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# Macrophage in the Role of Vascular Dysfunction Associated with Cigarette Smoke Induced Skeletal Muscle Fatigue

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Breanna Marie Trisko

Committee in charge:

Frank Powell, Chair
Randolph Hampton, Co-Chair
Ellen Breen
Kathleen French

2015

The Thesis of Breanna Marie Trisko is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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University of California, San Diego

2015

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LIST OF ABBREVIATIONS

C57Bl/6J = C57 Black 6J

$Ca^{2+}$ = Calcium

COPD = chronic obstructive pulmonary disease

CS = cigarette smoke

CSE = cigarette smoke extract

EDL = extensor digitorum longus

ERR-α = estrogen-related receptor-alpha

FDB = flexor digitorum brevis

i.p. = intraperitoneal

$O_2$ = oxygen

PGC-1α = peroxisome-proliferator-activated receptor-γ coactivator-1α

SkmVEGF -/- = skeletal myofiber specific VEGF knockout

SMA+ = smooth muscle actin+

TNF-α = tumor necrosis factor-alpha

VEGF = vascular endothelial growth factor

WT = wild type

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Ellen Breen for her support, guidance, and patience as my mentor for this project. From teaching me new techniques to editing my writing, she has taught me so much, and without her the completion of this project would not have been possible.

I would also like to acknowledge the contributions of Dr. Leonardo Nogueira. His work on the single fiber contractile properties, shown in Figure 4 of this thesis, was invaluable to my conclusions. This work is currently being prepared for submission as an abstract to be presented at the 2015 International Biochemistry of Exercise conference (Lima-Rosa et al., 2015).

The work presented in Figure 7 of this thesis is being prepared for submission in the Journal of Physiology. The thesis author was an author of this material.

Figures 1, 2, and 3 and Tables 1 and 2 are reprints of material published as an abstract presented at Experimental Biology Conference 2014 (Trisko et al., 2014).

The Introduction, Materials and Methods, Results, and Discussion sections of this thesis are being prepared, in part, for submission for publication in the Journal of Physiology. The thesis author was the primary investigator and author of this material.

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ABSTRACT OF THE THESIS

Macrophage in the Role of Vascular Dysfunction Associated with Cigarette Smoke Induced Skeletal Muscle Fatigue

by

Breanna Marie Trisko

Master of Science in Biology

University of California, San Diego, 2015

Professor Frank Powell, Chair
Professor Randolph Hampton, Co-Chair

Muscle dysfunction is thought to contribute to exercise intolerance in smokers with chronic obstructive pulmonary disease (COPD). In this study, cigarette smoke (CS) was hypothesized to impair skeletal muscle fatigue resistance via a decrement in vascular function associated with a change in the macrophage cell profile. To test this hypothesis, adult C57Bl/6J mice were exposed, via a nose-only system, to daily periods of CS (5 cigarettes/period for 2 periods, 5 days/week) for 8 weeks and

compared to mice delivered CS-extract (weekly, i.p) and control, unexposed mice. A significant difference in fatigue resistance was seen in CS and CS-extract mice [Control: 337 ± 51 sec, CS: 177 ± 17 sec, CS-extract: 177 ± 18 sec, $p<0.05$)]. Lung air space size was not affected in either of the smoke-exposed groups. A decreased capillary to fiber ratio was observed in the soleus of CS and CS-extract mice [Control: 1.60 ± 0.11, CS: 1.26 ± 0.04, CS-extract: 1.05 ± 0.03 ($p<0.05$)]. Arterial remodeling, a changing in the number or size of arteries, was not detected in the gastrocnemius complex. Control mice showed a trend of a greater number of M2-polarized macrophages in the gastrocnemius than the CS and CS-extract groups. These data suggest that cigarette smoke components may directly induce muscle dysfunction independent of changes in lung function. Vascular dysfunction at the capillary level, associated with a decrease in pro-angiogenic M2 macrophages, may limit $O_2$ and nutrient availability during periods of muscle contraction and contribute to poor locomotor skeletal muscle function in smokers.

# INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a widespread disease and is the fourth leading cause of death worldwide (Collaborators USBoD, 2013). Smoke exposure is a known risk factor for developing COPD and the main causal agent in industrialized countries (Halbert et al., 2006; Eisner et al., 2010).

The primary pathology that occurs in patients with COPD manifests in the lungs. COPD is characterized by an irremediable limitation in airflow that can also be associated with airspace enlargement and inflammation (Rabe et al., 2007). It is also associated with several additional extrapulmonary conditions, or pathologies, that occur in peripheral organs (Schols et al., 1996; Jobin et al., 1998; Maltais et al., 1998; Rabinovich et al., 2001). Peripheral and respiratory skeletal muscle dysfunction are common systemic manifestations. Impaired skeletal muscle function negatively impacts both exercise tolerance and quality of life for patients with this disease (Gosselink et al, 1996). Furthermore, COPD-related muscle weakness and reduced muscle mass are reliable predictors of disease mortality (Swallow et al., 2007; Marquis et al., 2002).

Another common extrapulmonary manifestation of COPD is chronic systemic inflammation, and this is often characterized by high circulating levels of the inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) (Schols et al., 1996; Barreiro et al., 2008; Gosker et al., 2008). The levels of circulating TNF-α have been shown to increase over the course of smoke exposure, and elevated TNF-α levels in the muscle have been associated with cachexia, or muscle wasting, in COPD patients

(Di Francia et al., 1994; Gosker et al., 2008; Schols et al., 1996; Takabatake et al., 2000).

One mechanismm by which cigarette smoke exposure has been suggested to lead to skeletal muscle dysfunction, is through TNF-α mediated down-regulation of peroxisome-proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Tang et al., 2010). PGC-1α acts as a transcriptional co-activator with estrogen-related receptor-alpha (ERR-α) to regulate transcription of several target genes involved in mitochondrial biogenesis, muscle atrophy, metabolism and vascular supply (Liang and Ward, 2006). The exercise-induced, pro-angiogenic factor, vascular endothelial growth factor (VEGF), is one of the downstream target genes of PGC-1α that is transactivated through the conserved ERR-α sites in the promoter and first intron of the *VEGF* gene (Arany et al., 2008). Following a period of sub-chronic smoke exposure, increased serum levels of TNF-α, decreased levels of PGC-1α mRNA in the soleus and extensor digitorum longus (EDL), and decreased levels VEGF in the soleus have been reported (Tang et al., 2010). Additionally, the soleus of these mice exhibited muscle dysfunction that was similar to patients with COPD. The soleus from cigarette smoke-exposed mice exhibited impaired fatigue resistance, decreased capillary to muscle fiber ratio, and an oxidative to glycolytic fiber type transition (Tang et al., 2010). Moreover, conditional ablation of the VEGF gene in skeletal myofibers of mice have been shown to have arterial wall remodeling and an increased number of smooth muscle actin+ arteries in the 30-60 μm range. Interestingly, clusters of macrophage were localized near skeletal muscle arteries found in

gastrocnemius (a mixed fiber muscle type) of skeletal myofiber VEGF-deficient mice (Delavar et al., 2014).

Taken together, these results suggest that systemic inflammation may lead to skeletal muscle dysfunction through a mechanism that, in part, involves remodeling of the vascular system in locomotor muscle. Inflammatory cells, especially macrophages, have been suggested to play a role in regulating blood vessel remodeling (Simons and Ware, 2003; Schaper, 2009). Macrophages are a heterogeneous cell population, and under inflammatory conditions, these divergent cell types migrate into injured tissue where they perform different roles (Geissman et al., 2010). “Inflammatory,” or M1, macrophages are characterized by “classical” macrophage duties, which include cytotoxicity and antimicrobial activity, and are involved in immune responses to pathogens or damaged tissue. Conversely, “resident,” or M2, macrophages are responsible for wound healing and revascularlization (Auffray et al., 2007; Nahrendorf et al., 2007). Monocytes will differentiate into either cell type depending on the kind of inflammatory cytokine signal (Gordon, 2003; Gordon and Martinez, 2010; Mantovani et al., 2002). M1 macrophages are also characterized by secreting high levels of proinflammatory cytokines, one of the main ones being TNF-α (Dale et al., 2008). Additionally, VEGF has been shown to be a recruiting signal for M2-polarized macrophages (Linde et al., 2012).

The mechanism behind COPD-related skeletal muscle dysfunction and whether these changes are independent of a decrement in lung function have yet to be discerned. Understanding the mechanism behind skeletal muscle dysfunction separate

from the development of emphysema will be useful in devising molecular targets, such as TNF-α and PGC-1α, for therapeutic approaches. We hypothesize that the damaging effect of cigarette smoke exposure on peripheral skeletal muscle function is independent of a decrement in lung function and is caused by a change in the M1/M2 macrophage cell profile, which leads to a negative change in vascular function.

To test this hypothesis, a sub-chronic mouse model was established using adult C57Bl/6J mice that were exposed to cigarette smoke for eight weeks. This is a period of time in which cigarette smoke is not expected to cause destruction of the pulmonary alveolar wall sturcutres, or emphysema (Wright and Churg, 1990; Hautamaki et al., 1997; Gosker et al., 2008). This time point was chosen to limit the effects of potential impaired lung function on muscle performance. C57Bl/6J mice were chosen as a mouse model because they have been previously shown to develop emphysema and muscle dysfunction in response to cigarette-smoke exposure, and several mouse strains are resistant to the effects of cigarette smoke (Rinaldi et al., 2012). Cigarette smoke was delivered to mice via two methods: one group received cigarette smoke through the airways using a nose-only exposure system (twice daily, five times per week), the other group was administered cigarette smoke extract through an intraperitoneal (i.p.) injection once weekly. Muscle function and muscle and lung morphology in both groups of cigarette smoke-exposed mice were compared to control, unexposed mice. Skeletal muscle fatigue resistance was used to evaluate muscle function by measuring the time to reach 50% of maximal force when electrically stimulated to contract *in situ*. Hind limb endothelial function was assessed

by measuring blood flow perfusion using an *in vivo* optical imaging system. The phenotype of infiltrating macrophages and the morphology of lung and muscle were assessed using histological techniques.

## MATERIALS AND METHODS

**Animals**. Wild-type C57Bl/6J, age 10 weeks, were purchased from Jackson Laboratories (Bar Harbor, MA). The University of California, San Diego Institutional Animal Care and Use Committee approved the use of animals in this study.

**Cigarette Smoke Delivery.** Non-filtered 3R4F research cigarettes, purchased from Kentucky Tobacco Research and Development Center at the University of Kentucky, were used for both forms of cigarette smoke exposure. *Nose exposure.* Mice were exposed to cigarette smoke twice daily (5 cigarettes/period, 5 days/week), with a 30-minute fresh air exposure between sessions, via a nose-only exposure system (SCIREQ InExpose). *Cigarette Smoke Extract*. Cigarette smoke extract was injected once weekly, i.p. It was prepared by passing cigarette smoke through sterile PBS via a rodent ventilator to draw on a lit cigarette. This continued until the solution reached an OD of 0.86 at 320λ. Control group mice of the same strain and age were continuously exposed to room air.

**Skeletal Muscle *In Situ* Fatigue.** Mice were anesthetized with 1% isoflurane. The gastrocnemius complex was set to a length at which it generated maximal force ($L_0$), and stimulated to contract via the sciatic nerve (8 V, 0.25 trains/s, 200-ms train duration, 120 Hz). The fatigue index is the time at which the force generated fell to 50% of the initial force output. Oxygen saturation levels were above 95% for all mice throughout the measurements.

**Estimation of Air Space Enlargement via the Mean Linear Intercept Index.** Lungs were fixed in 4% paraformaldehyde at 25 $cmH_2O$ and embedded in paraffin. Digital images were taken at 10x magnification and were overlayed with a 1-$mm^2$ grid. Chord legnths were measured using a point-count morphometric technique per a modification of the Thurlbeck method for every second image such that 50% of the entire cross sectional area was counted.

**Measurement of Plasma Cotinine Levels.** Plasma cotinine levels were assessed by an ELISA (BQKITS, Inc.), following the manufacturer's protocols.

**Capillarity measurements.** The non-stimulated gastrocnemius complex was surgically removed from the mouse post-mortem, positioned vertically, mounted with OCT, and frozen in liquid nitrogen-cooled isopentane. Samples were stored -80°C. Cross-sections were cut at a 10 μm thickness and stained via the Capillary Lead-ATPase method. Images were obtained using a Hamamatsu Nanozoomer Slide Scanning System. Total capillary and fiber number were calculated using FIJI software.

**Single Fiber Contractility.** The flexor digitorum brevis (FDB) was dissected from mice post-mortem and, single fibers were isolated. These fibers were mounted on a force transducer and subsequently microinjected with FURA-2, a $Ca^{2+}$-sensitive

fluorescent probe. Contractions were induced by electrical stimulation, and tension and $Ca^{2+}$-transients were recorded concurrently.

**Real-time assessment of contraction-induced blood flow/perfusion of the gastrocnemius complex.** Mice were anesthetized with 1% isoflurane. The gastrocnemius complex was stimulated to contract via the sciatic nerve (8 V, 0.25 trains/s, 200-ms train duration, 120 Hz). Blood flow perfusion through the capillary bed of the gastrocnemius was assessed at the same time points. A syringe was used to deliver 40 μl of 20 nm near-infrared spheres (Qtracker 800 non-targeted quantum dots, Q21071P, Invitrogen, Molecular Probes) in 200 μl saline through a catheter in the tail vein at a constant rate over a two-minute period. Thirty seconds into delivery the gastrocnemius was stimulated to contract. Images of both the rest and electrically stimulated (ES) hindlimbs were taken using an optical imaging chamber (IVIS Spectrum, Caliper Life Sciences), background corrected by subtracting the autofluorescence from the rest "pre-stim" images, and assessed for levels of fluorescent signals.

**Skeletal muscle morphology.** Ten μm cryosections were fixed with 4% paraformaldehyde for 6 minutes at room temperature, rinsed with PBS, and incubated overnight with the following primary antibody concentrations in order to detect smooth muscle cells, arterial remodeling, and macrophage cells: α-smooth muscle antibody (1:200, SIGMA, A2547) and CD206 (1:100, AbD Serotec,

MCA2235A488T). Signals were detected with anti-mouse AlexaFluor 488 and anti-rat AlexaFluor 546 (1:1000, Invitrogen, Molecular Probes, Carlsbad, CA) and mounted with Fluoro-gel II antifade reagent with DAPI (4', 6-diamidino-2-phenylindole) (Electron Microscopy Sciences, 17985-50). Images were obtained with an Olympus FV100 confocal microscope.

**Statistical Analysis**. A One-way ANOVA was used to detect differences among smoke exposure methods and the control group. Repeated measures ANOVA used to detect difference in body weights over time. A Tukey post-hoc test was used to analyze specific differences between the 3 groups. $P < 0.05$ is considered significant. All data are presented as mean ± SEM.

RESULTS

**Table 1:** Plasma cotinine levels in cigarette smoke and cigarette smoke extract exposed mice are increased above the levels in unexposed, control mice.

| Group | Cotinine (ng/ml) |
|---|---|
| Control | 0.04 ± 0.02 |
| CS | 0.81 ± 0.41 * |
| CS-Extract | 1.19 ± 0.14 * |

**Serum cotinine levels.** Table 1 shows that plasma cotinine (a nicotine metabolite) levels are higher in mice exposed to "nose-only" cigarette smoke and injected with cigarette smoke extract than unexposed, control mice after eight weeks. *P < 0.05 from control group; Control n = 14, CS n = 7, CS-Extract n = 6. Values are mean ± SEM.

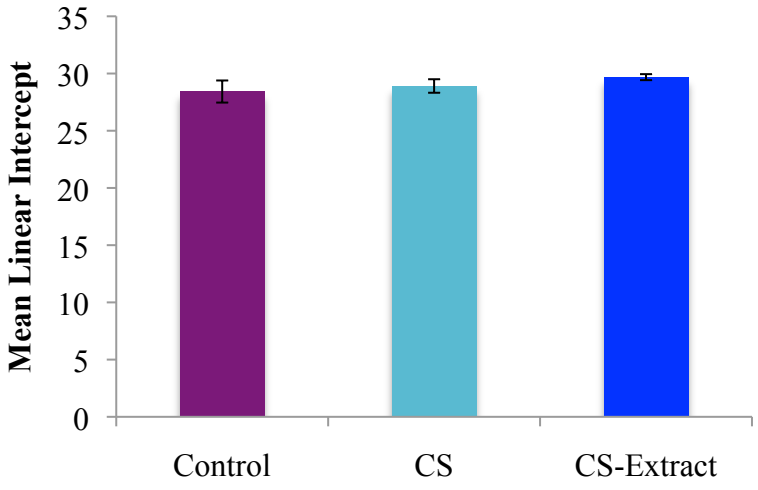



**Figure 1:** Lung airspace size is unchanged after eight weeks of exposure to cigarette smoke or delivery of cigarette smoke extract.

**Morphometric analysis of air space enlargement.** In Figure 1, lung air space size was assessed by measurement of the mean linear intercept. Histological sections

revealed air space size to be unchanged after eight weeks of exposure to cigarette smoke or cigarette smoke extract (Control: 28.42 ± 0.97, CS: 28.91 ± 0.59, CS-extract: 29.68 ± 0.26). Control n = 5, CS n = 5, CS-Extract n = 4. Values are mean ± SEM.

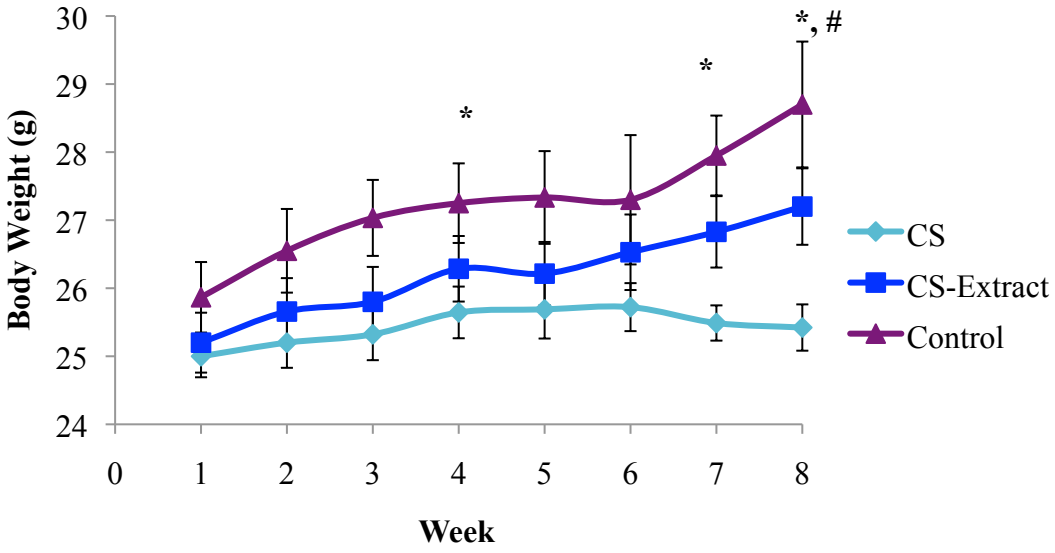



**Figure 2:** Cigarette smoke, but not cigarette smoke extract, prevents weight gain during the exposure period.

**Table 2:** Exposure to cigarette smoke does not affect hind limb muscle weights.

| Group | Muscle (g) / Body Weight (g) | | |
|---|---|---|---|
| | **Soleus** | **Plantaris** | **Gastrocnemius** |
| Control | 0.05 ± 0.01 | 0.07 ± 0.01 | 0.61 ± 0.02 |
| CS | 0.05 ± 0.01 | 0.11 ± 0.03 | 0.59 ± 0.02 |
| CS-Extract | 0.07 ± 0.02 | 0.08 ± 0.01 | 0.59 ± 0.01 |

**Whole body and locomotor skeletal muscle mass.** Figure 2 shows that body mass was 11% lower after eight weeks of exposure to cigarette smoke than that of the room air control group. Mice administered eight weeks of injections of cigarette smoke extract weighed 5% less then the control group [Control: 28.7 ± 0.9 g, CS: 25.4

± 0.3 g ($p < 0.05$), CS-extract: 27.2 ± 0.6 g ($p < 0.05$)]. *P < 0.05 CS group from control group; # P < 0.05 CS group from CS-Extract group. Control n = 6, CS n = 9, CS-Extract n = 7. Values are mean ± SEM. As seen in Table 2, no change in the muscle mass to body weight ratio [muscle (g) / body weight (g)] for the soleus, plantaris, or gastrocnemius was detected. Control n = 14, CS n = 9, CS-Extract n = 7. Values are mean ± SEM.

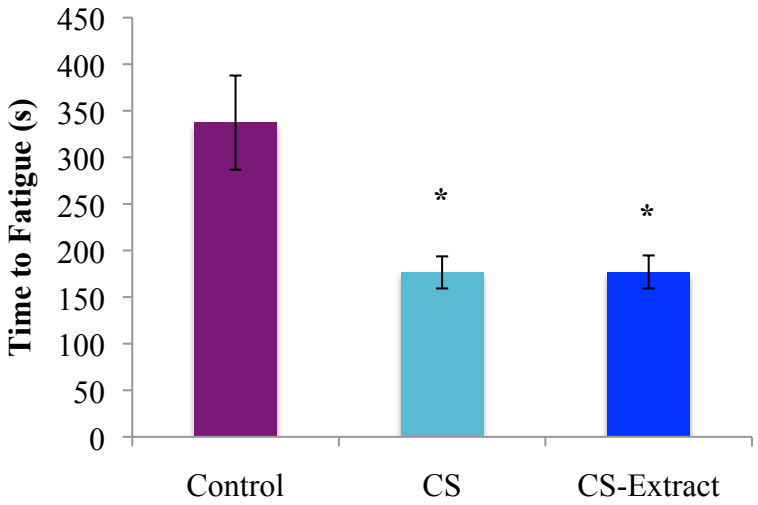



**Figure 3:** Gastrocnemius complex fatigue resistance is reduced following nose-only exposure to cigarette smoke or delivery of cigarette smoke extract.

***In situ* muscle fatigue.** *In situ* fatigue measurements of the gastrocnemius complex, as shown in Figure 3, reveal reduced fatigue resistance as indicated by a 52% decreased in the time to reach fatigue (a 50% decreased in the initial force) in the both cigarette smoke and cigarette smoke extract groups at the eight-week time point [Control: 337.3 ± 50.6 sec, CS: 177 ± 17.2 sec ($p < 0.05$), CS-extract: 177 ± 17.7 sec ($p < 0.05$)]. *P < 0.05 from control group; Control n = 10, CS n = 6, CS-Extract n = 6. Values are mean ± SEM.

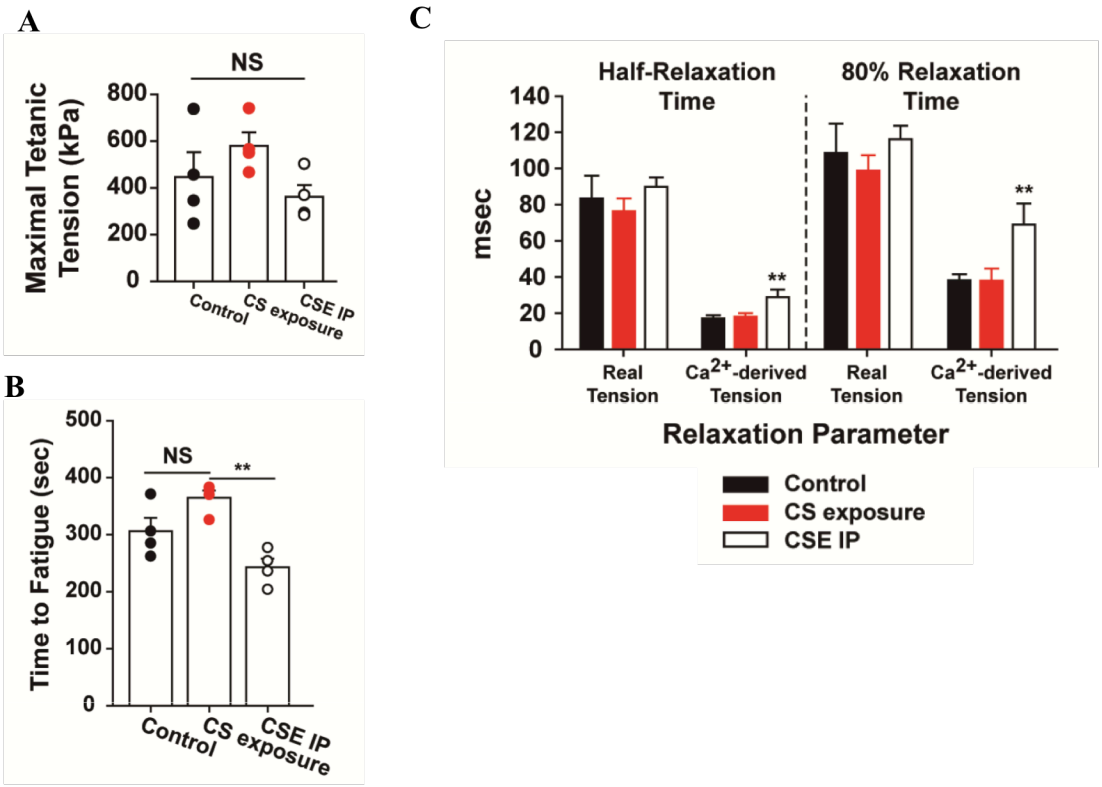



**Figure 4:** Chronic insults with compounds found in cigarette smoke extract reduce muscle fatigue resistance at the single fiber level by diminishing the ability of sarcoplasmic reticulum to uptake $Ca^{2+}$. (A) Tetanic tension evoked by stimulating muscle contraction at 150 Hz. (B) Time to 50% of maximal force. (C) The relaxation parameters obtained from the isometric tension developed during the 100 Hz contractions (real tension) and from the $Ca^{2+}$-derived tension.

**Single fiber contractile properties.** As seen in Figure 4A, maximal tension achieved by single fibers from the FDB was unchanged after eight weeks of cigarette smoke exposure. However, as shown in Figure 4B, the fatigue resistance of fibers from the cigarette smoke extract group was lower than the control and cigarette smoke groups [Control: 306 ± 13 sec, CS: 364 ± 13 sec, CS-extract: 243 ± 15 sec ($p < 0.05$)]. In Figure 4C, half relaxation time and 80% relaxation time are defined as the time

necessary to reach 50% and 80% of the peak tension, respectively. Calcium-derived tension was increased by 50% at both measurements. **P < 0.05 from control group and CS group; Control n = 4, CS n = 4, CS-Extract n = 4. Values are mean ± SEM.

**Muscle capillarity.** As shown in Figure 4, the capillary to fiber ratio in the soleus isolated from mice exposed to eight weeks of cigarette smoke and cigarette smoke extract is decreased by 21% and 34% respectively [Control: 1.60 ± 0.11, CS: 1.26 ± 0.04 (p < 0.05), CS-extract: 1.05 ± 0.03 (p < 0.05)]. *P < 0.05 from control group; Control n = 9, CS n = 6, CS-Extract n = 4. Values are mean ± SEM.

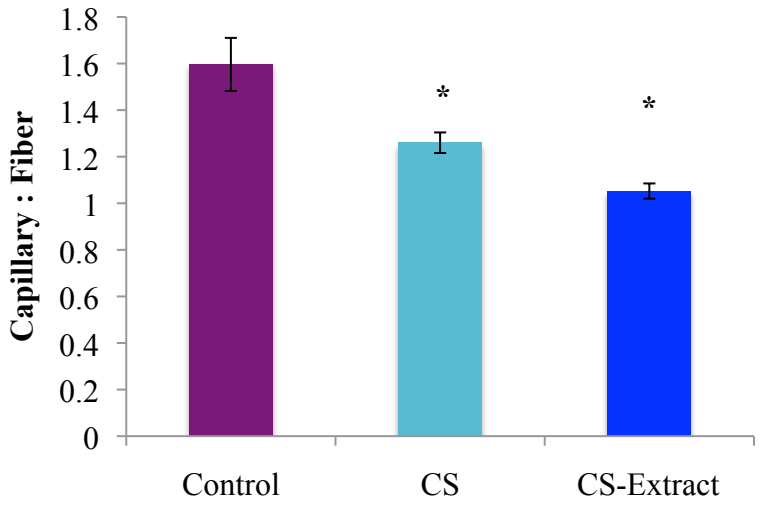



**Figure 5:** Cigarette smoke and cigarette smoke extract exposure result in a reduced capillary to fiber ratio in the soleus.

**Arterial remodeling.** As shown in Figure 6, neither the number of smooth muscle actin+ arteries per cross sectional area (Figure 6A, p = 0.07), nor the size distribution of the arteries (Figure 6B), in the gastrocnemius complex is altered after

eight weeks of cigarette smoke or cigarette smoke extract exposure [Control: 14.61 ± 1.04, CS: 11.62 ± 1.07 (p = 0.07), CS-extract: 9.96 ± 1.59 (p = 0.07)]. Control n = 5, CS n = 7, CS-Extract n = 4. Values are mean ± SEM.

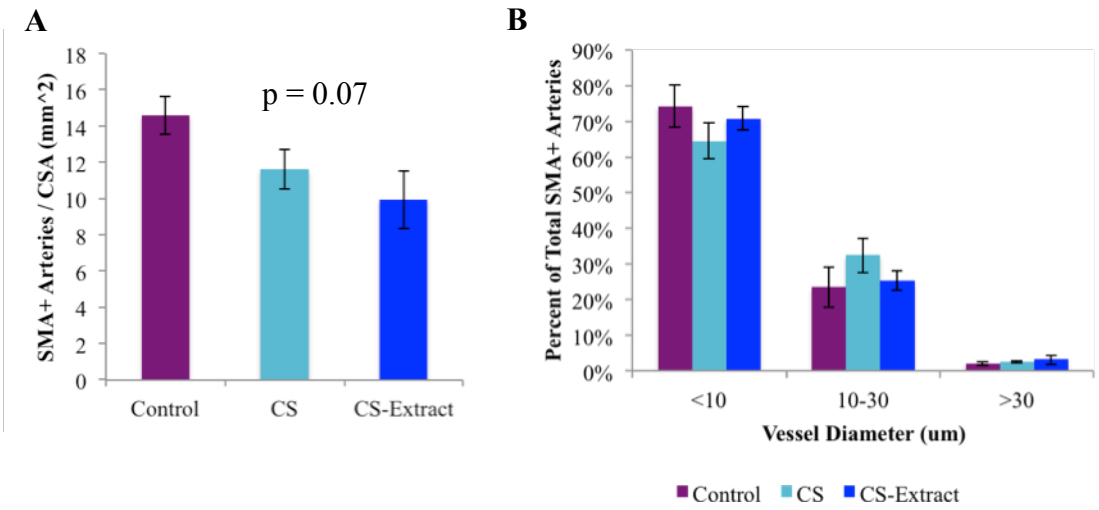



**Figure 6:** There is no change in density (A) or size distribution (B) of the arteries located in the gastrocnemius complex after eight weeks of exposure to cigarette smoke or delivery of cigarette smoke extract.

**Contraction induced blood flow.** While we have yet to measure skeletal muscle blood perfusion in cigarette smoke exposed mice, we have collected data using our conditional skeletal myofiber-specific VEGF gene deletion model. These data indicate a 50% decrease in perfusion of the hind limb skeletal muscle stimulated to contract *in situ* compared to that of contralateral, resting hind limb muscle. $P < 0.01$ from WT group; WT n = 5, Skm VEGF -/- n = 5). Values are mean ± SEM.

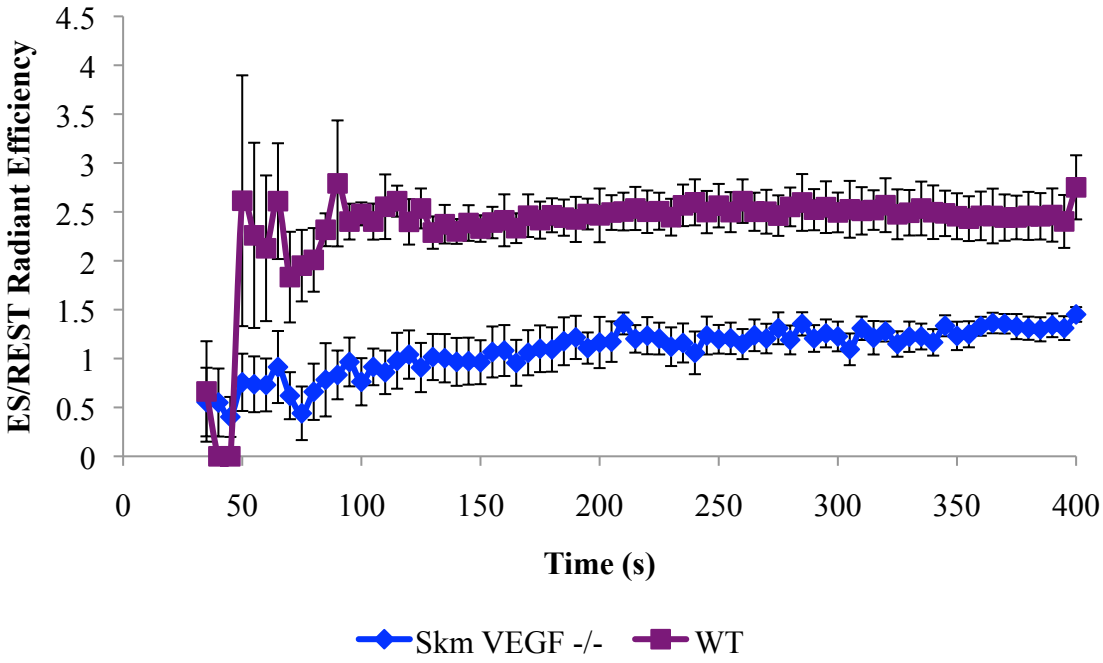



**Figure 7:** Mice with conditional gene deletion of VEGF in skeletal myofibers show a reduced ratio of blood perfusion of the hind limb muscles when stimulated to contract *in situ*.

**Number of potential pro-angiogenic macrophages.** As seen in Figure 8, there was a trend ($p = 0.07$) showing a reduction in the number of CD206+, or M2-polarized, macrophages present in the gastrocnemius complex after eight weeks of cigarette smoke and cigarette smoke extract exposure. Control n = 6, CS n = 6, CS-Extract n = 3. Values are mean ± SEM.

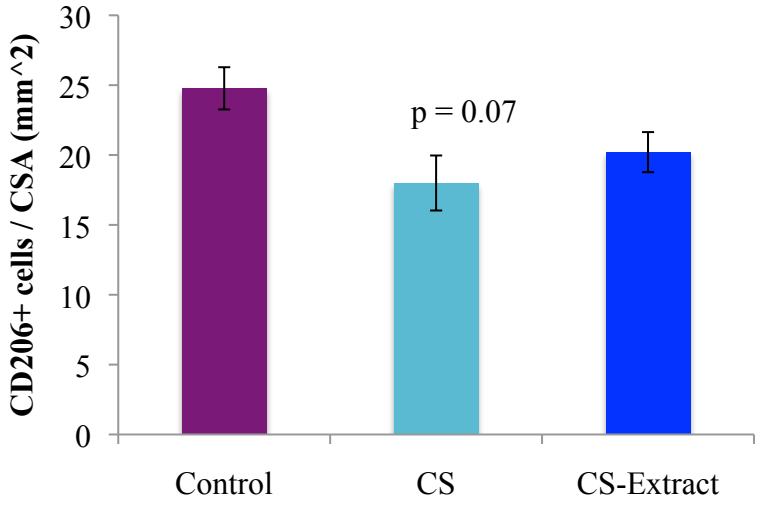



**Figure 8:** A trend of fewer M2-polarized macrophages in the gastrocnemius complex is observed in after eight weeks of daily exposure to cigarette smoke or weekly delivery of cigarette smoke extract.

Acknowledgements:

The material presented in Figure 4 is currently being prepared for submission as an abstract to be presented at the 2015 International Biochemistry of Exercise conference (Lima-Rosa et al., 2015).

The work presented in Figure 7 of this thesis is being prepared for submission in the Journal of Physiology. The thesis author was an author of this material.

Figures 1, 2, and 3 and Tables 1 and 2 are reprints of material published as an abstract presented at Experimental Biology Conference 2014 (Trisko et al., 2014).

## DISCUSSION

This study suggests that cigarette smoke induced skeletal muscle dysfunction may be caused in part by a decrement in the vasculature at the capillary level of the muscle. Futhermore, a loss of capillaries is associated with a decrease in M2-polarized, pro-angiogenic macrophages. After eight weeks of cigarette smoke and cigarette smoke extract exposure, *in situ* fatigue resistance of the gastrocnemius complex was impaired. This change in muscle function was not accompanied by, or was independent of, a decrement in lung function or loss of muscle mass. However, when single fibers of the FDB were stimulated to contract *ex vivo*, differences arose between the cigarette smoke and cigarette smoke extract groups. While there were no differences in maximal tension among the three groups, cigarette smoke extract showed an impaired resistance to fatigue and slower relaxation and $Ca^{2+}$-uptake compared to the cigarette smoke exposed and control mice.

The skeletal muscle dysfunction of both the cigarette smoke and cigarette smoke extract groups was accompanied by changes in the vasculature structure, specifically a decreased capillary to fiber ratio in the soleus. This loss of capillaries was not accompanied by a change in the number of arteries present in the gastrocnemius complex (the soleus, plantaris, and gastrocnemius). Previous, studies in our laboratory have shown that whole body cigarette smoke exposed mice express less VEGF in the soleus. Furthermore, conditional deletion of the VEGF gene in the skeletal myofiber has been shown to result in decreased perfusion of the gastrocnemius complex stimulated to contract *in situ*, and these mice demonstrate

changes in the size and number of small arteries in gastrocnemius complex (Delavar et al., 2014). As there were no significant changes in the arteries, a decrecase in VEGF levels alone cannot be responsible for the muscle dysfunction observed. Alternatively, M2-polarized macrophages have been shown to support and protect the satellite cells that form new capillaries (Chazaud et al., 2003). This data suggests that the trend of decreased number of CD206+, M2-polarized macrophages observed in the gastrocnemius complex in cigarette smoke and cigarette smoke extract exposed mice may be correlated with the capillary regression associated with skeletal muscle dysfunction.

**Impairment of *in situ* fatigue resistance**

Cigarette smoke and cigarette smoke extract exposed mice showed a similar reduction in their ability to resist fatigue when stimulated to contract *in situ* (Figure 3). No decrement in lung function was observed (Figure 1), so this had no impact on muscle dysfunction. Cigarette smoke exposed mice showed a significant decrease in body weight compared to controls (Figure 2), but this was not accompanied by a decrease in muscle mass to body weight ratios (Table 2). This data suggests that muscle wasting was not a contributing factor to the impairment in fatigue resistance.

**Differences in *ex vivo* single fiber fatigue resistance and contractile properties**

It is only at the single fiber level that differences between cigarette smoke and cigarette smoke extract were observed. While cigarette smoke exposed mice and

controls showed no differences, the FDB fibers of cigarette smoke extract exposed mice showed decreased fatigue resistance and slower relaxation and $Ca^{2+}$-uptake compared to the other groups (Figure 4). Cigarette smoke extract and cigarette smoke exposed groups received similar amounts of smoke exposure as shown by similar levels of plasma cotinine (Table 1), a nicotine metabolite, so differences in muscle function cannot be attributed to this. Nicotine, then, would not be the sole cigarette component leading to muscle dysfunction.

This suggests that the insult to the muscle is greater with cigarette smoke extract. The lungs have been shown to provide a protective filter for toxins (Mori et al., 1973), and this data suggests that they may play the same role in filtering some of the toxins of cigarette smoke. As this difference in fatigue resistance and $Ca^{2+}$ handling becomes apparent *ex vivo*, when the muscle is disconnected from the vasculature, this suggests that cigarette smoke extract exposure, has a negative effect on muscle contractile properties, regardless of changes in vasculature. *Ex vivo* fatigue measurements of the whole muscle soleus and EDL of mice exposed to cigarette smoke also show a decrement in fatigue resistance in the soleus only (Tang et al., 2010). However, impairment of fatigue compared to controls in these muscles and at the single-fiber level was considerably less (20% and 21% decrease, respectively) than that of the *in situ* fatigue measurements (52% decrease), suggesting that vascular dysfunction still plays an important role.

**Decrement in vascular function**

Impairment in fatigue resistance was accompanied by negative changes in vasculature in both cigarette smoke and cigarette smoke extract exposed groups at the capillary level only. Capillary regression was observed in the soleus of both groups of smoke exposed mice (Figure 5); however, there was no change in the number or size distribution of smooth muscle actin+ arteries in the gastrocnemius complex (Figure 6). It has been suggested that changes in the peripheral circulation, such as those observed, may limit muscle function due to reduced oxygen and nutrient availability to the skeletal myofibers (Duscha et al., 1999; Wagner et al., 1990).

When VEGF is conditionally deleted in the skeletal myofiber a decrease in blood flow perfusion is observed when the gastrocnemius complex is stimulated to contract *in situ* (Figure 7). However, it is also associated with an increase in the number of smooth muscle actin+ arteries (Delavar et al., 2014). As neither of the smoke exposure groups showed this compensation in the arteries, this suggests that a decrease in VEGF levels alone cannot be responsible for the observed impairment in fatigue resistance. In fact, mice can compensate, in part, for a loss of VEGF by both arteriogenesis and an enhanced metabolic profile that can be seen in *ex vivo* fatigue measurements (Delavar et al., 2014).

In contrast, cigarette smoke exposed and cigarette smoke extract exposed mice showed a trend of decreased levels of CD206+, M2-polarized macrophages in the gastrocnemius complex compared to controls (Figure 8). Previous studies have shown that M2-polarized macrophages serve a protective and supportive role for the satellite

cells that make new capillaries in skeletal muscle (Chazaud et al., 2013). The association between decreased capillary to fiber ratio in the oxidative soleus and the decreased levels of M2-polarized macrophages in the gastrocnemius suggests that cigarette smoke induced skeletal muscle dysfunction may be caused, in part, by an inability to support the creation of new capillaries due to a decrease in number of the supportive M2-polarized macrophages.

**Summary**

Muscle fatigue resistance was diminished in the oxidative muscles *ex vivo* (Tang et al., 2010) and at the single fiber level (Figure 4), decreasing approximately 20% compared to controls. The dysfunction was exacerbated when fatigue resistance was measured *in situ*, where the muscles were connected to the vasculature, decreasing by 52% compared to controls. Vascular dysfunction at the capillary level was correlated with a decrease in M2-polarized macrophages, but there was no arterial compensation as observed in VEGF conditional knockouts in the skeletal myofiber (Delavar et al., 2014).

These data suggest that a possible mechanism for the capillary regression associated with cigarette smoke exposure may be due to a decrease in M2-polarized macrophages. This subpopulation of macrophages has been shown to serve a protective and supportive role for the satellite cells that create new capillaries (Chazaud et al., 2013). Future experiments to confirm this conclusion could include measuring blood flow in cigarette smoke exposed mice, determining the M1/M2 ratio

in the gastrocnemius complex, assessing the number of macrophages recruited to the muscle, and elucidating the mechanism by which M1 macrophages transition to M2 macrophages under chronic inflammatory conditions.

REFERENCES

Arany, Z., Foo, S.Y., Ma, Y., Ruas, J.L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S.M., Baek, K.H., Rosenzweig, A., Spiegelman, B.M. (2008). HIF independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature *451*,1008–1012.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Saranicki, S., Cumano, A., Lauvau, G., Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 317: 666-670.

Barreiro, E., Schols, A.M., Polkey, M.I., Galdiz, J.B., Gosker, H.R., Swallow, E.B., Coronell, C., Gea, J. (2008). Cytokine profile in quadriceps muscles of patients with severe COPD. Thorax 63, 100–107.

Collaborators USBoD. (2013). The state of US health, 1990-2010: burden of diseases, injuries, and risk factors. JAMA : the journal of the American Medical Association *310*, 591-608.

Chazaud, B., Sonnet, C., Lafuste, P., Bassez, G., Rimaniol, F.P., Autheir, F.J., Dreyfus, P.A., Gheradi, R.K. (2003). Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. J Cell Biol. *163(5)*, 1133-43.

Dale, D.C., Boxer, L., Liles, W.C. The phagocytes: neutrophils and monocytes. (2008). Blood. *112(4)*, 935–945.

Delavar, H., Nogueira, L., Wagner, P.D., Hogan, M.C., Metzger, D., Breen E.C. (2014). Skeletal myofiber VEGF is essential for the exercise training response in adult mice. Am J Physiol Regul Integr Comp Physiol. *306*, R586-R595.

Di Francia, M., Barbier, D., Mege, J.L., Orehek, J. (1994). Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. *150*, 1453–1455.

Duscha, B.D., Kraus, W.E., Keteyian, S.J., Sullivan, M.J., Green, H.J., Schachat, F.H., Pippen, A.M., Brawner, C.A., Blank, J.M., Annex, B.H. (1999). Capillary denity of skeletal muscle: a contributing mechanism for exercise intolerance in class II-III chronic heart failure independent of other peripheral alterations. J Am Coll Cardiol *33*, 1956-1963.

Eisner, M.D., Anthonisen, N., Coultas, D., Kuenzli, N., Perez-Padilla, R. (2010). An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. Am J Respir Crit Care Med *182,* 693–718.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., Ley, K. (2010) Development of monocytes, macrophages, and dendritic cells. Science *327*, 656-661.

Gosselink, R., Troosters, T., Decramer, M. Peripheral muscle weakness contributes to exercise limitation in COPD. (1996). Am J Respir Crit Care Med *153*, 976–980.

Gosker, H.R., Langen, R.C., Bracke, K.R., Joos, G.F., Brusselle, G.G., Steele, C., Ward, K.A., Wouters, E.F., Schols, A.M. (2008). Extrapulmonary manifestations of COPD in a mouse model of chronic cigarette smoke exposure. Am J Respir Cell Mol Biol *40*, 710–716.

Gordon, S. (2003). Alternative activation of macrophages. Nat Rev Immunol. *3*, 23-35.

Gordon, S., Martinez, F.O. (2010). Alternative activation of macrophages: mechanism and functions. Immunity. *32*, 593-604.

Gosker, H.R., Langen, R.C., Bracke, K.R., Joos, G.F., Brusselle, G.G., Steele, C., Ward, K.A., Wouters, E.F., Schols, A.M. (2008). Extrapulmonary manifestations of COPD in a mouse model of chronic cigarette smoke exposure. Am J Respir Cell Mol Biol *40,* 710–716.

Halbert, R.J., Natoli, J.L., Gano, A., Badamgarav, E., Buist, A.S., Mannino, D.M. (2006). Global burden of COPD: systematic review and meta-analysis. Eur Respir J *28*, 523–532.

Hautamaki, R.D., Kobayashi, D.K., Senior, R.M., Shapiro, S.D. (1997). Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. Science *277,* 2002–2004.

Jobin, J., Maltais, F., Doyon, J.F., LeBlanc, P., Simard, P.M., Simard, A.A., Simard, C. (1998). Chronic obstructive pulmonary disease: Capillarity and fiber-type characteristics of skeletal muscle. J Cardiopulm Rehabil *18*, 432–437.

Liang, H., Ward, W.F. PGC-1$\alpha$: a key regulator of energy metabolism. (2006). Advan in Phsyiol Edu. *30*, 145-151

Lima-Rosa, F.L., Trisko, B., Hogan, M.C., Breen, E., Nogueira, L. (2015). Chronic treatment with cigarette smoke extract exacerbates the fatigue-induced slowing of relaxation and $Ca^{2+}$-uptake in single mouse fibers." IBEC. Abstract.

Linde, N., Lederle, W., Depner, S., van Rooijen, N., Gutschalk, C.M.., Mueller, M. M. (2012). Vascular endothelial growth factor-induced skin carcinogenesis depends on recruitment and alternative activation of macrophages. J. Pathol. *227*, 17-28.

Maltais, F., Jobin, J., Sullivan, M.J., Bernard, S., Whittom, F., Killian, K.J., Desmeules, M., Belanger, M., LeBlanc, P. (1998). Metabolic and hemodynamic responses of lower limb during exercise in patients with COPD. J Appl Physiol 84, 1573–1580.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.Trends Immunol. *23*, 549-555.

Marquis, K., Debigaré, R., Lacasse, Y., LeBlanc, P., Jobin, J. (2002). Midthigh muscle cross-sectional area is a better predictor of mortality than body mass index in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med *166,* 809–813.

Mori, K., Matsumoto, K., Gans, H. (1973). On the in vivo clearance and detoxification of endotoxin by lung and liver. Ann Surg. *177(2)*: 159-163.

Nahrendorf, M., Swirski, F.K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.L., Libby, P., Weissleder, R., Pittet, M.J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med 204*, 3037-3047.

Rabe, K.F., Hurd, S., Anzueto, A., Barnes, P.J., Buist, S.A., Calverley, P., Fukuchi, Y., Jenkins, C., Rodriguez-Roisin, R., van Weel, C. (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med *176*, 532–555.

Rabinovich, R.A., Ardite, E., Troosters, T., Carbo, N., Alonso, J., Gonzalez de Suso, J.M., Vilaro, J., Barbera, J.A., Polo, M.F., Argiles, J.M., Fernandez-Checa, J.C., Roca, J. (2001). Reduced muscle redox capacity after endurance training in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med *164*, 1114–1118.

Rinaldi, M., Maes, K., De Vleeschauewer, S., Thomas, D., Verbeken, E.K., Decramer, M., Janssens, W., Gayan-Ramirez, G.N.G. (2012). Long-term nose-only cigarette smoke exposure induces emphysema and mild skeletal muscle dysfunction in mice. Dis. Model. Mech. *5(3)*, 333-341.

Schols, A.M., Buurman, W.A., Staal van den Brekel, A.J., Dentener, M.A., Wouters, E.F. (1996). Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. Thorax *51*, 819–824.

Simons, M. & Ware, J. A. Therapeutic angiogenesis in cardiovascular disease. *Nature Rev. Drug Discov.* **2**, 863–872 (2003)

Schaper, W. Collateral circulation: past and present. *Basic Res. Cardiol.* **104**, 5–21 (2009)

Swallow, E.B., Reyes, D., Hopkinson, N.S., Man, W.D., Porcher, R. (2007). Quadriceps strength predicts mortality in patients with moderate to severe chronic obstructive pulmonary disease. Thorax *62*, 115–120.

Takabatake, N., Nakamura, H., Abe, S., Inoue, S., Hino, T., Saito, H., Yuki, H., Kato, S., Tomoike, H. (2000). The relationship between chronic hypoxemia and activation of the tumor necrosis factor-alpha system in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med *161*, 1179–1184.

Tang, K., Wagner, P.D., Breen, E.C. (2010). TNF-α-mediated reduction in PGC-1α may impair skeletal muscle function after cigarette smoke exposure. J Cell Physiol *222*, 320-327.

Takeda, Y., Costa, S., Delamarre, E., Roncal, C., Leite de Oliveira, R., Squadrito, M.L., Finisguerra, V., Deschoemaeker, S., Bruyere, F., Wenes, M., Hamm, A., Serneels, J., Magat, J., Bhattacharyya, T., Anisimov, A., Jordan, B.F., Alitalo, K., Maxwell, P., Gallez, B., Zhuang, Z.W., Saito, Y., Simons, M., De Palma, M., and Mazzone, M. (2011). Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature *479*, 122-126.

Trisko, B., Nogueira, L., Wagner, P.D., Breen, E.C. (2014). Cigarette smoke impairs the *in situ* fatigue resistance of locomotor skeletal muscle in mice. *FASEB J. 28*, 1102.18

Wagner, P.D., Roca, J., Hogan, M.C., Poole, D.C., Bebout, D.C., Haab, P. (1990). Experimental support for the theory of diffusion limitation for maximum oxygen uptake. Adv Exp Med Biol. *277*: 825-33.

Wright, J.L., Churg, A. (1990). Cigarette smoke causes physiologic and morphologic changes of emphysema in the guinea pig. Am Rev Respir Dis *142*, 1422–1428.