Respiratory muscle weakness in the Zucker diabetic fatty rat

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The obesity epidemic is considered one of the most serious public health problems of the modern world. The World Health Organization defines overweight (BMI ≥ 25) as abnormal or excessive fat accumulation. Prevalence has nearly doubled in the last 25 years with more than 10% of the global, adult population now considered obese (45). Obesity is a major risk factor for Type 2 diabetes mellitus (21, 32, 35), cardiovascular disease (20, 24), osteoarthritis (3), and cancer (12, 23). Although obesity is often preventable and treatable with physical activity, compliance is a major obstacle owing, at least in part, to dyspnea and exercise intolerance. This is further propagated by obesity-induced impairments in muscle metabolism, resulting in reductions in muscle strength and mass (33, 37, 40). However, despite the importance of exercise therapy, the mechanism behind these morphological changes and the resulting exercise intolerance remains unknown.

Skeletal muscle wasting occurs in many chronic diseases [e.g., cancer, chronic obstructive pulmonary disease (COPD), heart failure] and with age. Loss of skeletal muscle mass (i.e., cachexia) is exceedingly prevalent in cancer patients, distinct from treatment side effects (e.g., from chemotherapy, surgery) (22, 41). Skeletal muscle loss has been identified as a strong, independent predictor of mortality in patients with COPD (14) and heart failure (4). Wasting is also correlated with reductions in exercise performance, dyspnea, and reduced quality of life (1, 17, 27). Similarly, in aging, loss of skeletal muscle reduces exercise performance (42), an outcome that is exacerbated when combined with obesity (33). Importantly, loss of skeletal muscle mass also occurs in adolescent obesity in animal models (37, 43), thought to be the result of disuse and increased sedentary behavior. Obesity is associated with worsened disease pathogenesis and exercise intolerance, further supporting the urgency with which we need to identify the mechanism of skeletal muscle wasting.

Myostatin is a member of the transforming growth factor-β family and is a negative regulator of skeletal muscle growth. In health, myostatin is expressed primarily in skeletal muscles, although expression is also detected in the heart and adipose tissue (29, 36). Overexpression of myostatin results in significant loss of skeletal muscle (46), while genetic deletion results in increased muscle mass (44), with no impact on overall health. Antimyostatin strategies ameliorate muscle wasting in aging (31), Duchenne’s muscular dystrophy (10), and heart failure (18). In heart failure, where cachexia is a major contributor for exercise intolerance, cardiac-specific deletion of myostatin prevents the development of peripheral muscle wasting, suggesting that, in pathological states, skeletal muscle is not a main contributor to serum myostatin levels (18). Myostatin levels are also elevated in the serum of obese children, as well as in muscle fibers of insulin-resistant obese women (15, 19). In obese, transgenic mice, activation of the myostatin pathway is found in both adipose and skeletal muscle (2). Taken together, these data support a potential role for myostatin signaling in the development of obesity-induced skeletal muscle loss.

Current investigations of skeletal muscle loss in obesity are restricted almost exclusively to limb muscles. However, obese patients also have reduced inspiratory performance, suggesting skeletal respiratory muscles may be affected (13). Furthermore, respiratory muscle dysfunction is an underdiagnosed cause of dyspnea, a prominent symptom of exercise intolerance observed in obese patients (28). The diaphragm is a skeletal muscle with both voluntary and involuntary inputs and is the main muscle involved in inspiration. Importantly, the diaphragm provides a unique opportunity to investigate a skeletal muscle.
mature, at pH 7.4. A triangular section of muscle was removed from the costal portion of the right hemidiaphragm with rib and central tendon intact. 5-0 silk suture ties were secured to the central tendon and ribs and attached to a force transducer (Grass Instruments, Warwick, RI) and stationary hook, respectively. Stainless-steel stimulating electrodes were then placed on either side of the muscle section connected to the transducer. Tissue baths were then placed so that the samples were immersed in Krebs-Henseleit buffer, maintained at 27°C and pH 7.4. Following a 45-min thermodiuretation period, muscle strips were stretched using a micromanipulator and stimulated at 100 Hz and 250-ms train duration; the resultant force production was measured. This was repeated until force production plateaued. This was determined to be optimal length, and the muscle length was measured from the bony insertion point on the ribs to the central tendon attachment point. Once determined, muscles were stretched to optimal length, and force production and rate of force production were determined during a force-frequency test. Contractions were stimulated every 30 s with 250-ms train duration at frequencies of 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 Hz. Isometric contractility data were collected on AcqKnowledge software (Biopac Systems, Goleta, CA) and analyzed using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Force production and contractility were normalized to physiological cross-sectional area by dividing muscle weight (mg) by the product of optimal length (cm) and assumed muscle density (1.06 g/ml).

**Histological analysis.** Diaphragm strips were excised and relaxed in 50 mM KCl and fixed in 10% neutral buffered formalin (VWR International, Mississauga, ON, Canada) for 24 h. Samples were then dehydrated in xylene (Fisher Scientific, Ottawa, ON, Canada) and embedded in paraffin wax. Five-micrometer sections were mounted on charged 1.2-mm Superfrost slides (Fisher Scientific) and stained using Picrosirius red. Sections were imaged using an Olympus FSX 100 light microscope and images were acquired using Cell Sense software (Olympus, Tokyo, Japan). Percent fibrosis was expressed as the ratio of collagen-positive areas (red) to total muscle area in six random fields for each animal.

**Determination of gene expression.** RNA was prepared from samples with TRIzol reagent, according to the manufacturer’s instructions.

### Table 2. The ZDF phenotype imposes deleterious alteration in morphometric and hemodynamic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lean</th>
<th>ZDF</th>
<th>( \Delta )</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphometrics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>307±21</td>
<td>386±20*</td>
<td>&lt;0.001</td>
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<tr>
<td>EDL weight, mg</td>
<td>149±4</td>
<td>125±12*</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>Soleus weight, mg</td>
<td>147±12</td>
<td>130±12*</td>
<td>0.017</td>
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<tr>
<td>Fasting blood measurements</td>
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<td></td>
<td></td>
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<tr>
<td>Glucose, mmol/l</td>
<td>4.4±0.7</td>
<td>12.2±5.2*</td>
<td>0.001</td>
<td></td>
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<tr>
<td>Insulin, pmol/l</td>
<td>258±207</td>
<td>952±510*</td>
<td>0.003</td>
<td></td>
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<tr>
<td>Hemodynamic parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>112±3</td>
<td>133±7*</td>
<td>0.024</td>
<td></td>
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<tr>
<td>Diastolic BP, mmHg</td>
<td>84±2</td>
<td>95±6</td>
<td>0.153</td>
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<tr>
<td>( dp/dt_{max}, ) mmHg/s</td>
<td>7410±214</td>
<td>8507±687*</td>
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<tr>
<td>( dp/dt ) at LVP40, mmHg/s</td>
<td>6350±140</td>
<td>6700±540*</td>
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<tr>
<td>( dp/dt ) at 1/s, mmHg/s</td>
<td>-9163±287</td>
<td>-8630±923*</td>
<td>0.612</td>
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<tr>
<td>( dp/dt_{max}, ) MAP, 1/s</td>
<td>-98±4</td>
<td>-79±3*</td>
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<tr>
<td>EDP, mmHg</td>
<td>4.3±0.5</td>
<td>11.5±2.0*</td>
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<tr>
<td>HR, bpm</td>
<td>344±8</td>
<td>317±16</td>
<td>0.173</td>
<td></td>
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</table>

Morphometric and serum values are expressed as means ± SD. Hemodynamic values are expressed as means ± SE. \( n \), number of animals; ZDF, Zucker diabetic fatty rat; EDL, extensor digitorum longus; BP, blood pressure; \( dp/dt_{max}, \) maximal rate of change of systolic pressure during systole; \( dp/dt_{max}, \) maximal rate of change of diastolic pressure during diastole; LVP, left ventricular pressure; MAP, mean arterial pressure; EDP, end diastolic pressure, HR, heart rate. * \( P < 0.05 \) compared to lean, as determined by a two-tailed Student’s \( t \)-test.

### Table 1. Sequence Information

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>GenBank Accession Number</th>
<th>( T_m ), °C</th>
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<td>Myostatin</td>
<td>5'-ACGCTACACGCGAAACAACTC-3'</td>
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<tr>
<td>( \beta )-actin</td>
<td>5'-CACTAGTCAAGGCGAAAGTCCTC-3'</td>
<td>NM_031144.3</td>
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<tr>
<td>GAPDH</td>
<td>5'-TGATTTCACCGCCGCAAGT-3'</td>
<td>NM_017008.4</td>
<td>51</td>
</tr>
</tbody>
</table>

\( T_m \), melting temperature.
(Invitrogen, Life Technologies, Burlington, ON, Canada). Concentrations of isolated RNA were quantified using a spectrophotometer (NanoDrop, ND1000, Thermo Scientific, Mississauga, ON, Canada) prior to cDNA synthesis. Generation of cDNA was completed using SuperScript II reverse transcriptase (Invitrogen, Life Technologies), according to the manufacturer’s instructions. Amplification was completed using PCR (35 cycles) with primers for myostatin and housekeeping genes, as described in Table 1. Amplification products were run on ethidium bromide-stained agarose gels and quantified.

Protein biochemistry. Following excision, muscle samples were immediately frozen in liquid nitrogen and stored at −80 °C prior to analysis. Heparinized blood samples were collected via cardiac puncture, centrifuged, and plasma was stored at −80 °C prior to analysis. Muscles were homogenized using glass mortar and pestle in homogenizing buffer with a protease inhibitor cocktail. Protein samples were diluted with standard Laemmli sample buffer and normalized to actin to ensure equal protein loading. Plasma samples were diluted with standard Laemmli sample buffer and normalized to actin to ensure equal protein loading. Plasma samples were diluted with a modified sample buffer (3.3 g/l SDS, 3.3 g/l CHAPS, 3.3 g/l NP-40, 0.1 mol/l dithiothreitol, 1.4 mol/l urea, and 50 mmol/l Tris·HCl, at pH 6.8) and boiled for 5 min. Samples were separated electrophoretically via SDS-PAGE (5% stacking, 12.5% resolving gel) and either stained with Coomassie or transferred to nitrocellulose membrane (Hybond 0.1 mol/l dithiothreitol, 1.4 mol/l urea, and 50 mmol/l Tris·HCl, at pH 7.4). Membranes were blocked (Roche, Indianapolis, IN) and incubated with primary antibodies overnight at 4°C: anti-myostatin antibody (generated in-house to detect only the active form of myostatin); anti-myosin heavy-chain all fibers (A4.1025), slow fibers (BA-D5), and fast fibers (N3.36) (Developmental Studies Hybridoma Bank, Iowa City, IA); skeletal troponin I fast (FI32) and slow (MYNT-S) fibers; p-SMAD3 (Ser-465/467) (cat. 3101; Abcam, Toronto, ON, Canada); SMAD2 (cat. 5339; Cell Signaling, Danvers, MA); p-SMAD3 (cat. 9520; Cell Signaling); SMAD3 (cat. 9523; Cell Signaling); and β-actin (cat. ab8227; Abcam). Membranes were washed and subsequently incubated with corresponding secondary alkaline phosphate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). All antibody dilutions and membrane washes were completed in 1% PBS with Tween. Signal was detected through chemiluminescence (Immun-Star, Bio-Rad, Mississauga, ON, Canada) and a digital imaging system (Gel Logic Pro 6000, Carestream, Rochester, NY). Densitometry quantification was completed using open-access ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis. Values presented are means ± SE, unless otherwise indicated. Graphical and statistical analyses were completed using GraphPad (Prism 6; GraphPad Software, La Jolla, CA). The distribution of cross-sectional area was analyzed using a Kolmogorov-Smirnov test for one-dimensional probability distributions, and two-tailed, unequal Student’s t-tests were used for all comparisons, with the exception of maximal occlusion pressures, which was analyzed using a one-tailed Student’s t-test. Differences were considered significant at P < 0.05.

RESULTS

Characterization of the ZDF phenotype. Prior to investigating the effects of respiratory muscle dysfunction in the ZDF rat, we first confirmed the development of overt diabetes in our model. ZDF rats had increased body weight, increased fasting blood glucose levels, and decreased insulin sensitivity compared with lean controls (Table 2). ZDF rats also had peripheral muscle atrophy with decreased weights of slow (soleus) and fast- (extensor digitorum longus; EDL) twitch skeletal muscles (Table 2). Hemodynamic parameters confirmed the presence of hypertension with increased systolic blood pressure in ZDF rats with no difference in the maximal rate of contraction (dp/dt\text{max}) (Table 2). No differences were noted in either diastolic blood pressure or the maximal rate of relaxation (dp/dt\text{min}). However, reductions were observed when dp/dt\text{min} was normalized to mean arterial pressure (MAP), to account for the observed increase in afterload (i.e., systolic blood pressure; Table 2). Increased end-diastolic pressure (EDP) with decreased dp/dt\text{min} is indicative of diastolic dysfunction (Table 2).
Evidence of diaphragm weakness and atrophy in the absence of in vitro functional impairment. We chose to investigate the function and morphology of the diaphragm, a constitutively active muscle to remove disuse atrophy as a potential confounding variable as, while physical activity has not been specifically investigated in the ZDF strain, previous studies have identified that the obese Zucker strain has decreased spontaneous activity and wheel activity, compared with lean controls (38, 39). Functional analysis of the diaphragm revealed that baseline esophageal pressures were not different between ZDF and lean controls during eupneic breathing (Fig. 1A); however, maximal pressures following a 30-s tracheal occlusion were significantly reduced in ZDF rats ($P = 0.0313$) (Fig. 1, B–D). To determine whether diaphragmatic weakness was the result of intrinsic muscle impairment, we compared in vitro diaphragm contractile function between ZDF and lean controls. In vitro assessment revealed no differences in diaphragm fatigability or force production between ZDF and lean controls (Fig. 2).

We then examined the morphology of the diaphragm via histology and protein biochemistry. We analyzed histological sections of the diaphragm using Picrosirius red staining, which revealed increased fibrosis ($P = 0.0001$) (Fig. 3, A and B), and atrophy ($P < 0.0001$) (Figs. 3, A and C) in ZDF rats. Protein analysis revealed decreased myosin heavy-chain content in the diaphragm of ZDF rats ($P < 0.001$), with no evidence of fiber type shifting (Fig. 4). Interestingly, a proteomic study profiling myostatin-induced changes in C2C12 cells reported ~50% of all mRNA changes were within the myosin heavy-chain family with up to 80% reduction in expression, which is consistent with our contention that increases in myostatin signaling contributes to diaphragmatic atrophy (26). Furthermore, there is a linear relationship between specific force and myosin concentration, such that proportional loss of myosin heavy chain and physiological cross-sectional area, as reported here, do not result in a change in specific force (11). Taken together, these data suggest increased muscle atrophy via myosin heavy-chain loss in the diaphragm of ZDF rats.

Upregulation of the myostatin pathway in ZDF animals. On the basis of previously reported findings of increased myostatin expression in chronic diseases (18), we chose to investigate myostatin signaling in the ZDF rat. Western blot analysis revealed increased myostatin protein levels in the serum of ZDF rats ($P = 0.017$), compared with lean controls (Fig. 5A). This was further supported by gene analysis, which revealed
increased myostatin mRNA expression in the diaphragm \((P = 0.030)\), slow-twitch skeletal muscle (soleus) \((P = 0.005)\), retroperitoneal \((P = 0.03)\), and subcutaneous adipose depots (Fig. 5B). Subcutaneous adipose tissue levels of myostatin mRNA are not presented in Fig. 5B due to undetectable levels in control animals. No differences were noted in the expression of myostatin in the heart, fast-twitch skeletal muscle (EDL), or epididymal adipose depot.

Myostatin, and other members of the TGF-β family, have been previously shown to activate myogenic transcription factors (e.g., MyoD) via activation of the SMAD2 and SMAD3 phosphorylation pathway (43). Thus, we chose to also investigate SMAD2/3 protein content and phosphorylation levels in the diaphragm of ZDF rats. While there were no differences in the protein levels of SMAD2 or SMAD3 between ZDF and lean controls, phosphorylation of SMAD2 was increased 32% in ZDF rats \((P = 0.048)\) (Fig. 6). No differences were observed in the phosphorylation of SMAD3 (Fig. 6). These data suggest that, in the diaphragm, muscle atrophy may be induced by increased myostatin expression (both locally and systemically), resulting in increased SMAD2 phosphorylation and subsequent activation of myogenic transcription factors.

**DISCUSSION**

Here, we have identified atrophy as the cause for respiratory muscle dysfunction in an obese, diabetic model, a novel finding not previously reported. We demonstrated that, in the obese ZDF rat, the diaphragm undergoes detrimental morphological changes, including atrophy, fibrosis, and decreased myosin heavy-chain levels (Figs. 3 and 4), resulting in diaphragmatic weakness (Fig. 1). These changes are accompanied by activation of the myostatin pathway (Figs. 5 and 6). Importantly, while myostatin expression is increased in the diaphragm, expression is also increased in two different adipose depots (retroperitoneal and subcutaneous) and in the serum (Fig. 5). Previous work from our laboratory has also demonstrated depot-specific responses in the adipose tissue in an obese, diabetic model (8). This implies that myostatin may have a systemic role in obesity and diabetes, potentially originating from a nonskeletal muscle source.

While nonskeletal muscle sources of myostatin are known, their functional significance in pathophysiology and health has yet to be determined. Myostatin expression in adipocytes was originally identified, alongside skeletal muscle, suggesting it has a physiological role in health (29). In addition to increased muscle mass, myostatin knockout mice display reductions in fat accumulation, increased insulin sensitivity, and decreased blood glucose levels when fed either a standard chow or a high-fat diet (30). However, inhibition of myostatin signaling in adipose tissue, via activin IIb, has no effect on body composition, insulin sensitivity, or glucose levels at baseline or on a high-fat diet (30). The majority of studies on adipose-derived myostatin focus on obesity. Obesity is a disease borne of positive energy balance and is, thus, not an ideal model for the study of healthy, adipose physiology. We would expect to find that adipose-derived myostatin is involved in adipose-skeletal muscle signaling. It would seem logical to hypothesize that the function of this isoform could be to balance fuel storage (i.e., adipose) and fuel utilization (i.e., muscle size), which would be most relevant in periods of limited food availability where large muscle size would be disadvantageous.
However, there are no studies examining myostatin signaling in a model where food supply is limited. While the physiological significance of cardiac- and adipose-derived myostatin remains elusive, pathological dysregulation occurs in heart failure (18), and obesity, as reported in this article. In heart failure, while cardiac-derived myostatin does not contribute to the systemic regulation of skeletal muscle mass in health, cardiomyocytes overproduce myostatin, resulting in skeletal muscle wasting (18). This represents a commonality with our study as, following pathological insult, myostatin production becomes deranged, resulting in upregulation of systemic contribution from nonskeletal muscle sources. This suggests that, in our model, local production of myostatin from the diaphragm maintains muscle mass, but following pathological insult, nonskeletal muscle sources (i.e., adipose) become deranged, resulting in systemic overproduction and worsened pathology. Additional work must be completed to determine the functional significance of nonskeletal muscle-derived myostatin to determine potential therapeutic targets for reversing pathology-induced skeletal muscle wasting.

Exercise intolerance and dyspnea in obese patients is further exacerbated due to changes in respiratory mechanics. Excessive adipose deposition around the thoracic cage and abdomen reduces end expiratory lung volume, expiratory reserve volume, forced expiratory volume, functional residual capacity, and total lung capacity (9, 34). Although respiratory muscle function has not been fully investigated in obese patients with Type 2 diabetes, preliminary data indicate obese diabetic women have reduced respiratory muscle strength and reduced maximal inspiratory pressure compared with obese, nondiabetic controls (5). Indeed, we also see changes in respiratory mechanics in this study, which are due, at least in part, to morphological changes in the diaphragm (Figs. 1 and 3). In our study, we show that the diaphragm undergoes atrophy and fibrosis, which is contributing, at least in part, to reduced pressure generation in response to tracheal occlusions (Figs. 1 and 3). However, Zucker rats have blunted ventilatory responses to hypoxia (16). A decreased ventilatory response to hypoxia can be attributed to either a central (i.e., decreased neural drive) or peripheral (i.e., diaphragm weakness/dysfunction or mechanical alterations) issue or any combination of the two. While there was no difference in timing or breathing frequency between lean and ZDF at baseline or during occlusions, this does not exclude the possibility of impairments in

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**Fig. 4.** Myosin heavy-chain content in ZDF diaphragm. Representative Coomassie blue gel of lean and ZDF diaphragm tissue (A). Myosin heavy-chain protein expression in lean and ZDF diaphragms (B). While myosin heavy-chain protein content was reduced in ZDF diaphragms, there was no evidence of fiber type shifting in either myosin heavy-chain or skeletal troponin I (C). \( *P < 0.05 \) compared with lean, as determined by a two-tailed Student’s \( t \)-test \((n = 4 \text{ or } 5)\).
the other component of central drive, it does suggest a reduced neural drive may not be present in our model. Indeed, our model may not directly be comparable to the work of Farkas and Schlenker (16), as they used older animals with a more severe obese phenotype.

Exercise intolerance and dyspnea are the major obstacles, and an actual limitation, to rehabilitation. Improvements in ventilatory capacity are essential for empowering many obese patients with the ability to engage in an exercise rehabilitation program. Hence, overcoming respiratory limitations to exercise tolerance is vital to implementation of proven exercise intervention strategies. We believe respiratory muscle dysfunction is a major factor limiting exercise capacity, yet we know almost nothing about this muscle in obesity. Our work is the first to identify respiratory muscle dysfunction in obesity and provide insight into a potential mechanism of action through adipose-derived myostatin. This highlights an overlooked but central muscle that requires a targeted approach to maximize exercise-associated benefits, reduce adiposity, and improve quality of life. Further investigation into obesity-induced respiratory muscle dysfunction will provide knowledge that could be readily translated to patients and physicians treating obese, prediabetic and Type 2 diabetes mellitus patients. Additional studies will examine the potential to induce regression of respiratory muscle dysfunction at more advanced stages of diabetes and with age. This is important, as many patients seek medical treatment in advanced stages of the disease, as primary and secondary complications arise symptomatically. The findings of this study highlight a need to complete our understanding of the muscle-related pathophysiology of obesity and highlight a potential therapeutic target in the diaphragm, especially considering antimyostatin preclinical trials are already under way.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
REFERENCES


