* 2011;112:2307-2316.
- 37 Lee J, Taneja V, Vassallo R: Cigarette smoking and inflammation: cellular and molecular mechanisms. *J Dent Res* 2012;91:142-149.
- 38 Glass DJ: Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat Cell Biol* 2003;5:87-90.
- 39 Rodrigues CJ, Rodrigues AJ, Bohm GM: Effects of aging on muscle fibers and collagen content of the diaphragm: a comparison with the rectus abdominis muscle. *Gerontology* 1996;42:218-228.
- 40 Orozco-Levi M, Lloreta J, Minguella J, Serrano S, Broquetas JM, Gea J: Injury of the human diaphragm associated with exertion and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1734-1739.
- 41 Pagano AF, Arc-Chagnaud C, Briocoe T, Chopard A, Py G: Muscle Resting and TGF- β Inhibitor Treatment Prevent Fatty Infiltration Following Skeletal Muscle Injury. *Cell Physiol Biochem* 2019;53:62-75.
- 42 Stauber WT, Smith CA, Miller GR, Stauber FD: Recovery from 6 weeks of repeated strain injury to rat soleus muscles. *Muscle Nerve* 2000;23:1819-1825.
- 43 Kariks J: Diaphragmatic muscle fibre necrosis in SIDS. *Forensic Sci Int* 1989;43:281-291.
- 44 Tolep K, Higgins N, Muza S, Criner G, Kelsen SG: Comparison of diaphragm strength between healthy adult elderly and young men. *Am J Respir Crit Care Med* 1995;152:677-682.
- 45 Junqueira LCU, Bignolas G, Brentani RR: A simple and sensitive method for the quantitative estimation of collagen. *Anal Biochem* 1979;94:96-99.