Chronic Tobacco Smoke Exposure Negatively Impacts Morphological Characteristics of Peripheral Motor Axons and Neuromuscular Junctions in the Diaphragm of Mice

Alexander John Willms

Division of Experimental Medicine
McGill University, Montreal

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# TABLE OF CONTENTS

## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>3</td>
</tr>
<tr>
<td>RÉSUMÉ</td>
<td>5</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>7</td>
</tr>
<tr>
<td>PREFACE AND CONTRIBUTION OF AUTHORS</td>
<td>8</td>
</tr>
<tr>
<td><strong>CHAPTER 1: INTRODUCTION</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>CHAPTER 2: LITERATURE REVIEW</strong></td>
<td>13</td>
</tr>
<tr>
<td>1. Tobacco Smoke Prior to Disease Onset</td>
<td>13</td>
</tr>
<tr>
<td>1.1 Prevalence and Statistics of Chronic Tobacco Smoking</td>
<td>13</td>
</tr>
<tr>
<td>1.2 Development of Tobacco Smoke-Related Diseases</td>
<td>13</td>
</tr>
<tr>
<td>2. Implications of TS-Induced Diaphragm Impact</td>
<td>14</td>
</tr>
<tr>
<td>2.1 Introduction to COPD</td>
<td>14</td>
</tr>
<tr>
<td>2.1.1 Diaphragm Characteristics in COPD</td>
<td>15</td>
</tr>
<tr>
<td>2.1.1.1 COPD Impact on Diaphragm Phenotype</td>
<td>16</td>
</tr>
<tr>
<td>2.1.1.2 COPD Increases Diaphragm Workload</td>
<td>16</td>
</tr>
<tr>
<td>2.1.1.3 Hyperinflation-Induced Mechanical Disadvantage in the Diaphragm</td>
<td>17</td>
</tr>
<tr>
<td>2.1.2 TS-Induced Diaphragm Dysfunction and Mechanical Ventilation Weaning Success in COPD</td>
<td>18</td>
</tr>
<tr>
<td>2.1.3 TS-Induced Diaphragm Dysfunction and COPD Progress</td>
<td>18</td>
</tr>
<tr>
<td>3. TS Impact on the Diaphragm</td>
<td>20</td>
</tr>
<tr>
<td>3.1 Tobacco Smoke-Induced Diaphragm Phenotype</td>
<td>21</td>
</tr>
<tr>
<td>3.1.1 Fibre Type Distribution</td>
<td>21</td>
</tr>
<tr>
<td>3.1.2 Muscle Atrophy</td>
<td>22</td>
</tr>
<tr>
<td>3.1.2.1 Fibre Cross-Sectional Area and Whole Muscle Mass</td>
<td>22</td>
</tr>
<tr>
<td>3.1.2.2 Protein Degradation and Synthesis Signalling</td>
<td>23</td>
</tr>
<tr>
<td>3.1.3 Contractile Function and Muscle Fatigability</td>
<td>24</td>
</tr>
<tr>
<td>3.1.4 Differences Between TS-Induced Diaphragm and Locomotor Muscle Phenotype</td>
<td>26</td>
</tr>
<tr>
<td>4. Putative Mechanisms of Tobacco Smoke-Induced Skeletal Muscle Alterations</td>
<td>29</td>
</tr>
<tr>
<td>4.1 Oxidative Stress</td>
<td>29</td>
</tr>
<tr>
<td>4.2 Motor Unit Remodeling and Chronic Denervation</td>
<td>30</td>
</tr>
<tr>
<td>4.2.1 NMJ Impact Leads to Motor Unit Remodeling and Chronic Denervation</td>
<td>31</td>
</tr>
<tr>
<td>4.2.2 Motor Unit Remodeling and Denervation Impact on Skeletal Muscle Phenotype</td>
<td>33</td>
</tr>
<tr>
<td>4.2.2.1 Fibre Type Distribution</td>
<td>33</td>
</tr>
<tr>
<td>4.2.2.2 Protein Degradation, Synthesis, and Muscle Atrophy</td>
<td>35</td>
</tr>
<tr>
<td>4.2.2.3 Contractile Function and Muscle Fatigability</td>
<td>36</td>
</tr>
<tr>
<td>4.2.3 NMJ Degradation Leads to Motor Unit Remodeling and Chronic Denervation</td>
<td>37</td>
</tr>
<tr>
<td>4.2.3.1 Mechanisms of NMJ Degradation</td>
<td>38</td>
</tr>
<tr>
<td>4.3 TS-Induced NMJ Impact</td>
<td>40</td>
</tr>
</tbody>
</table>
ABSTRACT

Introduction: Globally, chronic tobacco smoke (TS) exposure is the leading cause of preventable disease, and is the primary risk factor for the development of the top three causes of death: chronic obstructive pulmonary disease (COPD), cardiovascular disease, and cancer. Importantly, TS-related diseases are accompanied by similar skeletal muscle abnormalities, which contribute to poor outcomes and prognosis. Recent findings have implicated TS-induced denervation in TS-related locomotor muscle abnormalities. Given the important physiological role of the diaphragm, and that muscle abnormalities in tobacco smokers and people with TS-related diseases manifest differently in the diaphragm than in locomotor muscle, the objective of this study was threefold: (1) to further assess the impact of TS exposure on the diaphragm; (2) to resolve whether the TS-induced neuromuscular junction (NMJ) impact observed in locomotor muscle also occurs in the diaphragm; and (3) to investigate the progressive impact of TS exposure on morphological features of the motor axon and NMJ. Methods: 15-wk-old male C57Bl/6 wild-type mice were randomly assigned to an 8- or 16-wk period of either TS or control air exposure. Body mass was weighed at the beginning and end of the exposure periods. Immediately following sacrifice, epididymal fat, spleen, and liver were harvested and weighed in mice undergoing 16-wk exposures. Diaphragm, gastrocnemius, plantaris, soleus, TA, and EDL muscles were harvested and weighed immediately following sacrifice in all mice. The diaphragm was used to analyze the morphology of motor axons and NMJs using immunofluorescent staining and confocal microscope imaging. Results: 8 and 16 weeks of TS exposure attenuated body growth (p < 0.0005). Furthermore, 8 weeks (p < 0.0001), but not 16 weeks, of TS exposure reduced muscle mass normalized to their respective controls in all muscles harvested. In the diaphragm, 8 and 16 weeks of TS exposure increased the fraction of NMJs lacking axonal input (p < 0.05) or pre-synaptic
nerve terminal structures (p < 0.005), the latter identified as denervated structures. Pre-synaptic axon diameter was reduced following 16 (p < 0.05), but not 8, weeks of TS exposure. 8 and 16 weeks of TS exposure led to reduced compactness of AChR structures (p < 0.005), in the absence of AChR fragmentation. **Conclusions:** Chronic TS exposure leads to reduced muscle mass of 8-week TS-exposed mice, as well as abnormal morphology of diaphragm motor axons and NMJs in 8- and 16-wk TS-exposed mice. Increased exposure length resulted in an exacerbation of the impact of TS on reduced pre-synaptic axon growth and AChR compactness in 16-wk versus 8-wk TS exposed mice. These findings illustrate that chronic TS exposure results in specific morphological abnormalities in motor axons and NMJs in the diaphragm muscle.
RÉSUMÉ

Introduction : L’exposition chronique à la fumée de cigarette représente la cause majeure de maladies évitables ainsi que le facteur principal menant au développement des trois causes majeures de mortalité : maladie pulmonaire obstructive chronique, maladies cardiovasculaires, et cancers. De manière importante, les maladies liées à la fumée de cigarette sont accompagnées d’anomalies similaires du muscle squelettique qui contribuent à un mauvais pronostique. Des résultats récents suggèrent que la dénervation induite par la fumée de cigarette est impliquée dans les anomalies locomotrices musculaires. Étant donné l’important rôle physiologique du diaphragme et la différente manifestation des anomalies musculaires entrainées par la fumée de cigarette au niveau du diaphragme et des muscles locomoteurs, cette étude avait trois objectifs : (1) évaluer l’impact de la fumée de cigarette sur le diaphragme ; (2) déterminer si l’effet de la fumée de cigarette observé sur la jonction neuromusculaire des muscles locomoteurs est similaire pour le diaphragme ; et (3) étudier l’impact progressif de l’exposition à la fumée de cigarette sur la morphologie des jonctions neuromusculaires et des axones moteurs. Méthodes : Des souris C57Bl/6 âgées de 15 semaines ont été assignées de manière aléatoire à une exposition de 8 ou 16 semaines à la fumée de cigarette ou à l’air. Les souris ont été pesées au début et à la fin de la période d’exposition. Immédiatement après le sacrifice, le diaphragme, le soleus, le tibialis anterior et l’extensor digitorum longus ont été récoltés et pesés pour toutes les souris, alors que la graisse épididymale, la rate et le foie n’ont été récoltés et pesés seulement pour les souris soumises à une exposition de 16 semaines. Le diaphragme a été utilisé pour analyser la morphology des axones moteurs ainsi que des jonctions neuromusculaires par marquage immunofluorescent et microscopie confocale. Résultats : L’exposition à la fumée de cigarette de 8 et 16 semaines a atténué la croissance (p < 0.0005). De plus, une diminution de la masse musculaire (normalisée
par la masse musculaire des contrôles respectifs) a été observée après 8 semaines (p < 0.0001), mais pas 16 semaines, d’exposition à la fumée de cigarette. Au niveau du diaphragme, la fraction de jonctions neuromusculaires n’ayant pas d’axones (p < 0.05) ou de structures de terminaison pré-synaptiques (signe de dénervation, p < 0.005) a été augmentée après 8 et 16 semaines d’exposition. Le diamètre des axones pré-synaptiques était réduit après 16 semaines (p < 0.05), mais pas après 8 semaines, d’exposition à la fumée de cigarette. La densité des récepteurs à l’acétylcholine était diminuée après 8 et 16 semaines d’exposition à la fumée de cigarette (p < 0.05) en l’absence de fragmentation de ces récepteurs. Conclusion : L’exposition chronique à la fumée de cigarette entraîne une réduction de la masse musculaire (après 8 semaines d’exposition) ainsi qu’une morphologie anormale des axones moteurs et des jonctions neuromusculaires du diaphragme (après 8 et 16 semaines d’exposition). L’augmentation de la durée d’exposition résulte en une exacerbation de l’effet de la fumée de cigarette sur la croissance des axones pré-synaptiques et la densité des récepteurs à l’acétylcholine. Ces résultats illustrent que l’exposition chronique à la fumée de cigarette résulte en des anomalies morphologiques spécifiques des axones moteurs et des jonctions neuromusculaires du diaphragme.
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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis was written by Alexander Willms and revised by Dr. Russell T. Hepple.

Chapter 3 is a manuscript for an experimental article:

Title: Chronic Tobacco Smoke Exposure Negatively Impacts Peripheral Motor Axon and Neuromuscular Junction Morphology in the Diaphragm of Mice

Authors & Affiliations: Willms, Alexander\textsuperscript{1,2}; Míguez, Kayla\textsuperscript{2}; Leduc-Gaudet, Jean-Phillipe\textsuperscript{1,2}; Baglole, Carolyn J.\textsuperscript{1,2}; Hussain, Sabah N.A.\textsuperscript{1,2}; Hepple, Russell T.\textsuperscript{3}

\textsuperscript{1}Faculty of Medicine, McGill University, Montréal, QC, Canada
\textsuperscript{2}Meakins Christie Laboratories and Research Institute of the McGill University Health Centre, Montréal, QC, Canada
\textsuperscript{3}Department of Physical Therapy, College of Health & Health Professions, University of Florida, Gainesville, FL, USA

Contributions: Harvesting and processing of tissues was conducted by Alexander Willms, with the help of Kayla Míguez and Jean-Phillipe Leduc-Gaudet. All other aspects of the project and the manuscript preparation were completed by Alexander Willms, with the help of several lab mates, in particular Kayla Míguez. Dr. Russell T. Hepple was the principle investigator of the project and contributed towards study design, results interpretation, and manuscript preparation.
CHAPTER 1: INTRODUCTION

Smoking is a persistent and prominent population health concern, with ~18% of the North American population engaging in chronic tobacco smoking (Statistics Canada, 2015; CDC, 2015). Exposure to tobacco smoke (TS) negatively impacts the health of not only individuals who engage in tobacco smoking, but also those being exposed to second-hand TS (USDHHS, 1984; CDC, 2015). Chronic TS exposure majorly burdens healthcare systems globally by increasing the risk of developing debilitating diseases, including the top current causes of death in North America: chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD) and various forms of cancer (USDHHS, 1984; Jones et al., 2010). Furthermore, while the prevalence of severe disease as a result of second-hand TS exposure has been reduced significantly over time, any level of exposure to TS has been shown to increase the risk of developing TS-related diseases (CDC, 2015). Moreover, smoking is the leading cause of preventable disease and death globally, and is estimated to have contributed to approximately 21% of all deaths in Canada between 2000 and 2010 (Jones et al., 2010). As such, it is important that health research continues to investigate the mechanisms by which chronic TS exposure impacts health and wellness, aiding in developing preventative therapies that seek to mitigate TS-related health consequences.

Notably, research investigating the pathophysiology of the aforementioned TS-related diseases found that patients with these different pathologies share a common locomotor skeletal muscle phenotype, which contributes to poor patient prognosis (Drexler et al., 1992; Whittom et al., 1998; Gosker et al., 2007; Heitmann et al., 2009; Kitzman et al., 2014; Patel et al., 2014; Jones et al., 2015; Wallengren et al., 2015; Toth et al., 2016). Further investigation into skeletal muscle phenotype of these diseases, resolved that the diaphragm was affected fundamentally differently when compared to locomotor muscles (Ottenheijm et al., 2008). Based on these findings, and the
negative impact that chronic TS exposure has on lung morphology and breathing dynamics, it is particularly important to examine the distinctive phenotype in respiratory muscles in the context of muscle functionality in TS-related diseases. Indeed, diaphragm weakness increases (1) ratings of severe breathlessness (dyspnea), (2) exercise intolerance, (3) impaired transdiaphragmatic pressure generation capacity, and (4) risk of mortality in patients with COPD (Killian & Jones, 1988; Gray-Donald et al., 1996; Yamaguti et al., 2009; Haruna et al., 2010; Elbehairy et al., 2016) and congestive heart failure (Mancini et al., 1992; Meyer et al., 2001; Ribeiro et al., 2009; Yamada et al., 2016). As such, understanding and preventing the mechanisms contributing to diaphragm abnormalities, and subsequent dysfunction, in TS-related diseases, is crucial to mitigating poor outcomes in patients.

Recent studies have discovered that diaphragm abnormalities associated with TS-related diseases arise prior to the onset of disease following a history of chronic tobacco smoking. These abnormalities include: (1) changes in fibre type distribution (Barreiro et al., 2010), (2) upregulation of the ubiquitin-proteasome proteolytic pathway, and (3) contractile dysfunction (Elbehairy et al., 2016; Bowen et al., 2017). However, despite the fact that chronic TS exposure has been shown to negatively impact the diaphragm phenotype, which leads to significant consequences for individuals with TS-related diseases, the mechanisms of action of TS exposure on the diaphragm are largely unknown. Understanding these mechanisms is crucial to developing therapeutic interventions for individuals suffering from respiratory disability in TS-related diseases.

The neuromuscular junction (NMJ) is the physiological unit comprising structures that dictate neuron-muscle communication, and has been shown to be particularly vulnerable throughout various conditions associated with skeletal muscle impact (Rüdolf et al., 2016). Damage to peripheral motor axons and NMJ structures leads to motor unit remodeling and chronic
denervation over time, leading to significant detriment to skeletal muscles in the context of aging (Jang & Van Remmen, 2010; Valdez et al., 2010; Chai et al., 2011; Hepple & Rice, 2016) and motor neuron disease (Murray et al., 2010; Jang & Van Remmen, 2010; Comley et al., 2015; Boido & Vercelli, 2016). Interestingly, several factors support the theory that TS-induced impact on peripheral motor axons and NMJs contributes to TS-related diaphragm dysfunction. Firstly, animals and humans experiencing these conditions, as well as animals undergoing experimentally-induced NMJ degradation, present with a similar skeletal muscle phenotype as those seen in TS-related diseases, including (1) changes in fibre type distribution (Kanda & Hashizume, 1998; Lexell & Taylor, 1991; Andersen et al., 1999; Baloh et al., 2007; Carter et al., 2010; Rowan et al., 2012; Purves-Smith et al., 2012; Aare et al., 2016; St-Jean-Pelletier et al., 2017), (2) muscle atrophy associated with up-regulation of the ubiquitin-proteasome proteolytic pathway (Lexell & Taylor, 1991; Rowan et al., 2016) and (3) contractile dysfunction (Ansved & Larsson, 1989; Brown & Hasser, 1996; Butiföker et al., 2011; Greising et al., 2015; Ward et al., 2015). Secondly, a history of chronic tobacco smoking has been shown to result in an exacerbation of age-related loss of total lean mass and leg lean mass (van den Borst et al., 2011). As such, it is possible that tobacco smoking partly contributes to skeletal muscle abnormalities by accelerating age-related processes, such as degradation of NMJs and motor unit remodeling. Finally, recent findings from our lab discovered that 16 weeks of TS exposure induced significant denervation in the tibialis anterior (TA) in mice assessed at 12 months of age (Míguez, 2017, McGill University graduate thesis; Kapchinsky et al., under review with the Journal of Physiology).

The primary objectives of this graduate thesis were (1) to further characterize the impact of chronic TS exposure of diaphragm characteristics, (2) evaluate the impact of chronic TS exposure on the morphological characteristics of peripheral motor axons and NMJs in the
diaphragm of mice, and (2) to evaluate the progressive nature of this impact. To accomplish these objectives, wild-type mice were exposed to either 8 or 16 weeks of TS alongside age-matched controls who were exposed to ambient air. Following exposure periods, morphological characteristics of peripheral motor axons and NMJs were assessed in the diaphragm and compared between groups. Furthermore, other physiological characteristics, such as body mass progression, fat mass, and selective organ and muscle mass, were assessed.

The hypotheses guiding this thesis are twofold. Firstly, we hypothesize that chronic TS exposure results in significant impact to the morphology of diaphragm motor axons and NMJs, and that these morphological abnormalities manifest similarly as in other conditions with skeletal muscle changes induced by motor axon and NMJ impact, such as aging. Secondly, we hypothesize that TS-induced motor axon and NMJ impact is progressive in nature, and that TS-induced morphological changes to these structures will be exacerbated in mice exposed to 16-wks of TS compared to those exposed for 8 weeks.
CHAPTER 2: LITERATURE REVIEW

1. Tobacco Smoke Prior to Disease Onset

1.1 Prevalence and Statistics of Chronic Tobacco Smoking

Chronic tobacco smoking is a pertinent global health issue, negatively impacting the health of individuals who are regularly exposed to tobacco smoke (TS), and resulting in massive expenditures for health care systems (Smith et al., 2001; USDHHS, 2004). In 2015, more than 17% of Canadian and United States residents were current tobacco smokers (Statistics Canada, 2015; CDC, 2015). Furthermore, a history of chronic tobacco smoking is estimated to have contributed to approximately 21% of all-cause mortality over the past decade (Jones et al., 2016). While there have been substantial reductions in the number of people severely affected by second-hand TS exposure, any level of involuntary TS inhalation puts an individual at risk of developing life-threatening diseases, such as cardiovascular disease (CVD), coronary heart disease, and lung cancer (CDC, 2006). Given the high prevalence of this detrimental habit, and the severity of the associated health consequences, it is imperative that health research focuses on methods to manage TS-related health consequences.

1.2 Development of Tobacco Smoke-Related Diseases

TS negatively impacts a number of physiological systems, reducing both the quality of life and the overall health status of individuals who are chronically exposed to TS. A cross-sectional epidemiological study by van den Borst and colleagues (2011) demonstrated that chronic exposure to TS accelerates the development of a number of negative physiological characteristics, including a reduction in overall body weight, fat mass, and lean body mass. These findings have been
confirmed in numerous chronic TS exposure animal models (Tang et al., 2010; Caron et al., 2013; Krüger et al., 2015; Cielen et al., 2016b; Bowen et al., 2017).

As the leading cause of preventable disease in North America, a history of chronic tobacco smoking is a primary risk factor for the development of a wide variety of diseases, including chronic obstructive pulmonary disease (COPD) and several CVDs and cancers (USDHHS, 2004; Zaher et al., 2004). Furthermore, approximately 85% of all COPD and lung cancer-related deaths, and 20% of all coronary heart disease and stroke-related deaths, are associated with a history of chronic tobacco smoking (Zaher et al., 2004). As such, investigating the mechanisms by which TS drives physiological damage that contributes to the progression of TS-related diseases, is important to understanding and preventing TS-related pathophysiology.

2. Implications of TS-Induced Diaphragm Impact

2.1 Introduction to COPD

Chronic tobacco smoking is widely accepted as the primary risk factor for the development of COPD (Riesco et al., 2017). ~80% of COPD patients have a history of chronic tobacco smoking, and 25% of individuals who chronically smoke tobacco develop COPD (Zaher et al., 2004). The number of pack years an individual has engaged in chronic tobacco smoking is independently associated with (1) the severity of COPD, (2) the development of emphysema and chronic bronchitis in conjunction, (3) increased frequency of exacerbations in COPD patients over 50-years-old, (4) decline in general lung function (Riesco et al., 2017), and (5) mortality in the general population after the age of 70 (Elbehairy et al., 2016; Nash et al., 2017). Despite these facts, ~35-45% of COPD patients actively smoke, compared to ~17% of the general population in the USA (CDC, 2015). Importantly, compromised skeletal muscle function in COPD patients has been characterized as an independent predictor of co-morbidities and mortality in this patient
population. As such, it is important to evaluate the progressive impact, and resulting consequences of skeletal muscle dysfunction in patients with COPD.

Skeletal muscle dysfunction is associated with poor outcomes for COPD and CVD patients, and has been shown to develop prior to the onset of TS-related diseases in people with a history of chronic TS use. These TS-related skeletal muscle phenotypes, described in section 3, have been shown to manifest throughout the progression of COPD, and are independent predictors of mortality in this population (Decramer et al., 1997; Marquis et al., 2002; Swallow et al., 2007; Patel et al., 2014). Furthermore, up to 45% of COPD patients continue to smoke tobacco (Riesco et al., 2017), indicating that TS-related skeletal muscle impact continues to evolve throughout the pathology of this subset of patients. Given the diaphragm’s role in maintaining breathing dynamics, it is important to evaluate the impact that TS-related diaphragm dysfunction has on people with TS-related diseases. This is particularly important in COPD patients, who struggle with what is considered a primarily pulmonary respiratory disease. As such, the importance of evaluating how TS impacts respiratory muscles is twofold: (1) to discern the degree to which TS-induced respiratory muscle changes contribute to respiratory symptoms and dysfunction throughout TS-related diseases, and (2) to understand how said changes influence the diaphragm’s ability to cope with and recover from pulmonary pathophysiology.

2.1.1 Diaphragm Characteristics in COPD

In order to understand the role that TS-induced diaphragm alterations have on patients with COPD, it is first important to understand how the diaphragm functions in this patient population. The performance of the diaphragm in COPD patients is fundamentally different from that of healthy individuals due to overt impairments in lung morphology and function resulting from chronic TS exposure and disease progression.
2.1.1.1 COPD Impact on Diaphragm Phenotype

As discussed in section 3, the TS-related phenotype of the diaphragm tends to be exacerbated in patients with COPD. Compared to healthy controls, the diaphragm of COPD patients present with increased proportions of muscle fibres the co-express two different isoforms of myosin heavy chain (MHC) protein, and an oxidative fibre type shift (Nguyen et al., 2000; Levine et al., 2003; Doucet et al., 2004), which is a result of the increased workload in COPD diaphragm. Diaphragm mean fibre CSA is not reduced in patients with mild-to-moderate COPD (Doucet et al., 2004), but is reduced in patients with severe COPD, independent of fibre type (Sanchez et al., 1985; Levine et al., 1997). Furthermore, while diaphragm fibres present with reduced contractile force production capacity per unit area (specific force) (Levine et al., 2003), diaphragm fatigue resistance seems to be either unaffected or increased in patients with COPD (Laghi & Tobin, 2003; Levine et al., 2003).

2.1.1.2 COPD Increases Diaphragm Workload

The burden on the diaphragm and other respiratory muscles is increased in patients with COPD due to hallmark symptoms that compromise pulmonary function. These symptoms include impaired gas exchange, inspiratory and expiratory airflow limitation, as well as severe dyspnea. In response, the diaphragm is forced to increase its contribution towards dictating breathing dynamics. This is illustrated by the fact that resting breathing rate is increased by up to 50% in patients with COPD, in an effort to increase the amount of oxygen that is breathed in over time (Laghi & Tobin, 2003). Furthermore, the diaphragm of COPD patients has been shown to generate up to three times more pressure during resting breathing in comparison to healthy subjects, increasing the workload of the diaphragm, which is characteristic of COPD (Laghi et al., 1998; Jubran & Tobin, 1997). In summary, the workload of the diaphragm in COPD patients is increased,
as this muscle generates more force (and transdiaphragmatic pressure), and contracts more frequently in order to increase the volume and frequency of breathing respectively. These functional changes are induced to compensate for impaired respiratory function and gas exchange efficiency.

2.1.1.3 Hyperinflation-Induced Mechanical Disadvantage in the Diaphragm

While the progression of lung pathophysiology requires an increased workload from the diaphragm, COPD pulmonary pathophysiology simultaneously put the diaphragm at a mechanical disadvantage. COPD is characterized by dynamic lung hyperinflation. Lung hyperinflation occurs because patients are unable to adequately expire air from their lungs before needing to inhale, due to chronic expiratory flow limitation. As a result, patients chronically breathe in before fully breathing out, and over time, recurrent gas trapping causes patients to breathe at higher lung volumes (De Troyer, 1997). This results in mechanical disadvantage for the diaphragm. As the lung volume increases, these structures impede on the diaphragm, decreasing the muscle’s functional range of motion. As a result, diaphragm myofibres shorten, and diaphragm curvature decreases, leading to reduced diaphragm tension generation capacity, and a reduced maximal transdiaphragmatic pressure generation capacity (De Troyer, 1997). In fact, Cassart and colleagues (1997) found that the area of the diaphragm responsible for creating pressure in the thoracic cavity encompasses 40% of total diaphragm area in patients with COPD, compared to 60% in healthy, age-matched subjects. As a result, the diaphragm is less able to compensate for lung pathophysiology in COPD patients experiencing lung hyperinflation.
2.1.2 TS-Induced Diaphragm Dysfunction and Mechanical Ventilation Weaning Success in COPD

A subset of patients with COPD, who are experiencing life-threatening respiratory exacerbations require mechanical ventilation, whereby a machine and tubing system is used to artificially induce pulmonary respiration (Tobin et al., 2009). Once it is deemed feasible, these patients are weaned off of mechanical ventilation systems and extubated, whereby mechanical ventilation systems and tubes are removed from patients. While some patients are able to begin breathing without experiencing exacerbations, others undergo severe pulmonary distress during the weaning process, and must resume mechanical ventilation (Tobin et al., 2009). Comparing diaphragm function in patients who fail to extubate from mechanical ventilators may illustrate how inspiratory muscle functionality contributes to patients’ abilities to rehabilitate from pulmonary disability.

It has been found that patients who fail to extubate from mechanical ventilators tend to demonstrate higher respiratory muscle energy expenditure due to rapid, shallow breathing throughout the weaning trial (Jubran & Tobin, 1997; Purro et al., 2009). As such, breathing dynamics seem to differ between patients who are and are not successful in weaning off of mechanical ventilators. However, findings have concluded that neither muscle fatigue, inspiratory muscle weakness, nor an inability to generate enough transdiaphragmatic pressure, contribute to failing to wean off of mechanical ventilators (Similowski et al., 1991; Jubran & Tobin, 1997; Purro et al., 2009).

2.1.3 TS-Induced Diaphragm Dysfunction and COPD Progression

Reduced diaphragm strength is a major limiting factor for patients with COPD (Ottenheijm et al., 2008), and is a primary outcome of chronic TS exposure (Elbehairy et al., 2016; Bowen et
As described previously, diaphragm weakness exacerbates exercise intolerance and dyspnea in patients with COPD (Killian & Jones, 1988). As well, reduced inspiratory muscle strength and associated transdiaphragmatic pressure generation capacity, has been shown to be an independent predictor of survival in patients with COPD (Gray-Donald et al., 1996; Yamaguti et al., 2009; Haruna et al., 2010; Elbehairy et al., 2016).

The functional capacity of COPD patients is also impacted by inspiratory muscle weakness, which impedes peak oxygen uptake and transport to peripheral muscles, as well as 6 minute walk distances compared to patients with healthy inspiratory muscle function (Wolpat et al., 2016). Importantly, 6-minute walk time in patients with COPD is an independent predictor of survival (Pinto-Plato et al., 2004). Therefore, TS-induced diaphragm weakness significantly impacts COPD populations through (1) exacerbating COPD symptoms such as dyspnea, (2) reducing functional capacity and exercise tolerance in patient populations, and (3) impairing the diaphragm’s ability to generate maximal transdiaphragmatic pressure. The final point is of particular importance, as the COPD diaphragm is expected to generate more pressure and increase air intake in response to impaired pulmonary gas exchange, and is already placed at a mechanical disadvantage due to COPD-related lung hyperinflation.

Chronic TS use is the primary risk factor for COPD, and diaphragm dysfunction severely contributes to co-morbidities and mortality in this patient population. As such, it is important to evaluate the impact that chronic TS exposure has on the development of diaphragm characteristics that are associated with disability and mortality in patients with COPD and other TS-related diseases.
3. **TS Impact on the Diaphragm**

Interestingly, it has been shown that diseases that arise from a history of chronic TS exposure (TS-related diseases) share certain skeletal muscle characteristics. Particularly, a fast fibre type shift (Drexler et al., 1992; Whittom et al., 1998; Gosker et al., 2007; Ottenheijm et al., 2008; Kitzman et al., 2014; Toth et al., 2017), muscle atrophy (Heitmann et al., 2009; Jones et al., 2015; Wallengren et al., 2015), and contractile abnormalities (Drexler et al., 1992; Maltais et al., 2014; Smith et al., 2016) are commonly characterized throughout TS-related diseases. Furthermore, many of these phenotypic and functional characteristics are found in chronic tobacco smokers who are otherwise healthy (Larsson & Örlander, 1984; Montes de Oca et al., 2008), as well as in animal models of chronic tobacco smoking (de Paepe et al., 2008; Gosker et al., 2009; Caron et al., 2013; Cielen et al., 2016a; Cielen et al., 2016b; Bowen et al., 2017). Importantly, these TS-induced skeletal muscle alterations are associated with reduced quality of life and increased risk of morbidity and mortality in patients with TS-related diseases (Gray-Donald et al., 1996; Swallow et al., 2007; Maltais et al., 2014; Patel et al., 2014).

When combined with the significant negative impact that chronic TS exposure has on the pulmonary system, TS-induced respiratory muscle dysfunction can have particularly detrimental consequences. In fact, insult on respiratory muscle function has been shown to exacerbate symptoms of exercise intolerance and dyspnea in patients with COPD (Gray-Donald et al., 1996; Yamaguti et al., 2009; Haruna et al., 2010; Elbehairy et al., 2016) and congestive heart failure (Mancini et al., 1992; Ribeiro et al., 2009; Yamada et al., 2016). Furthermore, respiratory muscle weakness, and associated reductions in transdiaphragmatic pressure generation capacity, are independent predictors of poor prognosis in both patients with COPD (Killian & Jones, 1988) and
congestive heart failure (Meyer et al., 2001). As such, it is crucial to identify and understand the effects of TS on respiratory muscle dysfunction.

3.1 Tobacco Smoke-Induced Diaphragm Phenotype

The majority of studies examining the effect of TS on skeletal muscle have focused on limb muscle. However, a few studies have described TS-induced diaphragm phenotypic alterations and dysfunction, using otherwise healthy smokers (Elbehairy et al., 2016) or animal models of chronic TS exposure (Barreiro et al., 2010; Martins et al., 2017; Bowen et al., 2017).

3.1.1 Fibre Type Distribution

In the locomotor muscles of human tobacco smokers (Örlander et al., 1979) and animal models of chronic TS exposure (Nakatani et al., 2003; De Paepe et al., 2008; Gosker et al., 2009; Tang et al., 2010; Rinaldi et al., 2012; Barreiro et al., 2010; Krüger et al., 2015), it has been consistently characterized that fibre type distribution shifts towards a fast glycolytic, and away from a slow oxidative profile. This fast fibre type shift in locomotor muscles has been shown to become exacerbated with the progression of COPD (Gosker et al., 2007), and is an independent predictor of mortality in this patient population (Patel et al., 2014).

At this time, there is no human data that has observed the impact of chronic TS exposure on diaphragm fibre type distribution; however, a limited amount of research has explored animal models. While no change in diaphragm fibre type distribution has been observed in mice after up to 20-weeks of chronic TS exposure (Bowen et al., 2017), a 24-week TS exposure on guinea pigs induced a mild 5% increase and concomitant decrease in the proportion of type 2 and type 1 fibres in the diaphragm, respectively. Using immunofluorescent staining of MHC fast- and slow-expressing muscle fibres, both studies (Barreiro et al., 2010; Bowen et al., 2017) only measured
and purported type 1 and type 2 muscle fibre abundances. Importantly, these studies failed to investigate measures that are crucial to gauging the nuances of skeletal muscle changes associated with fibre type shifts, including (1) abundances of type 2a, 2x, and 2b MHC isoforms individually, (2) MHC co-expressing fibre types, and (3) the degree of fibre type grouping (Rowan et al., 2011). As such, it is difficult to extrapolate such findings to a definitive shift in fibre type distribution.

### 3.1.2 Muscle Atrophy

Muscle atrophy, defined as the wasting or loss of muscle tissue, is a consequence of a net protein deficit in skeletal muscles, resulting from an imbalance between protein synthesis and degradation dynamics (Ottenheijm et al., 2008). A number of characteristics can be analyzed to determine the degree of skeletal muscle atrophy present, including activity levels of protein degradation (proteolytic) and protein synthesis enzymes, protein content, muscle fibre cross-sectional area (CSA), and whole muscle mass.

#### 3.1.2.1 Fibre Cross-Sectional Area and Whole Muscle Mass

Chronic TS exposure induces significant reductions in muscle fibre CSA and whole muscle mass in locomotor muscles. Otherwise healthy tobacco smokers exhibit significantly smaller lean body mass, lean leg mass, (van den Borst et al., 2011), and mean CSA of vastus lateralis muscle fibres (Larsson & Örlander, 1984). Furthermore, 16 to 24 week chronic TS exposures have induced both significant whole muscle atrophy (Tang et al., 2010; Caron et al., 2013; Krüger et al., 2015; Cielen et al., 2016a; Cielen et al., 2016b), and muscle fibre atrophy (De Paepe et al., 2008; Krüger et al., 2015; Cielen et al., 2016b) in hindlimb mouse muscle.

Interestingly, no significant differences in diaphragm fibre CSA have been detected in response to TS, in the absence of overt TS-related pathology. Animals undergoing 20 to 24 weeks...
of chronic TS exposure illustrated no change in diaphragm fibre CSA (Barreiro et al., 2010; Bowen et al., 2017). Furthermore, no changes in diaphragm CSA have been seen in patients with mild-to-moderate COPD (Doucet et al., 2004), and diaphragm muscle fibre atrophy is only a consistent finding in patients with severe COPD (Sanchez et al., 1985; Levine et al., 1997). Interestingly, specific contractile force generation capacity (maximum force ÷ mean fibre CSA) is impaired in both TS-exposed mice (Bowen et al., 2017) and in mild-to-moderate COPD patients (Ottenheijm et al., 2005). As such, muscle fibre CSA does not seem to be an appropriate early indicator of TS-related diaphragm dysfunction. Comparing the effects of chronic TS exposure on protein metabolic pathways may contribute to understanding why TS has differential effects on locomotor muscle and the diaphragm.

3.1.2.2 Protein Degradation and Synthesis Signalling

Research on the effect of chronic TS exposure on protein metabolism in locomotor muscle has discovered an upregulation of mRNA levels of pro-degradation enzyme muscle atrophy F-box (MAFbx, also known as Atrogin-1) in otherwise healthy smokers compared to healthy, age-matched controls (Peterson et al., 2007). Similarly, animals who underwent as little as 8 weeks of chronic TS exposure were found to have a significant upregulation of mRNA encoding transcription factor forkhead box-containing protein O3 (FoxO3), and pro-degradation enzymes muscle ring finger 1 (MuRF1), and MAFbx (Caron et al., 2013). These same mice had increased levels of MAFbx and ubiquitin-conjugated proteins following 24 weeks of chronic TS exposure (Caron et al., 2013). Chronic TS exposure seems to also downregulate protein synthesis in locomotor muscles. This is characterized by decreased basal protein synthesis, and an upregulation of myostatin muscle growth inhibitor in the muscle of otherwise healthy smokers (Peterson et al., 2007). Furthermore, chronic TS exposure has been shown to reduce the ratio of active Akt to total
Akt protein in the skeletal muscle of mice, a measure indicative of TS-related downregulation of protein synthesis (Caron et al., 2013). Collectively, these findings suggest that chronic TS exposure downregulates protein synthesis and upregulates proteolytic pathways, contributing to net protein catabolism in locomotor muscle.

Findings suggest that TS-induced protein degradation occurs in the diaphragm muscle as well. MAFbx protein expression was upregulated by approximately 65% in the diaphragm of mice exposed to 20-weeks of chronic TS exposure; however, this same mouse model illustrated no changes in MuRF1 protein expression (Bowen et al., 2017). Interestingly, in contrast to locomotor muscle, chronic TS seems to elicit an increase in protein synthesis in mouse diaphragm. In the diaphragm muscle, it has been found that 15 days of chronic TS exposure induces a significant increase in the concentration of activated phosphorylated mammalian target of rapamysin (p-mTOR), a protein contributing to the upregulation of protein synthesis through upstream Akt activation (Carlos et al., 2014). Compared to controls, MHC contractile protein concentrations in the diaphragm of mice were unchanged in response to 20 weeks of chronic TS exposure (Bowen et al., 2017). More research must be done using one model that measures proteolysis, protein synthesis, and contractile protein concentrations in the diaphragm following a chronic TS exposure to determine the various impact that TS has on diaphragm protein metabolism.

### 3.1.3 Contractile Function and Muscle Fatigability

Various studies have purported different findings regarding contractile properties in the locomotor muscle of otherwise healthy smokers. For the most part, examination of maximum strength, isometric and dynamic strength, and short-term dynamic endurance in the vastus lateralis (Örlander et al., 1979; Larsson & Örlander, 1984) and quadriceps (Morse et al., 2007; Wüst et al., 2008) of otherwise healthy tobacco smokers illustrates a lack of dysfunction in any of these
characteristics. Likewise, models of chronic TS exposure that have assessed both extensor digitorum longus (EDL) and soleus muscles in vitro detected no significant changes in contractile properties or function (Rinaldi et al., 2012; Cielen et al., 2016a; Cielen et al., 2016b; Bowen et al., 2017) following as much as 24 weeks of chronic TS exposure. However, one study found that chronic TS exposure does reduce the capacity of the quadriceps to generate maximal voluntary contractions in otherwise healthy smokers (Barreiro et al., 2010).

Locomotor muscles seem to be more fatigable in human smokers compared to healthy, age-matched non-smokers (Morse et al., 2007; Wüst et al., 2008). Interestingly, while most findings observe a lack of change in fatigability in the soleus and EDL (Tang et al., 2010; Rinaldi et al., 2012; Bowen et al., 2017) in response to chronic TS exposure, one study illustrated a 37% increase in soleus fatigability in mice following 16-weeks of chronic TS exposure. It should be noted that soleus muscles were stimulated at a higher electrical frequency (50Hz versus 40Hz), and for longer durations (500ms versus 250-330ms) in this study compared to others, potentially contributing to the disparity in findings.

Contrary to locomotor muscles, chronic TS exposure negatively impacts diaphragm contractile function. For instance, otherwise healthy tobacco smokers exhibit reduced maximal voluntary diaphragm activation capacity, diaphragm force generation capacity, and maximal transdiaphragmatic pressure generation capacity, when compared to healthy age-matched controls (Elbehairy et al., 2016). Furthermore, examination of animals exposed to 20 weeks of chronic TS illustrated a 15% reduction in diaphragm fibre bundle strength using ex vivo processing and analysis (Bowen et al., 2017), which is consistent with findings from human subjects with mild-to-moderate COPD (Ottenheijm et al., 2005).
Thus far, one TS animal study has measured (40Hz, 330ms stimulation/ 1 second for 2 minutes), and observed no changes in fatigability in the diaphragm of mice exposed to 20 weeks of chronic TS exposure (Bowen, 2017). Furthermore, diaphragm fatigability is not a limiting factor for patients with COPD (Mador et al., 2000), as this muscle is hypothesized to be even less fatigable under conditions of COPD compared to healthy individuals (Ottenheijm et al., 2008). As such, the major impact that chronic TS exposure has on diaphragm contractile properties is its negative impact on maximal force generation capacity, and associated reductions in maximal transdiaphragmatic pressure generation capacity.

3.1.4 Differences Between TS-Induced Diaphragm and Locomotor Muscle Phenotype

Given the stark contrast seen between TS-induced changes in diaphragm and locomotor muscle phenotypes, it is important to evaluate potential mechanisms to explain this phenomenon. One possibility is that TS-induced lung dysfunction results in adaptation of the diaphragm in the face of impaired pulmonary dynamics. Indeed, recent discoveries have resolved that otherwise healthy smokers experience increased dyspnea ratings, peripheral airway dysfunction, small airway inflammation, maldistribution of ventilation, and measures of airway resistance in comparison to healthy age-matched controls, which have been related to increased diaphragm workload (Elbehairy et al., 2016). This increased workload may be similar, albeit to a lesser extent, to diaphragm adaptations observed in patients with COPD.

Diaphragm muscle in mild-to-moderate COPD patients sustain a fundamentally different change in fibre type distribution compared to that seen in limb muscle. Importantly, an increased abundance of slow oxidative fibre types is observed in these patients, as assessed through (1) gel electrophoresis analysis of MHC type 1 and type 2 protein concentrations (Ottenheijm et al., 2006), (2) immunocytochemistry hematoxylin and eosin (H&E) detection of type 1 and 2a or 2x fibre
types on cross-sections (Levine et al., 2003), and (3) myofibrillar ATPase activity staining for type 1, 2a, and 2b fibre type abundance on diaphragm cross-sections (Doucet et al., 2004). While one study (Levine et al., 2003) quantified the abundance of MHC co-expressing type 1 and type 2a muscle fibres (no significant change was observed), all of these studies lacked a comprehensive assessment of fibre type shift, based on a lack of the criteria described in section 3.1.1. However, the degree of preferential slow fibre type abundance is associated with disease severity, as the diaphragm of patients with severe COPD have a higher proportion of oxidative fibres, and an increased proportion of MHC co-expressing muscle fibres, as assessed by immunocytochemistry of cross-sections (Nguyen et al., 2000).

COPD-related changes in diaphragm fibre type distribution are widely seen as an adaptive response similar to that seen in endurance training, whereby the increased work of breathing in the face of abnormal lung morphology and respiratory dysfunction associated with pathophysiology induces a higher demand on the diaphragm (Levine et al., 2002; Ottenheijm et al., 2008). Indeed, the extent of increased oxidative fibre type abundance is significantly related to impaired pulmonary function, as indicated by reduced FEV1, increased functional residual capacity (FRC), and increased total lung capacity (TLC) (Mercadier et al., 1998; Levine et al., 2003). Importantly, it has been shown that specific force of both type 1 and type 2a muscle fibres is negatively impacted in patients with mild-to-moderate COPD, and that type 1 fibres are more impaired than type 2a fibres (Ottenheijm et al., 2005). Given that diaphragm weakness and resulting impaired transdiaphragmatic force generation capacity contribute to dyspnea (Killian & Jones, 1988) and mortality in this patient population (Gray-Donald et al., 1996), it is clear that the adaptive change in fibre type distribution seen in COPD patients contributes to inspiratory muscle weakness, and is not strictly beneficial as previously thought.
Currently, the limited evidence on the independent effect of TS on fibre type distribution in the diaphragm indicates that, at most, a mild increase, and concomitant decrease, in the abundance of fast-twitch and slow-twitch fibres respectively, takes place following a 6-month-long exposure in rodents (Barreiro et al., 2010), with no change in fibre type distribution having been reported in response to shorter exposures (Bowen et al., 2017). In comparison, a pronounced fast fibre type shift in locomotor muscle (Nakatani et al., 2003; De Paepe et al., 2008; Gosker et al., 2009; Tang et al., 2010; Rinaldi et al., 2012; Barreiro et al., 2010; Krüger et al., 2015). This raises the possibility that TS-induced lung dysfunction and subsequent adaptation of the diaphragm in response to an increased workload, contributes to the differences in TS-related fibre type distribution between the diaphragm and locomotor muscles, prior to the onset of COPD. Importantly, differences between TS-related fibre type distribution in the diaphragm and locomotor muscle may explain the contrast between these muscles throughout measures of TS-related maximum contractile generation capacity and fatigability. Indeed, if the diaphragm is undergoing an endurance-like stimulus in response to an increased workload, then this muscle would adapt to become more fatigue resistant and become less able to generate maximal force. The progression of lung pathology resulting in a slow oxidative shift in the diaphragm (Nguyen et al., 2000) is hypothesized to contribute to the muscle’s reduced force generation capacity, one of the most limiting features of the diaphragm in COPD (Ottenheijm et al., 2008).

Finally, TS-related pulmonary dysfunction may also account for the lack of atrophy in the diaphragm in response to chronic TS exposure (Doucet et al, 2004; Barreiro et al, 2010; Bowen et al, 2017), in contrast to limb muscles (Larsson & Örlander, 1984; Cielen et al, 2016b) of both animals and humans. Importantly, mTOR protein activity is upregulated in the diaphragm of animals undergoing chronic TS exposure (Carlos et al., 2014), while it is downregulated in
locomotor muscles (Krüger et al., 2017). Given that mTOR signaling has been shown to be upregulated in the presence of muscle hypertrophy (Bassel-Duby & Olson, 2006) and mediates protein synthesis, it is possible that mTOR is upregulated in the diaphragm in response to the compensatory increased activity of this muscle in the presence of poor breathing dynamics in otherwise healthy smokers (Elbehairy et al., 2016). This increased diaphragm activity and upregulation of mTOR-mediated protein synthesis may also describe the lack of fibre atrophy seen in the diaphragm of animals and humans with a history of chronic TS exposure, in contrast to limb muscle. This is supported by the fact that age-related fibre atrophy has been shown in 65+-year-old sedentary men, but not in age-matched men who are physically active (St-Jean Petellier, 2017), indicating that regular activity can attenuate fibre atrophy in conditions of muscle deterioration. While speculative, TS-induced breathing impairments that result in compensatory increased diaphragm activation and hypertrophy is a possible mechanism to explain differences in TS-related fibre atrophy when comparing the diaphragm and locomotor muscles.

Given the above information, it would be interesting to comprehensively evaluate the effects that chronic TS exposure has on the workload of diaphragm, and relate these findings to diaphragm fibre type distribution, muscle atrophy markers, and force generation capacity in the absence of overt disease. However, at this time, the findings from various studies suggest that the diaphragm begins to adapt to TS-related lung dysfunction prior to the onset of overt disease.

4. **Putative Mechanisms of Tobacco Smoke-Induced Skeletal Muscle Alterations**

4.1 **Oxidative Stress**

Chronic TS exposure has been shown to induce oxidative stress by directly exposing the body to oxidants contained in tobacco smoke through the bloodstream (Barreiro et al., 2010). Through this mechanism, TS-related oxidative stress has been implicated in (1) major respiratory
complications, such as emphysema and chronic inflammation-related lower airway destruction, which are associated with the progression of COPD (Tuder et al., 2003; Pinho et al., 2007; Menegali et al., 2009; Yoshida & Tuder, 2007), (2) coronary artery atherosclerosis (Church & Pryor, 1985), and (3) skeletal muscle weakness and dysfunction occurring prior to the onset of respiratory changes (Barreiro et al., 2010; Carlos et al., 2014). Therefore, it is important to evaluate TS-induced oxidative stress as a potential mechanism for the development of TS-related diaphragm muscle abnormalities.

When compared to non-smoking controls, otherwise healthy smokers and animals exposed to chronic TS exhibit increased markers of oxidative stress in various skeletal muscles. These markers include protein carbonylation activity and lipid peroxidation, both of which were significantly upregulated in the diaphragm of rodents exposed to just 7 days of chronic TS (Ardite et al., 2006; Barreiro et al., 2010; Carlos et al., 2014) prior to increased oxidative stress in the lungs (Carlos et al., 2014). As such, it is clear that TS-induced oxidative stress impacts skeletal muscle, including the diaphragm, independent of its effects on the respiratory system.

4.2 Motor Unit Remodeling and Chronic Denervation

Neuromuscular junction (NMJ) impact as a potential mechanism of TS-induced skeletal muscle and diaphragm alterations is a novel hypothesis. The primary reason for investigating impact to the NMJ as a potential mechanism of TS-induced diaphragm alterations lies in the fact that conditions involving NMJ impact, such as aging and motor neuron diseases, exhibit skeletal muscle phenotypic changes similar to those seen in chronic tobacco smokers.

A motor unit is made up of a motor neuron and the collective group of muscle fibres it innervates. These physiological structures dictate and adjust muscle movement through the modulation of recruitment patterns and action potential firing rates (Hepple & Rice, 2016). Indeed,
motor neuron properties are so closely linked to skeletal muscle function that these properties define the contractile characteristics, and the MHC isoform, expressed by the muscle fibres that each motor neuron innervates (Buller et al., 1960; Jakobsson et al., 1988). Therefore, all of the muscle fibres that are part of one motor unit are of the same fibre type as well.

A crucial component of motor units are the NMJs. NMJs are characterized as the structures involved in direct communication between lower motor neurons and their target skeletal muscle fibres through synaptic neurotransmission. The NMJ is composed of the pre-synaptic axon terminal, the muscle endplate, also known as the post-synaptic membrane, and perisynaptic Schwann cells (PSC) (Jones et al., 2016). Overlap between the pre-synaptic terminal and muscle endplate, as well as maintenance of these structures and axonal sprouting by the PSCs, is crucial to healthy synaptic function (Hepple & Rice, 2016). Importantly, the NMJ has been illustrated as a point of vulnerability, and damage to the NMJ contributes to muscle wasting throughout physiological aging and muscle wasting disorders, such as motor neuron disease (Fahim & Robbins, 1982; Deschenes et al., 2010; Rudolf et al., 2016). As such, the NMJ is frequently investigated as a point of impact when assessing the development of muscle wasting.

4.2.1 NMJ Impact Leads to Motor Unit Remodeling and Chronic Denervation

NMJs have been shown to fragment and lose function throughout the development of advanced age (Oda 1984; Gutmann & Hanzlikova, 1996; Wang et al., 2005), and in neuromuscular pathologies such as amyotrophic lateral sclerosis (ALS) (Telerman-Toppet & Coers, 1978; Gordon et al., 2009; Perez-Garcia & Burden, 2012), Charcot-Marie-Tooth disease (CMT) (Telerman-Toppet & Coers, 1978), spinal muscle atrophy (SMA) (Johnson et al., 1975; Ramzan et al., 2015; Boido & Vercelli, 2016), and post-polio syndrome (PPS) (Trojan et al., 1991; Maselli et al., 1995; Boyer et al., 2010; Tiffreau et al., 2010). This NMJ deterioration leads to motor unit remodelling,
defined as the phenomenon whereby motor neurons that are adjacent to a muscle fibre with a deteriorated NMJ proceed to sprout collateral motor axon branches and thereby re-innervate the denervated muscle fibre in a compensatory response (Balice-Gordon, 1997; Jang & Van Remmen, 2010; Hepple & Rice, 2016). As such, motor neurons with the capacity to re-innervate muscle fibres with deteriorated NMJs continually respond to this phenomenon by generating axon branches and innervating a gradually increasing number of muscle fibres, creating larger motor units. While this occurs periodically throughout adulthood, these repeated cycles of denervation and re-innervation increase the load on motor neurons that are forced to innervate a large number of muscle fibres, eventually leading to motor neuron death and an exhaustion of re-innervation capacity, whereby axons can no longer sprout and re-innervate muscle fibres with deteriorated NMJs (Painter et al., 2014; Tomlinson & Irving, 1977; Doherty et al., 1993; Dalton et al., 2008; Aare et al., 2016). Over time, consistent motor neuron death results in chronic denervation, which is characterized by a lack of connectivity between the pre-synaptic axon terminal and the muscle endplate of an NMJ, resulting in compromised communication between these structures.

Hepple and Rice (2016) have published an in-depth review of age-related motor unit remodelling and chronic denervation-associated skeletal muscle phenotypic characteristics. Motor unit remodeling, as well as chronic denervation, lead to changes in skeletal muscle phenotype. These changes are associated with exercise intolerance, reduced quality of life, and mortality, stressing the importance of developing therapeutic interventions that address NMJ impact in muscle wasting conditions (Hepple & Rice, 2016; Rudolf et al., 2016). As such, the consequences of denervation on skeletal muscle phenotype, discussed in section 4.2.2, significantly burden individuals undergoing muscle wasting.
4.2.1.1 Experimental Models of Denervation

Two models of experimental denervation will be discussed throughout this literature review. Firstly, experimental models of surgical denervation are characterized by manual dissection of nerves. This process results in collateral motor neuron death, denervation, and exacerbation of re-innervation capacity. As such, this model tends to not mimic physiological conditions associated with denervation, such as aging or motor neuron diseases. In contrast, experimental models of sporadic denervation have been found to more accurately mimic the skeletal muscle phenotype of processes involving physiological denervation, such as sarcopenia. This transgenic mouse model over-expresses neurotrypsin protein, which is responsible for cleaving Agrin. Agrin is involved in the stability of acetylcholine receptor (AChR) clusters in the muscle endplate of the NMJ. This transgenic model of denervation is therefore driven by NMJ instability. Furthermore, re-innervation capacity is preserved in this experimental model of denervation (Butiföker et al., 2011; Aare et al., 2016; Spendiff et al., 2016).

4.2.2 Motor Unit Remodeling and Denervation Impact on Skeletal Muscle Phenotype

Motor unit remodelling and chronic denervation negatively impact skeletal muscle in similar ways as does chronic TS exposure. As such, comparing skeletal muscle characteristics in models of chronic TS exposure to models of motor unit remodeling and chronic denervation, may help to further justify investigating TS-induced neuromuscular impact as the mechanism driving skeletal muscle change observed in models of chronic TS exposure.

4.2.2.1 Fibre Type Distribution

As described in section 3.2.1, motor neuron properties dictate the fibre type of the myocytes that they are innervating. As such, motor unit remodeling and chronic denervation status heavily
influences fibre type distribution throughout skeletal muscles. Significantly reduced proportions in the number of fibres expressing only one isoform of MHC, with a concomitant increase in the abundance of MHC co-expressing muscle fibres have been discovered in animal models of denervation (Patterson et al., 2006), advanced age in rodent models (Snow et al., 2005; Carter et al., 2010; Rowan et al., 2011; Purves-Smith et al., 2012; Aare et al., 2016), and very old humans (Andersen et al., 1999; St-Jean-Pelletier et al., 2017). Importantly, age-related co-expression of MHC isoforms within one myocyte has been directly associated with Nav1.5 expression (Rowan et al., 2011), a marker of denervation in adult muscle (Yang et al., 1991). This increased proportion of denervation-induced MHC co-expressing muscle fibres has been confirmed in various models of denervation. For instance, the abundance of co-expressing muscle fibres has been shown to increase significantly in the diaphragm of mice that have undergone a surgical denervation of the phrenic nerve (Carraro et al., 1985; Gauthier & Hobbs, 1989), as well as in the soleus of mice undergoing sporadic denervation (Aare et al., 2016). Finally, humans with neuromuscular diseases such as ALS and other lower motor neuron diseases, exhibit regions of muscle that express an increased abundance of MHC co-expressing muscle fibres compared to healthy age-matched controls (Telerman-Toppet & Coers, 1978; Baloh et al., 2007).

Another hallmark of denervation-re-innervation cycling is fibre type grouping. This phenomenon occurs as a result of one motor neuron, with the capacity to re-innervated fibres with deteriorated NMJs, continually re-innervating surrounding fibres that sustain NMJ impact. As such, there an increased abundance of fibres become innervated by one motor neuron in a localized region. The final outcome results in increased motor units, and a regional grouping of muscle fibres that express the same MHC isoform (Karpati & Engel, 1968). This fibre type grouping phenomenon has been confirmed in senescent animal models (Aare et al., 2016) and very old
humans (Lexell & Taylor, 1991; Kanda & Hashizume, 1989). Experimental models of sporadic
denervation in the soleus (Butiföker et al., 2011; Aare et al., 2016) and surgical denervation of the
diaphragm (Carraro et al., 1985) illustrate significant fibre type grouping as well. Finally, grouping
of atrophic muscle fibres of the same type has been described in motor neuron diseases such as
ALS, CMT, and SMA (Telerman-Toppet & Coers, 1978; Lyons & Slater, 1991; Baloh et al., 2007;
Boido & Vercelli, 2016; Comley et al., 2016).

4.2.2.2 Protein Degradation, Synthesis, and Muscle Atrophy

Denervation-induced muscle atrophy was characterized as early as 1966 (Gutmann &
Hanzlikova, 1966), and a number of studies have since implicated motor unit remodeling and
chronic denervation in the upregulation of proteolytic pathways, as well as in whole muscle and
muscle fibre atrophy.

Animal models of surgical denervation have been shown to express increased activity of
MAFbx and MuRF1 (Bodine et al., 2001). Furthermore, senescent rats with myocytes expressing
higher levels of protein degradation-signalling ubiquitin ligases MuRF1 and MAFbx, also stained
positive for Nav1.5, implicating denervation in the age-related upregulation of proteolytic pathways
within skeletal muscle fibres (Rowan et al., 2011).

Very old humans (Scelsi et al., 1980; Lexell & Taylor, 1991), senescent animal models
(Klitgaard et al., 1989; Brown & Hasser, 1996; Butiföker et al., 2011; Rowan et al., 2011; Aare et
al., 2016), animal models of surgical denervation (Carrero et al., 1985; Finol & Lewis, 1981; Lewis
et al., 1996; Chen, S.P. et al., 2005), and humans with motor neuron diseases (Telerman-Toppet &
Coers, 1978; Luons & Slater, 1991; Baloh et al., 2007) have reported significant denervation-
related muscle fibre atrophy and angulation. Furthermore, in senescent animals expression of
Nav1.5 is increased in very small atrophic fibres, which were more abundant in comparison to
young animals (Rowan et al., 2011). These denervation-related atrophic myocyte patterns have been observed in senescent human muscle as well (Spendiff et al., 2016). Interestingly, models of sporadic denervation illustrate no difference in mean fibre CSA compared to controls (Butiföker et al., 2011; Spendiff et al., 2016), and while there is an increased abundance of small muscle fibres compared to control animals, this shift is significantly smaller than what is observed in senescent animals (Aare et al., 2016).

These findings implicate motor unit remodeling in alterations to muscle fibre CSA; however, significant reductions in fibre CSA seems to be a result of chronic denervation. This hypothesis is supported by the fact that mean CSA of fibres is unchanged in young animals undergoing sporadic denervation, but still have the capacity for compensatory re-innervation. This is in contrast to models of aging, surgical denervation, and motor neuron disease, where re-innervation capacity is depleted and mean CSA of fibres is significantly reduced, as discussed previously. Indeed, human aging has been shown to be accompanied by transient remodeling of the NMJ (Deschenes et al., 2010), and motor unit remodeling (Larsson, 1995), prior to observing significant skeletal muscle impact.

4.2.2.3 Contractile Function and Muscle Fatigability

Various models of motor unit remodeling and denervation have illustrated reduced contractile functionality in muscles undergoing these processes. A reduction in peak force generating capacity has been observed in muscles of senescent humans (MacLennan et al., 1980; Young et al., 1985), senescent animals (Ansved & Larsson, 1989; Brown & Hasser, 1996; Greising et al., 2015), and animals undergoing surgical denervation (Finol & Lewis, 1981; Lewis et al., 1996). Additionally, both senescent mice and mice undergoing sporadic denervation events present with reduced functional skeletal muscle capacity, indicated by insecure and hesitant gaits, reduced
stride length, and reduced grip strength when compared to young control mice (Butiföker et al., 2011). Furthermore, age-related decline in motor neuron integrity has been shown to relate to declines in strength in the quadriceps (Ward et al., 2015).

### 4.2.3 NMJ Degradation Leads to Motor Unit Remodeling and Chronic Denervation

NMJ degradation has been observed throughout a number of muscle wasting conditions. Furthermore, this impact has been observed prior to age- and motor neuron disease-related motor neuron death (Balice-Gordon, 1997; Frey et al., 2000; Fischer et al., 2004; Chai et al., 2011), and muscle wasting phenotype (Masseli et al., 1995; Fischer et al., 2004; Deschenes et al., 2010). These findings support the hypothesis that NMJ damage is the primary source of impact, that subsequently leads to cycles of motor unit remodeling, and eventually chronic denervation and skeletal muscle impairments that negatively impact functional capacity, quality of life, and survival throughout muscle wasting.

Animal model of sporadic denervation (Butiföker et al., 2011; Spendiff et al., 2016) provides evidence that NMJ impact drives motor unit remodeling and denervation. This model of denervation, described in 3.2.1.1, is genetically engineered to destabilize muscle endplate structures in the NMJ. Similar to advanced age, the NMJs in these experimental mice present with (1) fragmented post-synaptic structures, (2) denervation, and (3) reduced pre-synaptic and post-synaptic overlap in innervated NMJs. As described previously, these transgenic mice experience marked increase in the abundance of MHC co-expressing muscle fibres, indicative of motor unit remodeling, and shifts in fibre size distribution in the absence of change in mean fibre size (Butiföker et al., 2011; Spendiff et al., 2016).

Compromised NMJ integrity, similar to those seen in models of sporadic denervation, have been found throughout age-related models of muscle wasting. Throughout advanced age, NMJs
present with (1) fragmented pre- and post-synaptic structures (Andonian & Fahim, 1987; Lyons & Slater, 1991; Butiföker et al., 2011), (2) denervation (Butiföker et al., 2011), (3) reduced pre-synaptic and post-synaptic overlap in innervated NMJs, and (4) narrow innervating axons, and (5) axons that are more prone to sprouting (Balice-Gordon, 1997; Jang & Van Remmen, 2010; Chai et al., 2011; Samuel et al., 2012; & Valdes et al., 2012).

Early synaptic transmission defects have been detected in human (Masseli et al., 1995) and animal (Frey et al., 2000; Fischer et al., 2004) models of ALS, as well as in humans with PPS (Boyer et al., 2010; Tiffreau et al., 2010). Interestingly, ALS and motor neuron degenerative disorder mouse models present with significant denervation, which is inversely related to sprouting competency of motor neurons at the NMJ, and precedes significant motor neuron loss (Frey et al., 2000; Fischer et al., 2004). Based on these findings, NMJ impact drives motor unit remodeling and chronic denervation in patients with motor neuron diseases, and muscle wasting similar to that found with age. Furthermore, when sprouting competency is compromised in experimental models of these conditions, chronic denervation is more frequently observed due to a lack of re-innervation capacity.

**4.2.3.1 Mechanisms of NMJ Degradation**

Various mechanisms that drive NMJ degradation-induced skeletal muscle wasting and deterioration have been proposed. As discussed in sections 2.1.2 and 3.2.3.2, chronic TS exposure and denervation induce protein degradation in skeletal muscle as indicated by upregulation of transcript and/or protein expression of pro-degradation proteins such as MAFbx, MuRF1, and transcription factor FoxO3. Interestingly, MuRF1 protein has been found to be upregulated at the NMJ throughout muscle wasting, particularly at the motor endplate (Rudolf et al., 2013; Khan et al., 2014), and knockout of MuRF1 in animals undergoing surgical denervation illustrated marked
attenuation of denervation-induced AChR instability and muscle atrophy (Rudolf et al., 2013). Based on these findings, MuRF1 is crucial to denervation-driven degradation of NMJ AChR structures, and is a particular point of interest when examining NMJ impact under skeletal muscle deterioration conditions.

The agrin-muscle specific kinase (MuSK)-Lrp4 pathway has been investigated as a pathway that may be affected, leading to NMJ impact that is related to skeletal muscle deterioration throughout aging and motor neuron disease. Agrin, released by the motor neuron, binds with Lrp4 in the synaptic cleft, to subsequently activate MuSK protein, which is integral to stabilizing AChR and muscle endplate morphology (Tintignac et al., 2015). The over-expression of neurotrypsin protease, which cleaves agrin, is the basis of the mouse model undergoing sporadic denervation, which maintains phenotypic similarities to sarcopenia (Butiföker et al., 2011; Spendiff et al., 2016). Interestingly, while neurotrypsin deficiency did not attenuate sarcopenia phenotypes in senescent mice, senescent mice expressing cleavage-resistant agrin illustrated reduced sarcopenia when compared to other senescent mice (Butiföker et al., 2011). These findings indicated that this agrin contributes, but does not completely dictate, age-related skeletal muscle phenotype. The agrin-MuSK-Lrp4 pathway has also been implicated in motor neuron disease. Interestingly, Lrp4 autoantibody molecules have been found in high abundance in both ALS and CMT patients (Guyon et al., 1998).

Given the mounting evidence that NMJ impact may orchestrate motor unit remodeling and is consistently present throughout conditions of skeletal muscle deterioration, including aging and motor neuron diseases, evaluating NMJ integrity is a logical step when assessing any condition associated with skeletal muscle deterioration. As such, investing NMJ integrity in response to
chronic TS exposure has the strong potential to develop our understanding of a mechanism by which TS negatively impacts skeletal muscle and the diaphragm.

4.3 TS-Induced NMJ Impact

TS-induced impact at the NMJ is a novel hypothesis to explain respiratory muscle dysfunction in people with a history of chronic TS exposure. Support for this hypothesis is multidimensional. Firstly, skeletal muscles exhibit significant phenotypic similarities in response to chronic TS exposure and denervation-related stimuli. Furthermore, compared to non-smokers, age-related changes in body mass, lean body mass, and lean leg mass follow similar patterns throughout aging in chronic tobacco smokers, but are significantly more impaired throughout this process (van den Borst et al., 2011). This data indicates that chronic TS exposure may partially impact skeletal muscle by exacerbating age-related muscle atrophy, without deviating from the muscle frailty patterns seen throughout advanced age. As such, TS-induced exacerbation of age-related NMJ impact and motor unit remodeling would explain these phenomena. Secondly, previous findings from our lab, which are currently published in a McGill University Master’s degree thesis (Míguez, 2017), and under review for publication in the Journal of Physiology (Kapchinsky et al.), have implicated locomotor muscle NMJ denervation in whole muscle atrophy and impaired mitochondrial oxidative capacity of adult mice exposed to 16-weeks of chronic TS.

5. Hypotheses of the Current Study

Based on the rationale provided in section 4.3, there is strong support for the theory that TS-induced motor axon and NMJ impact is a mechanism that contributes to TS-related diaphragm abnormalities. As such, this study hypothesizes that chronic TS exposure of mice will significantly impact motor axon and NMJ morphology in mouse diaphragm, similar to the impact that is seen
in other conditions of neuromuscular degradation, such as aging, motor neuron disease, and experimental models of sporadic denervation. Furthermore, we hypothesize that this effect is progressive, and that TS-induced changes in motor axon and NMJ morphology following 8 weeks of exposure will be exacerbated following 16 weeks of chronic TS exposure.
CHAPTER 3: EXPERIMENTAL ARTICLE

8 and 16 weeks of Chronic Tobacco Smoke Exposure Negatively Impacts Morphological Characteristics of Peripheral Motor Axons and Neuromuscular Junctions in the Diaphragm of Mice

Introduction

Chronic tobacco smoking negatively affects the health of individuals who are regularly exposed to smoke, and is the leading cause of preventable disease in North America. Furthermore, chronic tobacco smoking is implicated as a primary risk factor for the development of the top three causes of death worldwide: chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD), and cancer (USDHHS, 1984; Zaher et al., 2004; Jones et al., 2010). As such, a history of chronic tobacco smoke (TS) exposure is estimated to have contributed to ~21% of all mortalities in Canada between 2000 and 2010 (Jones et al., 2010). Interestingly, individuals with the aforementioned TS-related diseases experience similar, significant skeletal muscle impact that has been shown to relate to disease progression and risk of mortality (Gray-Donald et al., 1996; Marquis et al., 2002; Swallow et al., 2007; Patel et al., 2014; Maltais et al., 2014).

Importantly, findings have implicated a history of chronic tobacco smoking in the development of skeletal muscle dysfunction in patients with TS-related diseases. This is highlighted by the fact that prior to the onset of overt pathological characteristics, otherwise healthy tobacco smokers (Larsson & Örlander, 1984; Montes de Oca, 2008), and experimental models of chronic TS exposure in animals (de Paepe et al., 2008; Gosker et al., 2009; Rinaldi et al., 2012; Caron et al., 2013; Cielen et al., 2016a; Cielen et al., 2016b; Bowen et al., 2017) share
muscle phenotypes with individuals who have TS-related diseases. These findings implicate TS-induced skeletal muscle dysfunction in the manifestation of muscle impairment associated with TS-related diseases. Furthermore, given that up to 45% of COPD patients actively smoke (Riesco et al., 2017), chronic TS exposure continues to exacerbate muscle dysfunction throughout the disease progression of many patients. When present in inspiratory muscles, this impact has unique, negative consequences for patients. Indeed, when present in the diaphragm, muscle dysfunction contributes to exacerbation of co-morbidities (Mancini et al., 1992; Gray-Donald et al., 1996; Ribeiro et al., 2009; Yamaguti et al., 2009; Haruna et al., 2010; Elbehairy et al., 2016; Yamada et al., 2016; Bowen et al., 2017) and mortality (Killian & Jones, 1988; Meyer et al., 2001) in patients with COPD and chronic heart failure. Despite the severe consequences of TS-induced skeletal muscle impact for patients, the mechanisms driving this impact are still largely unknown.

A number of factors provide the rationale for investigating TS-induced neuromuscular junction (NMJ) impact as a mechanism of TS-related diaphragm impact. These include the fact that: (1) the NMJ is a particularly vulnerable structure that has been shown to degrade in various muscle wasting conditions (Deschenes et al., 2010; Rudolf et al., 2013; Rudolf et al., 2016), (2) TS-related skeletal muscle phenotypes are similar to that seen in individuals undergoing processes that are characterized by NMJ degradation, such as aging (Oda 1984; Gutmann & Hanzlikova, 1996; Wang et al., 2005), and various motor neuron diseases (Johnson et al., 1973; Telerman-Toppet & Coers., 1978; Trojan et al., 1993; Baloh et al., 2007; Gordon et al., 2009; Perez-Garcia & Burden, 2012; Ramzan et al., 2015), and (3) findings from a recent study in our lab revealed that the tibialis anterior (TA) of 8-month-old mice undergoing 16 weeks of chronic TS exposure demonstrated a significant increase in the fraction of denervated NMJs (Míguez, 2017, McGill University graduate thesis; Kapchinsky et al., under review with the Journal of Physiology).
Resolving the mechanisms that drive TS-induced diaphragm dysfunction is crucial to optimizing preventative strategies against muscle impact that can compromise breathing dynamics, and lead to poor outcomes for patient populations, including increased risk of mortality (Killian & Jones, 1988).

We hypothesized that chronic TS exposure negatively impacts morphological features of motor axons and NMJs in the diaphragm, and that this impact would progressively worsen over longer exposure periods. To test these hypotheses, the current study analyzed a number of morphological characteristics of motor axons and NMJs in the diaphragm of mice exposed to 8 and 16 weeks of TS exposure, and related these findings to body characteristics of these animals.

**Methods**

**Animals**

104 ± 8.63 d old male C57Bl/6-elite wild-type mice (Charles River Laboratories, QC, Canada) were used for all experimental procedures. C57Bl/6-elite mice differ from C57Bl/6 mice only in that they maintain a virus antigen free (VAF)/ Elite® health profile as defined by Charles River Laboratories, which means that specific infectious agents are excluded from animal colonies throughout breeding and housing periods. Throughout the entirety of the experimental design, mice were housed 1-3 mice per cage and subject to all experimental procedures in the vivarium of the Research Institute of the McGill University Health Centre (RI-MUHC, Montreal, QC, Canada). All animal procedures were previously approved by the McGill University Animal Care Committee (protocol no. 5933, C. Baglole).
Tobacco Smoke Exposure

Mice were separated into four groups: 8-wk air (n=9), 8-wk tobacco smoke (TS) (n=9), 16-wk air (n=7), and 16-wk TS (n=8). Mice in the TS groups were passively exposed to the TS from 24 3R4F Filtered Research Cigarettes (University of Kentucky, Lexington, KY, USA) using a SciReq InExpose Whole Body Exposure Chamber system (SciReq, Montreal, QC, Canada), for 54 min, 2 times daily, 5 d/wk, for either 8 or 16 weeks. Mice in the air groups served as controls, and were exposed to ambient air in the same procedure room as the TS-exposed mice. For each exposure, all mice, including air mice, were transported within their respective cages from the animal housing room to the procedure room. This was done to keep both the effect of being transported to the procedure room and being separated from a source of water for the duration of the TS exposure period constant across all groups of mice. To quantify the intensity of the TS exposure, an electronic monitor was used to measure real-time particulate density within the exposure chamber (MicroDust Pro, Casella, Buffalo, NY, USA). On average, particulate matter density was maintained between 4.0 and 6.0 mg x m\(^{-3}\) x min\(^{-1}\) throughout each exposure. To examine the impact of TS exposure on changes in body mass throughout the exposure period, mice were weighed 4-8 times per month, from the beginning of the exposure period until sacrifice.

Animal Sacrifice

Following the 8-wk and 16-wk exposures, mice were sacrificed at 162 ± 10 d of age, and 226 ± 4 d of age, respectively. Mice within the TS groups were sacrificed 48 hours after their last smoke exposure. All mice were anesthetised using an inter-peritoneal injection of ketamine solution (1 mL x g of body weight\(^{-1}\)). Afterwards, mice in the 8-wk groups were sacrificed by CO\(_2\) asphyxiation, followed by cervical dislocation. However, mice in the 16-wk groups first underwent an in situ muscle contractility protocol on their right tibialis anterior (TA) (data not reported here).
Following this terminal procedure, while still heavily anesthetized, the 16-wk exposed mice were euthanized by cervical dislocation.

**Tissue Harvest/ Processing**

Following euthanasia of all mice, several muscles and organs were harvested, trimmed of excess fat and connective tissue, weighed, and processed accordingly. Epididymal fat tissue was also harvested and weighed from the mice in the 16-wk group. The gastrocnemius, extensor digitorum longus (EDL), and plantaris from both hind limbs of all mice were flash-frozen in liquid nitrogen (N₂) for subsequent protein and transcript analysis. The right TA and right soleus from all mice were mounted on histology blocks and flash frozen in isopentane cooled by liquid N₂. The left TA from mice in the 8-wk groups were flash-frozen in N₂(l) for subsequent protein and transcript analysis, while the left TA from mice in the 16-wk groups were placed in ice-cold stabilizing Solution A (2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 6.56 mM MgCl₂, 0.5 mM dithiothreitol, 50 mM K-MES, 20 mM imidazol, 20 mM taurine, 5.3 mM Na₂ATP, 15 mM phosphocreatine) to be used to assess mitochondrial respiratory function, as has been done previously (Spendiff et al., 2016). The left soleus from each mouse was placed in ice-cold 1 x phosphate buffer saline (PBS) solution to be used for subsequent neuromuscular junction (NMJ) analysis. After harvesting and weighing the diaphragm, the central tendon and crural region were removed and discarded to limit experiments to the costal diaphragm. Either the right or left posterior quarter of the costal diaphragm was randomly selected and placed in PBS solution on ice for subsequent NMJ analysis. The remaining posterior quarter was mounted in Cryomatrix embedding resin (Thermo Scientific, Waltham, MA, USA) and flash-frozen in isopentane cooled in liquid N₂. Muscles flash-frozen for protein and transcript analysis or histology were stored at -80 °C.
NMJ Immunofluorescent Staining, Imaging, and Analysis

Soleus and diaphragm muscle that was processed for NMJ analysis underwent a muscle mount NMJ immunofluorescent staining protocol similar to that used by Spendiff et al. (2016). After being harvested, weighed, and immediately placed in ice-cold 1 x PBS solution, muscles were washed in fresh 1 x PBS for 20 min. Using 2% paraformaldehyde (PFA) solution, the diaphragm and soleus were fixed for 4 h and 2 h, respectively, at room temperature (RT). Following fixation, the muscles were again washed in 1 x PBS (1 x 20 min, followed by 3 x 5 min). Following the washes, the muscles were placed in fresh 1 x PBS solution at 4 °C for 1 to 36 h, due to project scheduling. This length of time was randomized amongst the different groups of mice, to limit its effect on subsequent results. Before subsequent processing, diaphragm muscles were cut width wise into smaller pieces, while soleus muscles were carefully separated under a stereomicroscope into smaller bundles of fibres. All muscles were then placed in a blocking/permeabilization solution (5% normal goat serum, 5% BSA, and 2% Triton X-100 in PBS) overnight, while shaking at 4 °C. Muscles were then incubated for 24 h while shaking at 4 °C in blocking solution (5% normal goat serum, 5% BSA, in PBS) containing mouse anti-synaptophysin primary antibody (1:25 dilution, AB8049, Abcam, Cambridge, MA, USA) and rabbit anti-heavy neurofilament (NF200) primary antibody (1:200 dilution, N4142, Sigma, Germany). Afterwards, the muscles were washed in blocking solution (5 x 60 min) at RT. Muscles were then incubated overnight while shaking at 4 °C, in blocking solution containing AF488-conjugated α-bungarotoxin conjugated antibody (1:500 dilution, B13422, Life Technologies, Carlsbad, CA, USA), AF594 goat-anti-mouse IgG1 secondary antibody (1:500 dilution, A21125, ThermoFisher, Waltham, MA, USA), and Cy5 goat-anti-rabbit IgG (H+L) (1:200 dilution, A10523, ThermoFisher, Waltham, MA, USA). Muscles were subsequently washed in blocking solution (5
while shaking at RT, and stored in blocking solution overnight at 4 °C. The following morning, the pieces of diaphragm and bundles of soleus fibres were mounted onto respective slides in ProLong Gold Antifade Mountant (P36930, Life Technologies, Carlsbad, CA, USA). Stacked images (slice thickness = 1.00 μm) of immunolabeled NMJs were imaged using an LSM880 confocal microscope (Carl Zeiss, Oberkochen, Germany) with a 63x oil immersion objective. A pinhole size of 66.0 μm was used for each fluorescent channel. Maximum intensity projection NMJ images were analyzed using Fiji computer software. The method used to analyze NMJ fluorescent images was adapted from the protocol developed and used by Jones and colleagues (2016). As described in the methodological paper, binary images of muscle endplates for each NMJ are created using Fiji, based on the AChR image for each NMJ.

Statistical Analysis

All statistical analyses were done using GraphPad Prism 6.0h (GraphPad Software, La Jolle, CA, USA). All results are expressed as mean ± SD. All data was tested for significant outliers using a ROUT test (Q = 5 %), and outliers were subsequently excluded from analysis. Unless otherwise stated, statistical analysis was performed using a two-way ANOVA (exposure [TS vs. air] x exposure length) and a Tukey’s multiple comparisons test to compare each of the four groups of mice with one another. Significant findings comparing 8-wk air and 16-wk TS mice, or 8-wk TS and 16-wk air mice, were not reported.

Results

Animal Characteristics

Change in body mass growth, calculated according to mass taken immediately following sacrifice divided by mass taken just prior to the beginning of the experimental protocol, was
significantly lower in both 8- and 16-wk TS exposure groups (p < 0.0005). Furthermore, a multiple comparisons test revealed that, when independently compared to 16-wk air mice (1.275 ± 0.12), each of 8-wk air (1.104 ± 0.075) and 16-wk TS (1.097 ± 0.021) exhibited smaller body mass growth over their respective exposure periods (p < 0.01 and 0.005, respectively; Fig 1).

Epididymal fat, spleen, and liver were weighed in 16-wk air and 16-wk TS mice immediately after sacrifice. Differences in the mean absolute mass of these tissues between the 16-wk groups were analyzed using an unpaired t-test. Epididymal fat mass was significantly reduced in 16-wk TS mice (770.5 ± 278.05 g) compared to 16-wk air mice (1267.87 ± 682.09 g) (p > 0.05) (Fig 2a). No significant differences were observed in liver or spleen mass between 16-wk air mice and 16-wk TS mice (p = 0.100 and 0.645, respectively; Fig 2b, c).

To permit the use of a two-way ANOVA for the statistical analysis of the effect of age on diaphragm, gastrocnemius, plantaris, soleus, TA, and EDL muscle mass, muscle masses of both air groups were normalized to those in the 8-wk air group, which acted as a control (Fig 3). Doing so revealed that age did not impact normalized muscle mass (p = 0.188) (Fig 3). Likewise, to permit the use of a two-way ANOVA for the statistical analysis of the effect of TS exposure on muscle mass, muscles masses were normalized to their respective age-matched control mice. 8-wks of chronic TS exposure significantly reduced normalized muscle mass (p < 0.0001); however, 16 weeks of chronic TS exposure did not (p = 0.876) (Fig 4a, b).

**Diaphragm Motor Axon and NMJ Morphology**

Representative confocal images of diaphragm NMJs are provided in Figure 5a – c.
Motor Axon Morphology

The following morphological characteristics were assessed in 8-wk air (n=5), 8-wk TS (n=4), 16-wk air (n=6), and 16-wk TS (n=6) mice. Of the NMJs with axonal input, chronic TS exposure did not impact mean number of axonal inputs between groups (p = 0.69) (Fig 6a). Thus, chronic TS exposure did not result in an increased fraction of poly-neuronal innervated NMJs. In contrast, chronic TS exposure led to a significant increase in the fraction of NMJs lacking axonal input in both 8-wk and 16-wk exposed mice (p < 0.05) (Fig 5c, 6b). No significant differences were observed between any groups when comparing the mean number of terminal axon branches per NMJ, or the mean number of axonal branch points per NMJ. However, when independently compared to 16-wk air mice (1.80 ± 0.09 μm) mean axon diameter was significantly smaller in both 8-wk air (1.75 ± 0.11 μm) and 16-wk TS mice (2.48 ± 0.74 μm) (p < 0.05 for each measure), with no significant difference observed between 8- and 16-wk TS mice (p = 0.99) (Fig 5b, 7).

Pre-Synaptic Nerve Terminal and Motor Endplate Morphology

The following morphological characteristics were assessed in 8-wk air (n=4), 8-wk TS (n=4), 16-wk air (n=6), and 16-wk TS (n=6) mice. No significant differences were observed between any groups when observing the following characteristics: (1) mean AChR area, (2) mean pre-synaptic nerve terminal area, or (3) mean endplate area (Fig 8a - c). No significant differences were observed between any groups when comparing synaptic overlap, which is defined by the fraction of AChR area that overlaps with synaptophysin (Fig 9).

“Compactness” is defined by the percentage of endplate area overlapping with either synaptophysin (termed “synaptophysin compactness”) or AChR (termed “AChR compactness”). No significant differences were observed between any groups when comparing synaptophysin compactness (10a); however, reduced AChR compactness was driven by TS exposure in all groups.
(two-way ANOVA, p < 0.005). Furthermore, AChR compactness was significantly smaller in 16-wk TS mice (55.78 ± 3.33 %) compared to 16-wk air mice (62.46 ± 1.88 %) (Tukey’s multiple comparisons test; p < 0.005) (Fig 10b)

**AChR Fragmentation**

No significant differences were observed between any groups (n=6 for all groups) when assessing AChR cluster fragmentation. Fragmentation was assessed by both the mean number of AChR fragments per NMJ (Fig 11a), and the fraction of NMJs characterized by either one, two to four, or greater than four separate AChR fragments (Fig 10b).

**Denervation Characteristics**

In both 8-wk and 16-wk TS mice, chronic TS exposure led to a significant increase in the fraction of denervated NMJs (p < 0.005) (Fig 5c, 12), defined by the absence of pre-synaptic nerve terminals overlapping muscle endplate structures.

**Discussion**

In spite of the fact that TS-related impact on diaphragm increases the risk of co-morbidities (Mancini et al., 1992; Gray-Donald et al., 1996; Ribeiro et al., 2009; Yamaguti et al., 2009; Haruna et al., 2010; Elbehairy et al., 2016; Yamada et al., 2016) and mortality (Killian & Jones, 1988; Meyer et al., 2010) across multiple TS-related diseases, the mechanisms that drive TS-related diaphragm dysfunction are largely unknown. The current study measured morphological features of peripheral motor axons and NMJs in the diaphragm to assess TS-induced neuromuscular impact as a possible mechanism that contributes to TS-related diaphragm dysfunction. Furthermore, this study sought to gauge the progressive nature of this impact by comparing measurements following 8 and 16 weeks of chronic TS exposure.
The current study observed a significant impact following both 8 and 16 weeks of TS exposure on measures of: (1) reduced change in body mass over the exposure period, (2) a reduced mean number of axons innervating diaphragm NMJs, driven by an increase in the fraction of NMJs lacking axonal innervation, (3) reduced AChR “compactness”, defined by the percentage of endplate area overlapping with AChR area, and (4) an increased fraction of denervated NMJs (two-way ANOVA [exposure (air vs. TS), exposure length (8 weeks vs. 16 weeks)]). Furthermore, 8 weeks of chronic TS exposure led to significantly reduced muscle mass normalized to age-matched controls, whereas no effect was seen when measuring this parameter following 16 weeks of chronic TS exposure (two-way ANOVA [exposure (air vs. TS), muscle type (diaphragm, gastrocnemius, plantaris, soleus, TA, EDL)]). Finally, increased exposure length resulted in exacerbation of two morphological characteristics: (1) attenuation of pre-synaptic axon growth, as characterized by increased pre-synaptic axon diameter in 16-wk air compared to 8-wk air mice, with no change in this measure when comparing 16-wk TS to 8-wk TS mice (Tukey’s multiple comparisons test), and (2) exacerbation of reduced AChR compactness, based on the fact that a Tukey’s multiple comparisons post hoc tests revealed a significant reduction in 16-wk TS compared to 16-wk air mice, but only a trend when comparing 8-wk TS to 8-wk air mice.

Body Mass

Reduced body mass has been shown to relate to COPD severity (Vestbo et al., 2006). Importantly, chronic TS exposure has been shown to attenuate body mass growth in young mice (Gosker et al., 2009; Rinaldi et al., 2012; Cielent et al., 2016b), and reduce absolute body mass in older humans (van den Borst et al., 2011). The TS-related impact on body mass has been suggested to be driven by a number of factors, including slightly reduced food intake, reduced fat mass, and reduced skeletal muscle mass, as observed in tobacco smoking animal models (de Paepe et al.,
2008; Tang et al., 2010; Caron et al., 2013; Krüger et al., 2015; Cielen et al., 2016a; Cielen et al., 2016b) and older humans with chronic TS history (Chen et al., 2007; van den Borst et al., 2011). Body mass growth was unaffected in 8-wk TS mice compared to 8-wk air mice; however, body mass growth was attenuated in 16-wk TS mice compared to 16-wk air mice (Fig 1). These findings are generally in line with previous studies; however, most studies finding slower growth with TS exposure have examined TS-related body mass growth patterns following exposures longer than 8 weeks. As such, other studies have found that body mass growth is attenuated in very young mice following between 12 and 24 weeks of chronic TS exposure (Gosker et al., 2009; Rinaldi et al., 2012; Cielen et al., 2016b).

As discussed previously, lower fat mass is one factor that contributes to (1) attenuated body growth in very young tobacco smoking animal models (Cielen et al., 2016b), and (2) reduced whole body mass in older, otherwise healthy human tobacco smokers (van den Borst et al., 2011). In the current study, absolute epididymal fat mass was significantly smaller, by ~39%, in 16-wk TS mice compared to 16-wk air mice. Reduced food intake, which has been illustrated in both mice (Chen, H. et al., 2005; Chen et al., 2007; Cielen et al., 2016b) and rats (Ypsilantis et al., 2013) that are exposed to chronic TS exposure, most likely contributed to this TS-related reduction in fat mass; however, this parameter was not measured in the current study. Importantly, undernourishment has been shown to induce significantly impact skeletal muscle (Kim, 2013), and assessing markers of malnutrition was important to defining whether or not this phenomenon was occurring as a result of TS exposure. As such, absolute spleen and liver masses were compared between 16-wk mouse groups, as undernourished mice present with significantly reduced spleen and liver mass (Serafim et al., 2010; Kim, 2013). 16 weeks of chronic TS did not impact absolute spleen or liver mass. Therefore, while 16 weeks of chronic TS exposure led to significantly reduced
fat mass, which most likely contributed to reduced body mass growth, 16-wk TS-exposed mice
did not show overt signs of malnutrition.

Muscle Mass

Importantly, skeletal muscle dysfunction that is associated with chronic TS exposure,
including muscle atrophy, is implicated with poor clinical outcomes, exercise intolerance, and
reduced quality of life in patients with COPD (Decramer et al., 1996; Marquis et al., 2002; Swallow
et al., 2007; Maltais et al., 2014; Patel et al., 2014). While no TS-related impact on thigh
circumference was observed in studies examining young adult (~24-years-old) (Morse et al., 2007)
and adult (~44-years-old) (Örlander et al., 1979) humans, it has been shown that older (~73-years-
old) otherwise healthy tobacco smokers exhibit reduced total lean mass and lean leg mass
compared to age-matched controls (van den Borst et al., 2011). Therefore, TS-related skeletal
muscle atrophy in humans seems to manifest at older age. This is most likely due to the combined
impact of more chronic TS exposure and age on skeletal muscle. On the other hand, animal studies
on very young mice have illustrated mixed findings on the effect of chronic TS exposure on whole
muscle mass. While various models have observed reduced whole muscle mass following 8 to 32
weeks of TS exposure (de Paepe et al., 2008; Tang et al., 2010; Caron et al., 2013; Krüger et al.,
2015; Cielen et al., 2016a; Cielen et al., 2016b), other studies have seen no loss of muscle mass
following TS exposure periods of similar lengths (Gosker et al., 2009; Rinaldi et al., 2012).

Mean absolute muscle mass was recorded for all mice immediately following sacrifice
(Table 1). Using a two-way ANOVA, 8 weeks of TS exposure was determined to significantly
impact normalized muscle mass (Fig 4a); however, this effect was not observed following 16
weeks of TS exposure (Fig 4b). Muscle mass data available in the literature from TS mouse models
is conflicting – as such, the contradictory nature of this current study’s data mimics the mixed data
available in the literature. Given this phenomenon, the following reasoning could help to speculate underlying causes of the findings from the current study.

Given that 8-wk-old mice, the most common age used in previous studies, are still young and are growing rapidly, it is difficult to discern whether the mechanisms driving TS-related reduced muscle mass in previous studies is due to activation of muscle atrophy pathways, an inhibition of muscle growth, or a combination of both. As discussed previously, mouse skeletal muscle mass has been shown to develop until ~17 to 26-wks of age (Ontell et al., 1984; Alnaqeeb et al., 1986), and the current study illustrates that at 23-wks of age, muscle mass was already fully developed in the mice studied (Fig 3). Therefore, given that mice were ~15-wks-old upon commencement of the exposure period, it is likely that muscle mass was still growing throughout an early portion of the experimental procedures. Based on this fact, one speculative explanation for the conflicting effect of 8 versus 16 weeks of TS exposure on muscle mass, is as such: while both groups of mice were potentially stunted in their muscle mass development due to being exposed to chronic TS while their muscle is still growing, muscle mass of the 16-wk exposed mice was somehow able to recover from this early impact, while muscle mass of the 8-wk exposed mice was not. Factors that may have contributed to this phenomenon might include: (1) reduced appetite and food intake at the commencement of the exposure that was subsequently normalized once the mice were habituated to TS exposures, and/ or (2) early TS-induced insult to muscle was followed by a subsequent compensatory upregulation of muscle protein synthetic pathways. However, these speculations are not confirmed by the current study’s findings. A more in-depth analysis of the progressive effect that chronic TS exposure has on muscle growth and atrophy factors in the skeletal muscle of young mice is necessary to determining the differential impact that chronic TS exposure has on developing mice.
**Contribution of Growth to Body Characteristics**

In the current study, mice began undergoing experimental protocols at ~15-wks-old, and were sacrificed at ~23-wks-old and 32-wks-old following 8 and 16 weeks of exposure, respectively. The results pertaining to the impact of TS on body mass should be considered in the context of mice that are still growing. This is highlighted by the fact that body mass growth over the exposure period (body mass at sacrifice ÷ body mass at the start of exposure), was significantly larger in 16-wk air compared to 8-wk air mice (Fig 1). In contrast, comparison of 8-wk and 16-wk air group muscle masses normalized to 8-wk air muscle masses, revealed that age did not impact normalized muscle mass (p = 0.188) (Fig 3). Therefore, these findings indicate that at 23-wks-old, mice were still undergoing body mass growth at the end of the 8-wk exposure period; however, skeletal muscle mass had stabilized by this time. As such, fat mass growth most likely contributed to increased growth in 16-wk mice compared to 8-wk mice; however, the appropriate measures were not taken to make these comparisons.

**Impact on Motor Axon and NMJ Morphology**

The NMJ, being a particularly vulnerable structure (Rudolf et al., 2016), is impacted under various muscle wasting conditions, such as aging (Hepple & Rice, 2016) and various neuromuscular diseases (Boido & Vercelli, 2016; Comley et al., 2016; Rudolf et al, 2016). Importantly, NMJ impact has been detected prior to the onset of skeletal muscle abnormalities in aging (Deschenes et al., 2010), and loss of motor neurons in aging (Chai et al., 2011) and motor neuropathies (Fischer et al., 2004). Indeed, patients affected by the Poliomyelitis virus, which causes severe loss of motor neurons early in life, typically live relatively normal lives for many years before succumbing to the impact of so-called Post-Polio syndrome wherein muscle atrophy and weakness become evident (Boyer et al., 2010; Tiffreau et al., 2010). This phenomenon
underscores the highly effective plasticity of the motor unit that is normally present for much of adulthood in dealing with neuromuscular junction degeneration through re-innervation. Various morphological abnormalities of motor axons and NMJs are associated with skeletal muscle deficits observed throughout physiological aging, motor neuron pathologies, and an experimental model of sporadic denervation. For a more in-depth examination, various papers of key interest have assessed motor axon and NMJ impact under conditions of aging (Jang & Van Remmen, 2010; Valdez et al., 2010; Chai et al., 2011; Valdez et al., 2012; Hepple & Rice, 2016), motor neuron disease (Murray et al., 2010; Jang & Van Remmen, 2010; Boido & Vercelli, 2016; Comley et al., 2016), and experimental sporadic denervation (Butiföker et al., 2011; Spendiff et al., 2016). The findings of the current study suggest that motor axon and NMJ morphology are also vulnerable under conditions of chronic TS exposure, and that these structures respond similarly to this stress as is seen in other conditions associated with motor axon and NMJ impact.

**Motor Axon Morphology**

Peripheral motor axon impact throughout the previously described conditions, where NMJ impact is evidenced, present with relatively consistent morphological features. These include: (1) increased fraction of NMJs lacking axonal contact, (2) increased branching of motor axons, (3) relatively thin innervating axons, and (4) axons that are more prone to sprouting (Prakash & Sieck, 1998; Jang & Van Remmen, 2010; Valdez et al., 2010; Valdez et al., 2012; Deschenes et al., 2013).

Neither 8 nor 16 weeks of TS exposure led to significant poly-neuronal innervation in diaphragm NMJs, as indicated by similar mean number of axonal inputs in NMJs with axonal input between groups (Fig 6a). This finding is in contrast to studies of age-related motor axon characteristics, where an increased fraction of NMJs undergoing poly-neuronal innervation has been observed in very old mice (Valdez et al., 2010). In contrast, TS exposure significantly
increased the fraction of NMJs lacking any axonal input (Fig 5c, 6b). These findings are consistent with other studies on NMJ impact, which have observed an age-related increase in the fraction of endplates that are not contacted by motor axons (Valdez et al., 2010; Valdez et al., 2012).

Representative images of diaphragm motor axons and NMJs are provided in Figure 5a – c. The mean number of terminal branches and branch points of the motor axon were not significantly different between any of the groups observed (data not shown). This data contrasts with findings on the age-related change in branching characteristics, where it has been observed that 24-month-old mice contain axonal inputs in the diaphragm with significantly more branches compared to younger mice (Prakash & Sieck, 1988). Interestingly, this does not seem to be consistent amongst all muscles; while significant age-related axonal branching has also been observed in the EDL, this finding was not apparent in the soleus (Deschenes et al., 2013). These findings in the literature raise an important point about changes in motor axon, NMJ, and skeletal muscle integrity. Importantly, different muscles have been shown to be more susceptible to motor axon and NMJ impact, as well as associated skeletal muscle abnormalities in the context of aging and ALS (Valdez et al., 2012). As such, it is possible that differential susceptibility exists in different muscles in response to TS-induced motor axon, NMJ, and skeletal muscle impact as well. This point is discussed more in-depth in the Muscle-Specific Susceptibility to Motor Axon and NMJ Impact sub-section in the Limitations section of this paper.

Under air conditions, pre-synaptic axons were significantly larger in 16-wk mice compared to 8-wk mice, suggesting that healthy axonal growth occurred between the end of the 8-wk (at 23-wks-old) and 16-wk (~32-wks-old) exposure periods. Interestingly, 16 weeks of chronic TS exposure attenuated axonal growth, as indicated by similar pre-synaptic axon diameter measures between 8-wk TS and 16-wk TS mice. As such, pre-synaptic axon diameter was smaller in 16-wk
TS compared to 16-wk air mice (Fig 5b, 7). In summary, these findings implicate chronic TS exposure in the attenuation of pre-synaptic axon growth, and suggest that this may only arise following longer exposure periods, as 8-wk TS and 8-wk air mice maintained similar pre-synaptic axon diameters. While TS-induced stunted growth of pre-synaptic axon diameter is a novel finding, thinning of pre-synaptic axons has been observed in both aging and ALS mouse models (Jang & Van Remmen, 2010; Valdez et al., 2012).

**NMJ Morphology**

Impact at the NMJ is also accompanied by relatively consistent skeletal muscle abnormalities throughout muscle wasting conditions. These characteristics include reduced AChR “compactness”, driven by fragmentation of post-synaptic structures, reduced synaptic overlap, and denervation (Andonian & Fahim, 1987; Lyons & Slater, 1991; Frey et al., 2000; Fischer et al., 2004; Valdez et al., 2010; Butiföker et al., 2011; Chai et al., 2011; Valdez et al., 2012; Spendiff et al., 2016)

Similar values were observed when comparing absolute endplate, pre-synaptic nerve terminal, or AChR areas between all groups (Fig 8a - c). These findings are similar to those investigating age-related changes in NMJ morphology, where no differences in mean absolute size of endplate, pre-synaptic nerve terminal, or AChR areas have been observed (Deschenes et al., 2013). Interestingly however, age-related, muscle fibre type specific growth in AChR and pre-synaptic nerve terminal areas have been observed in mouse diaphragm (Prakash & Sieck, 1998). The current study lacks the appropriate measures to consider fibre type specific characteristics of motor axons and NMJs.
Synaptic overlap, described previously, was not impacted by chronic TS exposure in any of the groups (Fig 9). This is contrast to previous findings on aging mice, which have been found to contain an increased fraction of NMJs with reduced synaptic overlap.

No significant differences in synaptophysin compactness were observed as a result of chronic TS exposure (Fig 10a). This is consistent with findings on post-synaptic nerve terminal integrity with aging (Samuel et al., 2011; Deschenes et al., 2013). Reduced AChR compactness, or dispersal of AChR structures over a wider area, was driven by chronic TS exposure in the current study (two-way ANOVA). Multiple comparisons post hoc tests of AChR compactness revealed a significant reduction in 16-wk TS compared to 16-wk air mice, but only a trend when comparing 8-wk TS to 8-wk air mice (Fig 10b). These findings suggest that chronic TS exposure drives dispersal of AChR structures, and that the effect is progressive, becoming more significant after longer exposure lengths. Importantly, the current study observed no significant signs of AChR fragmentation as a result of chronic TS exposure (Fig 11a, b). In combination, these findings are particularly interesting, as previous studies have found that reduction in AChR compactness is driven by significant fragmentation in aged mice (Valdez et al., 2010; Valdez et al., 2012), mice with reduced muscle specific kinase (MuSK) signaling (Butiföker et al., 2011; Aare et al., 2016), and transgenic mice used to simulate motor neuron diseases (Lyons & Slater, 1991). As such, given that chronic TS exposure led to progressive dispersal of AChR structures in the absence of fragmentation, it is possible that this impact is a pre-cursor to fragmentation events, but that chronic TS exposure is a more mild stimulus that requires that longer periods of exposure to result in significant fragmentation of AChR structures. If correct, this would also implicate chronic TS exposure in the acceleration of fragmentation events occurring with age.
Chronic TS exposure significantly increased the fraction of NMJs with abandoned endplates (Fig 5c, 12). All 8-wk TS mice exhibited significant denervation, with up to 35% of NMJs being denervated in some individual mice. 67% of 16-wk TS mice exhibited significant denervation, with up to 20% of NMJs being denervated in some individual mice. Furthermore, denervation was not detected in any of the NMJs analyzed in the 8- and 16-wk air groups. As such, the current study illustrates that chronic TS exposure induced NMJ denervation in the diaphragm of most mice that are exposed. These findings on denervation are consistent with other models of muscle impact associated with NMJ degradation, including aged mice (Valdez et al., 2010; Valdez et al., 2012), mice with reduced MuSK activity (Aare et al., 2016), and transgenic mice used to simulate motor neuron diseases (Lyons & Slater, 1991; Valdez et al., 2012), which has been confirmed in human models of aging (Oda, 1984; Spendiff et al., 2016) and ALS (Fischer et al., 2004) as well.

Importance of TS-Induced Neuromuscular Impact in the Diaphragm

Patients with TS-related diseases experience severe muscle dysfunction that is associated with poor outcomes, and COPD-related diaphragm impairment leads to exacerbation of comorbidities, such as dyspnea and exercise intolerance (Mancini et al., 1992; Gray-Donald et al., 1996; Ribeiro et al., 2009; Yamaguti et al., 2009; Haruna et al., 2010; Elbehairy et al., 2016; Yamada et al., 2016) and increased risk of mortality (Killian & Jones, 1988; Meyer et al., 2001). Furthermore, the diaphragm muscle in COPD is forced to maintain a higher than normal workload to compensate for impaired breathing dynamics (Laghi & Tobin, 2003), while also being at a mechanical disadvantage due to chronic lung hyperinflation (De Troyer, 1997). As such, impaired diaphragm function as a result of TS-induced changes to motor axon and NMJ morphology would have significant effects on an already impaired muscle in patients with COPD. Furthermore, with
up to 45% of COPD patients actively smoking (Rinaldi et al., 2017), TS-induced neuromuscular impact and skeletal muscle dysfunction in the diaphragm is likely to continue to evolve throughout the progression of COPD pathology.

Synaptic connectivity is integral to skeletal muscle function, and motor neuron properties dictate the fibre type and contractile properties of the myocytes that they are innervating (Buller et al., 1960). Therefore, significant damage to NMJ structures results in severe consequences for affected skeletal muscle. NMJ degradation leads to motor unit remodeling, which is characterized by continuous degradation of NMJs and compensatory re-innervation of muscle fibres by surrounding axons (Hepple & Rice, 2016). While motor unit remodeling occurs periodically throughout adulthood without significant consequences, these repeated cycles of denervation and re-innervation result in consequentially large, overloaded motor units over time, which leads to motor neuron death (Tomlinson & Irving, 1977; Doherty et al., 1993; Dalton et al., 2008; Painter et al., 2014; Aare et al., 2016). Significant motor neuron death, and accompanied motor unit loss results in exhaustion of re-innervation capacity, whereby peripheral axons can no longer sprout and re-innervate muscle fibres with deteriorated NMJs. As such, muscle fibres become chronically denervated, whereby significant fractions of muscle fibres have NMJs with abandoned endplates that will not be re-innervated. This process is comprehensively described in the context of aging, in a review by Hepple & Rice (2016).

The current study is the first to resolve significant impact at the motor axon and NMJ in the diaphragm as a result of chronic TS exposure. While our findings illustrate that this impact can be seen in the absence of reduced muscle mass, this is in line with studies on age-related NMJ impact, which has been shown to precede skeletal muscle abnormalities (Deschenes et al., 2010). As such, NMJ impact is most likely a precursor to skeletal muscle impairment throughout age-
related muscle wasting. The current study indicates that chronic TS exposure likely has the ability to accelerate age-related motor unit remodeling and denervation, along with associated muscle dysfunction. This is illustrated in Figure 13, which is a hypothetical schematic that outlines TS-induced NMJ impact over time. As such, chronic TS exposure may not result in significant muscle wasting and dysfunction in young humans or mice; however, with advancing age TS-induced acceleration of motor unit remodeling is a likely mechanism that contributes to diaphragm, and global, skeletal muscle dysfunction in patients with TS-related diseases who are typically in the 7th decade of life or older.

**Limitations**

**Smoking Mouse Experiments**

There is currently a lack of consistency in both the procedural and reporting methods used by researchers that conduct animal tobacco smoking studies. Researchers generally report some combination of different data to illustrate what protocols were used. These data sets may include length and/ or frequency of exposures, number of cigarettes used throughout each exposure, digitalized data that tracked smoke particle density over time, or blood markers of TS exposure (i.e., the nicotine metabolite cotinine). Furthermore, not all data used to track smoking protocols are reported following experimental procedures, making it difficult to replicate and/ or compare smoke exposures with previous studies.

**Extrapolation of Results to Humans**

Animal chronic TS exposure studies, including this one, are difficult to extrapolate to human subjects. This is partly due to the vast differences in body composition, size, and metabolic rate between the two species. As well, mice in these studies are generally examined at an age that
is reflective of very young humans. Indeed, given that C57Bl/6 mice have an average lifespan of ~29-months-old (Kunstyr et al., 1975), beginning to expose 15-wk-old mice to TS for between 8 and 16 weeks in the current study, was approximately equal to a human beginning to smoke at ~10-years-old, with muscle biopsies taken at ~15 and 20 years of age. As discussed previously, the effects of chronic TS exposure on muscle most severely impact elderly patients with TS-related diseases such as COPD and chronic heart disease. As such, while the current study used an older cohort of mice compared to previous TS animal studies, these mice still do not reflect the age at which humans, who chronically smoke tobacco for decades, are most impacted by TS-induced respiratory muscle dysfunction. Furthermore, the mice used in the current study were still undergoing significant growth. This limits our ability to extrapolate TS-induced impact on skeletal muscle as a result of impaired growth, whole muscle atrophy, or both features simultaneously. As such, future research should examine the effects of chronic TS exposure on the diaphragm using studies that: (1) begin exposing mice that are fully grown, to eliminate the potential effect that chronic TS exposure has on growth, (2) expose mice to chronic TS for longer periods of time, to mimic the lifetime of smoking that humans affected by TS-related diaphragm dysfunction have engaged in, and (3) analyze morphological features of motor axons and the NMJ, as well as diaphragm characteristics, in older mice to more accurately illustrate the impact chronic TS exposure has on the diaphragm in conjunction with aging processes.

**Muscle-Specific Susceptibility to Motor Axon and NMJ Impact**

Previous, comprehensive examination of the effects of age and ALS conditions on muscle in a study by Valdez and colleagues (2012) revealed that various muscles show different levels of susceptibility to motor axon and NMJ damage. Indeed, this study found that extra-ocular muscles of mice undergoing aging and ALS conditions are almost completely saved from morphological
abnormalities of motor axons and NMJs that are observed in the EDL and soleus of the same animals. These features included, but were not limited to an increased fraction of NMJs that (1) contained thinned or swollen axons, (2) were fragmented, and (3) were denervated in response to aging. Importantly, there is also variability in the fraction of NMJs that are denervated when comparing different locomotor muscles such under these conditions. For instance, the EDL, TA, and soleus exhibit significantly different denervation responses compared to one another under conditions of both aging and ALS (Valdez et al., 2012). As such, without doing further assessments on the effect of chronic TS exposure on morphological characteristics of motor axons and NMJs in more muscles it is not possible to indicate whether the effects seen in the diaphragm in the current study would be found in all muscles of the same TS-exposed mice.

**Conclusion**

The objective of this study was to test the hypothesis that chronic TS exposure leads to significant motor axon and NMJ impact in the diaphragm of mice, and that this impact would progressively worsen from 8 to 16 weeks of chronic TS exposure. Our findings confirmed this hypothesis. Furthermore, these findings indicate that TS-induced motor axon and NMJ impact in the diaphragm most likely induce motor unit remodeling in the diaphragm, and that with continued TS exposure would further exacerbate motor unit remodelling that normally occurs with aging, potentially leading to early depletion of re-innervation capacity and resultant chronic denervation. As such, TS-induced neuromuscular impact most likely contributes to the diaphragm muscle abnormalities seen in patients with TS-related diseases that are associated with poor outcomes in these patients. Future investigation of TS-induced motor axon and NMJ impact in the diaphragm should seek to (1) evaluate older subjects, as these populations are most affected by TS-related skeletal muscle dysfunction throughout both age and TS-related diseases, and (2) seek to mitigate
TS-induced abnormalities at peripheral motor axons and the NMJ as a preventative approach to alleviating TS-induced skeletal muscle abnormalities that contribute to poor patient outcomes.
Figures

Figure 1. Change in body mass was significantly reduced as a result of chronic TS exposure (two-way ANOVA, p < 0.0005). Furthermore, change in body mass was significantly larger in 16-wk air mice compared to both 8-wk air and 16-wk TS mice (Tukey’s multiple comparisons test, p < 0.01 and p < 0.005, respectively).
Figure 2a - c. (a) Absolute epididymal fat mass was significantly reduced in 16-wk TS exposed mice compared to 16-wk air mice (unpaired t-test, p < 0.05). (b, c) No significant differences were observed between groups for spleen and liver mass (unpaired t-test, p = 0.65 and 0.101, respectively).
Fig 3. Age did not significantly impact muscle mass between air groups when normalized to controls (mean muscle mass of 8-wk air mice) (two-way ANOVA, p = 0.19).

a) 8-Week Muscle Mass Normalized to Controls

Fig 4a, b. (a) TS exposure significantly reduced muscle mass normalized to controls in 8-wk mice (two-way ANOVA, p < 0.0001). (b) TS exposure did not significantly impact muscle mass normalized to controls in 16-wk mice (two-way ANOVA, p = 0.87).
Figure 5a – c. Representative images of peripheral motor axons and NMJs in the diaphragm of mice. (a) A merged image of a healthy NMJ, with separate images of intact acetylcholine receptor (AChR, green), pre-synaptic nerve terminal (Synaptophysin, red), and motor axon (NF200, white) structures. (b) A peripheral motor axon with a large diameter (yellow arrow) relative to one with a reduced diameter (red arrow). (c) A merged image of an NMJ, with separate images detailing the presence of an intact AChR structure, but a lack of pre-synaptic nerve terminal (white arrow) and motor axon (green arrow) structures.
Figure 6a, b. (a) TS did not impact the mean number of axonal inputs in innervated NMJs in any group (two-way ANOVA, p = 0.69). (b) Fraction of NMJs lacking axonal input was significantly increased in TS-exposed mice from both 8 and 16-wk groups (two-way ANOVA, p < 0.05).
Figure 7. Mean absolute pre-synaptic axon diameter was significantly smaller in both 8-wk air and 16-wk TS mice when independently compared to 16-wk air mice (two-way ANOVA followed by Tukey’s multiple comparison test, each p < 0.05).

Figure 8a – c. No significant differences were observed between any groups when comparing mean absolute (a) endplate area, (b) pre-synaptic nerve terminal area, and (c) AChR area (two-way ANOVA, each p > 0.05).
Figure 9. No significant differences in synaptic contact were observed between groups (two-way ANOVA, p = 0.81).

Figure 10a, b. (a) No significant differences in synaptophysin compactness were observed between groups (two-way ANOVA, p = 0.13). (b) AChR compactness was significantly reduced in TS-exposed mice in both 8- and 16-wk groups (two-way ANOVA, p < 0.005), specifically in 16-wk TS compared to 16-wk air mice (Tukey’s multiple comparison test, p < 0.005).
Figure 11a, b. No significant differences were observed between any groups when comparing muscle endplate fragmentation, as characterized by (a) mean absolute number of AChR clusters per NMJ, or (b) fraction of NMJs with various degrees of AChR fragmentation (two-way ANOVA, p > 0.05).
Figure 12. Fraction of abandoned endplates was significantly increased in TS-exposed mice in both 8-wk and 16-wk groups (two-way ANOVA, p < 0.005).

Figure 13. A hypothetical schematic depicting chronic TS exposure leading to significant impact at the NMJ, accelerating age-related motor unit remodeling, exacerbation of re-innervation capacity, chronic denervation, and subsequent skeletal muscle dysfunction.
Conclusions and Implications

The current study is the first to assess the progressive impact of chronic TS exposure on morphological characteristics of peripheral motor axons and the NMJ in skeletal muscle, analyzing ~30 features of these structures in the diaphragm following 8 and 16 weeks of TS exposure. Furthermore, this study is the first to examine motor axon and NMJ characteristics in TS-exposed diaphragm muscle. As such, the current study offers novel insight into the impact that TS-induced motor axon and NMJ damage has on the diaphragm following different periods of exposure.

Under air conditions, pre-synaptic axons were shown to grow throughout the 16-week exposure period, as indicated by a significantly increased pre-synaptic axon diameter in 16-week air (~32-week-old mice) compared to 8-week air (~23-weeks-old) mice. Interestingly, this growth was attenuated throughout 16 weeks of chronic TS exposure, as 8-week TS and 16-week TS mice maintained similar pre-synaptic axon diameters. Furthermore, pre-synaptic axon diameter was smaller in 16-week TS mice compared to 16-week air mice. In summary, these findings implicate chronic TS exposure in the attenuation of pre-synaptic axon growth, and suggest that this may only arise following longer exposure periods, as 8-week TS and 8-week air mice maintained similar pre-synaptic axon diameters.

The current study’s findings illustrate that in response to chronic TS exposure, a higher fraction of NMJs in the diaphragm lacked axonal input, and contained endplates abandoned of overlapping nerve terminal structures. These findings implicate TS-induced denervation in the acceleration of age-related motor unit remodeling and early onset of motor neuron death, motor
unit loss, exacerbation of re-innervation capacity, and associated dysfunction in the diaphragm. This is based on the fact that transient NMJ degradation drives compensatory re-innervation of muscle fibres and motor unit remodeling (Hepple & Rice, 2016). Furthermore, this phenomenon would unfold concurrently with the development of debilitating TS-related pathologies, which independently compromise diaphragm function through impaired pulmonary dynamics and lung morphology. As such, the current findings suggest that TS-induced motor axon and NMJ impact in the diaphragm likely contributes to (1) respiratory muscle dysfunction in TS-related diseases, and (2) increased risk of co-morbidities and mortality in TS-related disease patients that experience respiratory muscle dysfunction.

A previous study in our lab examined the effect of 16 weeks of chronic TS exposure on hindlimb TA muscle in fully grown, adult mice (Míguez, 2017; McGill University graduate thesis; Kapchinsky et al.; under review with the Journal of Physiology). This previous study illustrated significant TS-induced denervation in mouse locomotor muscle, which was accompanied by global muscle atrophy in the diaphragm, gastrocnemius, plantaris, and soleus muscles. Together with the current study, these findings prompt a number of novel conclusions on the effect of chronic TS exposure on muscle. Firstly, TS-induced NMJ impact may occur in young tobacco smokers, in the absence of skeletal muscle dysfunction, which would only become significant throughout advanced age. This is evidenced by the fact that 16 weeks of chronic TS exposure in adult mice, but not in young mice, led to a significant reduction in respiratory and locomotor muscle mass when compared to age-matched controls. These findings further implicate TS-induced NMJ impact as an acceleration of age-related motor unit remodeling and associated skeletal muscle dysfunction. This is supported by the fact that TS-related skeletal muscle dysfunction primarily impacts older individuals with TS-related diseases. Secondly, chronic TS
exposure most likely impacts all skeletal muscle in the body, as several different muscles from these studies were impacted by TS-induced denervation. As such, TS-induced NMJ impact most likely contributes to abnormalities of all skeletal muscles in otherwise healthy tobacco smokers and patients with TS-related diseases. Importantly, locomotor muscles and respiratory muscles are each independently related to various co-morbidities and mortality in patients with COPD.

The current study implicates TS-induced motor axon and NMJ impact in TS-related diaphragm dysfunction for the first time, which can contribute to the development of preventative therapies targeting consequential diaphragm dysfunction in patients with TS-related diseases.

Future Directions

Tobacco smoking animal studies, including the current study, are limited in their ability to gauge the progressive impact that a lifetime of tobacco smoking, super-imposed on aging processes, has on human muscle. Given that TS-related diaphragm and locomotor muscle dysfunction most severely impacts older tobacco smoking individuals with TS-related diseases, it is important to evaluate chronic TS-induced motor axon and NMJ impact in diaphragm biopsy samples from these individuals, or in animal models that mimic these conditions. As such, future studies should increase the exposure length of chronic TS in mice, and evaluate skeletal muscle impact in advanced age. Such studies would provide more translatable findings to clinical populations impacted by TS-induced damage to neuronal and muscular structures.

Previous studies have comprehensively assessed how discrete age-related skeletal muscle characteristics are related to markers of denervation. For example, Rowan and colleagues (2011), implicated denervation in age-related muscle atrophy, up-regulation of the proteolytic proteasome pathway, and increased abundance of MHC co-expressing muscle fibres, by staining muscle cross-sections with Nav1.5, a marker of denervation (Yang et al., 1991). Future studies should conduct
similar assessments on muscle cross-sections in otherwise healthy smokers and patients with TS-related diseases to more comprehensively assess the impact that TS-induced motor axon and NMJ impact has on discrete skeletal muscle characteristics in these populations.

It is crucial to assess the mechanisms by which chronic TS exposure may be impacting motor axon and NMJ morphology. Given that TS-induced NMJ impact is a recent discovery, there is currently no research on proposed mechanisms that drive this incident. Future studies may focus on harmful events that are known to occur in muscle as a result of chronic TS exposure, including increased abundance of damaging oxidants (Carlos et al., 2014), increased inflammation (Martins et al., 2017), or up-regulation of the proteasome proteolytic pathway (Caron et al., 2013; Bowen et al., 2017). Indeed, recent findings have resolved that proteasome proteolytic pathway proteins are not only up-regulated in muscles, but also at the NMJ in various muscle wasting conditions (Rudolf et al., 2016). As such, examining deleterious pathways that are up-regulated in response to chronic TS exposure in the context of motor axon and NMJ morphology, may provide more insight into the mechanism by which TS impacts these neuromuscular structures.

Finally, future studies should focus on therapeutic interventions for TS-induced motor axon and NMJ damage. NMJ impact has been shown to precede age-related changes in skeletal muscle (Deschenes et al., 2013), and the current study provides evidence that motor axon and NMJ impact resulting from 16 weeks of chronic TS exposure occurs in the absence of reduced muscle mass in mice. Given these findings, interventions that promote the structural integrity of motor axons and NMJs prior to the onset of TS-induced skeletal muscle dysfunction may provide meaningful, preventative therapies for individuals impacted by TS-related skeletal muscle abnormalities.
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