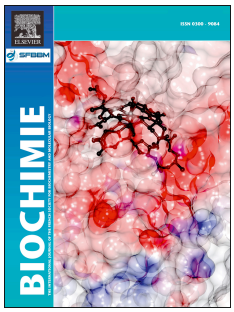

EFFECTS OF THE BETA$_2$ AGONIST FORMOTEROL ON ATROPHY SIGNALING, AUTOPHAGY, AND MUSCLE PHENOTYPE IN RESPIRATORY AND LIMB MUSCLES OF RATS WITH CANCER-INDUCED CACHEXIA

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ABSTRACT

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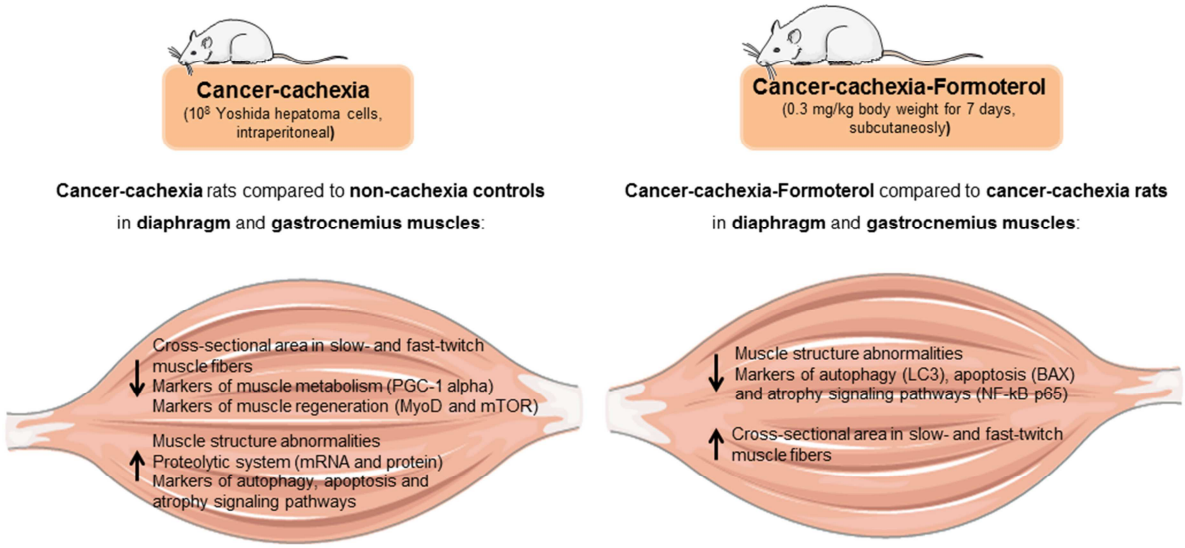
GRAPHICAL ABSTRACT



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ABSTRACT

Muscle mass loss and wasting are characteristic features of patients with chronic conditions including cancer. Beta-adrenoceptors attenuate muscle wasting. We hypothesized that specific muscle atrophy signaling pathways and altered metabolism may be attenuated in cancer cachectic animals receiving treatment with the $beta_2$ agonist formoterol. In diaphragm and gastrocnemius of tumor-bearing rats (intraperitoneal inoculum, 10^8 AH-130 Yoshida ascites hepatoma cells, 7-day study period) with and without treatment with formoterol (0.3 mg/kg body weight/day/7days, subcutaneous), atrophy signaling pathways (NF-κB, MAPK, FoxO), proteolytic markers (ligases, proteasome, ubiquitination), autophagy markers (p62, beclin-1, LC3), myostatin, apoptosis, muscle metabolism markers, and muscle structure features were analyzed (immunoblotting, immunohistochemistry). In diaphragm and gastrocnemius of cancer cachectic rats, fiber sizes were reduced, levels of structural alterations, atrophy signaling pathways, proteasome content, protein ubiquitination, autophagy, and myostatin were increased, while those of regenerative and metabolic markers (myoD, mTOR, AKT, and PGC-1alpha) were decreased. Formoterol treatment attenuated such alterations in both muscles. Muscle wasting in this rat model of cancer-induced cachexia was characterized by induction of significant structural alterations, atrophy signaling pathways, proteasome activity, apoptotic and autophagy markers, and myostatin, along with a significant decline in the expression of muscle regenerative and metabolic markers. Treatment of the cachectic rats with formoterol partly attenuated the structural alterations and atrophy signaling, while improving other molecular perturbations similarly in both respiratory and limb muscles. The results reported in this study have relevant therapeutic implications as they showed beneficial effects of the $beta_2$ agonist formoterol in the cachectic muscles through several key biological pathways. **Word count: 250**

KEY WORDS: cancer-induced cachexia; diaphragm and gastrocnemius; formoterol treatment; muscle atrophy signaling; proteolytic and autophagy markers; metabolic pathways

LIST OF ABBREVIATIONS

AKT: serine/threonine kinase 1

ANOVA: analysis of variance

ATP: adenosine triphosphate

BAX: BCL2 associated X protein

BCL-2: B-Cell CLL/Lymphoma 2

cAMP: cyclic adenosine monophosphate

COPD: chronic obstructive pulmonary disease

ERK: extracellular signal-regulated kinases

FoxO: forkhead box O

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

IkB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

LC: lung cancer

LC3B: microtube-associated protein 1 light chain 3

MAPK: mitogen-activated protein kinases

mTOR: mammalian target of rapamycin

MuRF-1: muscle ring finger protein-1

MyHC: myosin heavy chain

MYOD: myoblast determination protein 1

NF: nuclear factor

P62: nucleoporin p62

PGC: peroxisome proliferator-activated receptor gamma coactivator

PPAR: peroxisome proliferator-activated receptor

SPSS: statistical Package for the Social Science

TRIM32: tripartite motif-containing protein 32

TUNEL: terminal deoxynucleotidyl transferase-mediated uridine 5’-triphosphate nick-end labelling

UTP: uridine 5’-triphosphate

1. INTRODUCTION

Muscle mass loss and dysfunction are common systemic manifestations in patients with chronic conditions such as chronic obstructive pulmonary disease (COPD), heart and renal failure, diabetes, and cancer [1-3,21-23,61,62]. In patients with critical illness, severe muscle wasting also takes place very rapidly, impairing the patients' outcome [21]. Importantly, muscle wasting and atrophy of the lower limb muscles entails a reduction of the patients' quality of life and has been shown to predict morbidity and mortality regardless of the underlying condition [3,22,23,34,37,42,52,55].

Biological mechanisms such as increased oxidative stress, epigenetic regulation, metabolic derangements, sarcomere disruptions, contractile protein loss, autophagy, enhanced proteolysis, and specific signaling pathways have been demonstrated to be involved in the pathophysiology of muscle wasting associated with chronic diseases including cancer [2,4-6,8,14-17,24-27,35,36,43-46]. Mitogen-activated protein kinases (MAPK) and nuclear factor (NF)-κB are central regulators of a great variety of cellular processes including adaptive and maladaptive responses to cellular stress in skeletal muscles [32]. Activation of MAPK, NF-κB, and forkhead box O (FoxO) transcription factors was shown to mediate muscle wasting in an experimental model of cancer-induced cachexia [13,15,40]. Indeed, treatment of the cachectic mice with either NF-κB or MAPK inhibitors significantly improved muscle mass loss as a result of an attenuation of muscle proteolysis [15].

The ubiquitin-proteasome system has been shown to degrade muscle proteins in experimental models of cancer-induced cachexia [15,54,59,60] and disuse muscle atrophy [16]. Interestingly, the proteasome inhibitor bortezomib was shown to restore diaphragm muscle contractile function and myosin heavy chain content (MyHC) following coronary heart failure [59] and elastase-induced emphysema [60] in mice. In a model of cancer-induced cachexia, however, bortezomib did not significantly attenuate muscle mass or function loss [15].

In skeletal muscles, other cellular pathways can also be targeted for the treatment of cachexia. For instance, canonical beta-agonist signaling improves muscle metabolism through the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), that activates protein kinase A [49]. In skeletal muscles, the proportions of $beta_2$ receptors are significantly more abundant than those of $beta_1$- and $beta_3$-adrenoceptors [63]. Part of the beneficial effects induced by beta-agonist signaling involves a decrease in protein degradation, an increase in protein anabolism or both [49]. Interestingly, skeletal muscle growth can be achieved following treatment with beta-adrenoceptor agonists when administered at higher doses than those normally used for the treatment of airways diseases such as COPD [49]. In this regard, the long-acting $beta_2$ agonist formoterol, which has a rapid onset of action, while maintaining a long duration of action, has been shown to induce significant beneficial effects on skeletal muscles through several biological mechanisms [10-12,57]. Whether atrophy signaling may be attenuated by treatment with formoterol as another beneficial effect of $beta_2$-adrenoceptor therapy in muscles deserves further attention in models of cachexia.

On this basis, we hypothesized that signaling pathways that lead to muscle mass loss and altered metabolism may be attenuated in cancer cachectic animals receiving treatment with the $beta_2$ agonist formoterol. In the current investigation, experiments on the diaphragm muscle have also been conducted as the main respiratory muscle. This approach enabled us to elucidate whether the profile of the target biological events may be influenced by the activity of the muscles. Accordingly, the study objectives were defined as follows: 1) to analyze levels of expression of signaling pathways such as NF-κB, MAPK, and FoxO and proteolytic markers, 2) to determine expression levels of autophagy markers, 3) to assess levels of transcription factors involved in muscle mass maintenance and metabolism, and 4) to investigate structural features in both diaphragm and gastrocnemius muscles of cachectic rats

bearing the Yoshida ascites hepatoma cells [10-12,26,27,57,58] in response to treatment with formoterol.

2. MATERIALS AND METHODS

(Detailed information on all methodologies is described in the online supplementary material)

2.1. Animal experiments

2.1.1. Experimental design.

Male Wistar rats (5 weeks, 130-165 grams, Interfauna, Barcelona, Spain) were used for the purpose of the investigation. Animals were randomly subdivided into four groups (N=10/group): 1) non-cachexia controls, 2) non-cachexia controls treated with formoterol (non-cachexia control-F), 3) cancer-cachexia rats, and 4) cancer-cachexia rats treated with formoterol (cancer-cachexia-F). Cachexia was induced as a result of an intraperitoneal inoculum of 10^8 AH-130 Yoshida ascites hepatoma cells, which were obtained from tumors in exponential growth as previously described [56]. Formoterol treatment was administered daily during the study period (seven days) subcutaneously (0.3 mg/kg body weight/24 hours, dissolved in physiological solution) and the non-treated animals received the corresponding volume of physiological solution subcutaneously.

All animal experiments were conducted at *Facultat de Biologia, Universitat de Barcelona (Barcelona)*. This was a controlled study designed in accordance with both the ethical standards on animal experimentation in our institution (EU 2010/63 CEE and *Real Decreto* 53/2013 BOE 34, Spain) and the Helsinki convention for the use and care of animals. All experiments were approved by the Institutional Animal Research Committee (*Universitat de Barcelona*).

2.2. In vivo measurements in the animals

Food and water were administered to the rats for the entire duration of the study. All the animals were maintained at a temperature of 22 ± 2 °C with a regular light-dark cycle (lights were on from 08:00 a.m. to 08:00 p.m.) and had free access to food and water. Body weight was determined in all animals on day 0 and immediately prior to their sacrifice on day 7. Tumor weights were determined in all animals upon sacrifice. The percentage of body weight gain at the end of the study period was calculated as follows: [(body weight on day 7 – tumor weight on day 7) – body weight on day 0]/ body weight on day 0 x 100.

2.3. Sacrifice and sample collection

On day 7 after tumor transplantation, the rats were weighed and anesthetized with an intraperitoneal injection of 3:1 ketamine/xylazine mixture (Imalgene 1000, Rhone Merieux, France and Rompun®, Bayer AG, Leverkusen, Germany, respectively). In all animals, the pedal and blink reflexes were evaluated in order to verify total anesthesia depth. Each tumor was harvested from the peritoneal cavity, and the volume and cellularity were evaluated. The diaphragm and gastrocnemius muscles were obtained from all the rats. In all samples, a fragment of the muscle specimens was immediately frozen in liquid nitrogen and subsequently stored at -80ºC, while the remaining specimen was immersed in an alcohol-formol bath to be thereafter embedded in paraffin until further use. Frozen tissues were used to assess the expression of the target molecular markers, whereas paraffin-embedded tissues were used for the histological studies.

2.4. Muscle biology analyses

All the muscle biological experiments were performed in the same laboratory at *Hospital del Mar*-IMIM-*Universitat Pompeu Fabra* (Barcelona).

2.4.1. *Muscle fiber counts and morphometry*

Muscle fibers of diaphragm and gastrocnemius were identified on three-micrometer paraffin-embedded sections of all groups of mice. MyHC-I (slow-twitch fibers) and MyHC-II (fast-

twitch fibers) isoforms were identified using anti-MyHC-I and anti-MyHC-II antibodies (Sigma- Aldrich, Saint Louis, MO, USA), respectively, as previously described [14-18,50].

2.4.2. *Muscle structural abnormalities*

The area fraction of normal and abnormal muscle was evaluated on three-micrometer paraffin-embedded sections of the diaphragm and gastrocnemius muscles in all study groups following previously published methodologies [14-18,45,46,50].

2.4.3. *Terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate (UTP) nick-end labeling (TUNEL) assay*

In muscle paraffin-embedded sections, apoptotic nuclei were identified using the TUNEL assay (In Situ Cell Death Detection Kit, POD**,** Roche Applied Science, Mannheim, Germany) in both diaphragm and gastrocnemius muscles from all study groups following the manufacturer's instructions and previously published studies [4,15,17,50].

2.4.4. *Immunoblotting*

Protein levels of the different molecular markers analyzed in the study were explored by means of immunoblotting procedures as previously described [14-18,25,45,46,50,51].

The following primary antibodies were used: anti-mammalian target of rapamycin (mTOR; 1:1000, #2972S), anti-serine/threonine kinase 1 (AKT; 1:1000, #9272S), anti-phospho-AKT (1:750, 9271S), and anti-microtube-associated protein 1 light chain 3 (LC3; 1:1000, #2775S) antibodies from Cell signaling (Boston, MA, USA), anti-20S proteasome subunit C8 antibody (1:5000, BML-PW8110-0100) from Biomol (Plymouth Meeting, PA, USA), anti-protein ubiquitination antibody (1:5000, A-100) from Boston Biochem (Cambridge, MA, USA), anti-nucleoporin p62 (p62; 1:1000, P0067) antibody from Sigma-Aldrich (St. Louis, MO, USA), anti-myostatin (1:2000, A300-401A) antibody from Bethyl (Montgomery, TX, USA), anti-muscle ring finger protein-1 (MuRF-1; 1:2000, sc-27642), anti-atrogin-1 (1:1000, sc-166806), anti-tripartite motif-containing protein 32 (TRIM32;

1:500, sc-49265), anti-spectrin (1:500, sc-46696), anti-beclin-1 (1:200, sc-11427), anti-myoblast determination protein 1 (myoD; 1:500, sc-760), anti-peroxisome proliferator-activated receptor (PPAR)-alpha (1:500, sc-9000), anti-PPAR-gamma (1:500, sc-7196), anti-peroxisome proliferator-activated receptor gamma coactivator (PGC) 1-alpha (1:500, sc-13067), anti-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) p65 (1:500, sc-8008), anti-phospho- NF-kB p65 (1:500, sc-101749), anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IkB) –alpha (1:500, sc-847), anti-phospho-IkB-alpha (1:500, sc-7977), anti-phospho-forkhead box protein O1 (FoxO-1; 1:750, sc-7977), anti-extracellular signal-regulated kinases (ERK)1/2 (1:1000, sc-157), anti-phospho-ERK1/2 (1:500, sc-16982), anti-p38 (1:1000, sc-7149), anti-phospho-p38 (1:1000, sc-101759), anti- B-Cell CLL/Lymphoma 2 (BCL-2; 1:500, sc-7382), anti-BCL2 associated X protein (BAX; 1:500, sc-526), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000, sc-25778) antibodies from Santa Cruz (Santa Cruz, CA, USA), anti-FoxO-1 (1:1000, 04-1005) antibody from Millipore (Billerica, MA, USA), anti-FoxO-3 antibody (1:500, AP20683PU-N) from Acris (Aachen, Germany), and anti-phospho-FoxO-3 antibody (1:500, BS5019) from Bioworld Technology (St. Louis, MO, USA).

Optical densities of specific proteins were quantified using the software Image Lab version 2.0.1 (Bio-Rad Laboratories). Final optical densities obtained in each specific group of animals corresponded to the mean values of the different samples (lanes) of each of the study antigens. Values of total protein ubiquitination in a given sample were calculated by addition of optical densities (arbitrary units) of individual protein bands in each case. Values of optical densities (arbitrary units) of the activated proteins: NF-kB p65, IkB-alpha, ERK 1/2, p38 and AKT were calculated as the ratio of the phosphorylated protein to total protein content for each of these signaling markers. Values of optical densities (arbitrary units) of the activated proteins FoxO-1 and FoXO-3 were calculated as the ratio of total protein to phosphorylated protein content in each case. Values of optical densities (arbitrary units) of the

ratio of calpain-cleaved alpha II-spectrin were calculated as the ratio of calpain (145 kDa) to alpha II-spectrin (250 kDa). To validate equal protein loading across lanes, the glycolytic enzyme GAPDH was used as the protein loading control in all immunoblots (Figures S3-S6 and S8-S9).

2.4.5. RNA extraction

Total RNA was first isolated from snap-frozen skeletal muscle specimens using Trizol reagent following the manufacturer's protocol (Life technologies, Carlsbad, CA, USA). Total RNA concentrations were determined spectrophotometrically using the NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) as previously described [14,16,43-45].

2.4.6. *Gene expression was assessed using qRT-PCR.*

A single RT was performed from which all the target genes of the study were analyzed. First-strand cDNA was generated from mRNA using oligo(dT)$_{12-18}$ primers and the Super-Script III reverse transcriptase following the manufacturer's instructions (Life technologies). TaqMan based qPCR reactions were performed using the ABI PRISM 7900HT Sequence Detector System (Life technologies) together with commercially available gene expression assays. The probes corresponding to the following genes involved in proteolytic system were tested: *murf-1* (Rn00590197_m1, Life technologies), *atrogin-1* (Rn00591730_m1, Life technologies), and *trim32* (Rn01764787_m1, Life technologies). The housekeeping gene *gapdh* (Rn01775763_g1, Life technologies) served as the endogenous control for mRNA gene expression. Reactions were run in triplicates, and mRNA data were collected and subsequently analyzed using the Expression Suite software v1.1 (Applied Biosystems, Foster City, CA, USA), in which the comparative C_T method ($2^{-\Delta\Delta CT}$) for relative quantification was employed. Results are shown as the relative expression of that in the non-cachexia control group, which was set to equal to 1.

2.5. Statistical Analysis

The normality of the study variables was verified using the Shapiro-Wilk test. Equality of variance for the study variables was tested using the Levene's test. As established results are represented as mean (standard deviation) [41]. Comparisons between all study groups were analyzed using the two-way analysis of variance (ANOVA). Subsequently, *Tukey post-hoc* analysis was used to adjust for multiple comparisons among the different study groups. The sample size chosen was based on previous studies [14-18,45,46,50] and on assumptions of 80% power to detect an improvement of more than 20% in measured outcomes at a level of significance of $p \leq 0.05$. All statistical analyses were performed using the Statistical Package for the Social Science (Portable SPSS, PASW statistics 18.0 version for Windows, SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1. Physiological characteristics of the study animals

At the end of the study period, cancer-cachexia rats exhibited a significant reduction in final body weight and body weight gain parameters compared to non-cachexia controls (Table 1). Treatment with formoterol induced a significant improvement in body weight gain of cancer-cachectic rats (Table 1). The weights of diaphragm and gastrocnemius muscles were significantly reduced in cancer-cachexia rats compared to non-cachexia controls (Table 1). Treatment with formoterol elicited a significant improvement in the weight of the gastrocnemius (similar to that in non-tumor controls), while it did not significantly ameliorate the weight of the diaphragm in the cancer-cachectic rats (Table 1). No significant differences were observed in the tumor cell content or weight between cancer-cachexia rats with or without treatment with formoterol (Table 1).

3.2. Effects of formoterol on muscle phenotype

The proportions of slow- and fast- twitch fibers did not significantly differ in the study muscles between cancer-cachectic rats and non-cachexia control animals (Table 2 and Figure S1). The fiber cross-sectional areas significantly decreased in both diaphragm and gastrocnemius muscles of cancer-cachexia rats compared to non-cachexia controls (Table 2 and Figure S1). Specifically, in the cachectic rats compared to non-cachectic control animals, the sizes of slow- and fast-twitch fibers were reduced by 23% and 32%, respectively, in the diaphragm and by 28% and 21%, respectively, in the gastrocnemius (Table 2 and Figure S1). Treatment of the cachectic rats with formoterol induced a significant improvement of the sizes of the slow- and fast-twitch fibers in the study muscles: 19% and 30% (diaphragm, respectively) and 78% and 26% (gastrocnemius, respectively), compared to non-treated control animals (Table 2 and Figure S1). Recovery of fiber sizes reached levels observed in the non-cachectic control animals following treatment with formoterol only in the gastrocnemius, particularly in the slow-twitch fibers (Table 2 and Figure S1).

Muscle structural abnormalities were significantly greater in both diaphragm and gastrocnemius muscles of cancer-cachexia rats than in non-cachexia controls (Table 3 and Figure S2). Treatment with formoterol significantly improved the structural abnormalities in both muscles, especially in the diaphragm, of the cachectic rats, and levels were similar to those found in the non-cachectic control animals (Table 3 and Figure S2).

3.3. Effects of formoterol on atrophy signaling pathways in muscles

Activated NF-kB p65 levels significantly increased in diaphragm and gastrocnemius muscles of cancer-cachexia rats compared to non-cachexia controls, and formoterol treatment attenuated such an increase in both muscles, whose levels were similar to those in the control animals (Figures 1A and S3-4). Activated IkB-alpha levels were significantly lower in respiratory and limb muscles of cancer-cachectic rats than in non-cachexia controls (Figures 1B and S3-4). Protein levels of activated FoxO-3 significantly increased in both muscles of

cancer-cachexia rats compared to non-cachexia controls (Figures 1C and S3-4). Formoterol treatment did not induce any significant effect on activated IkB-alpha or FoxO-3 protein levels in the study muscles of the cancer-cachexia rats, and in the latter marker levels were similar to the controls (Figures 1B, 1C, and S3-4). No significant differences were observed in the levels of activated FoxO-1, ERK1/2, or p38 in any study muscle between cancer-cachexia rats and non-cachexia controls in any muscle among study groups (Figures 1D-F and S3-4).

3.4. Effects of formoterol on proteolytic markers in muscles

In diaphragm and gastrocnemius muscles, gene expression of MuRF-1 significantly increased in both muscles of cancer-cachexia rats compared to non-cachexia controls, while no significant differences were observed in MuRF-1 protein levels in any muscle among study groups (Figures 2A and S5). Expression of atrogin-1 was only significantly increased in gastrocnemius muscles of cancer-cachexia rats compared to non-cachexia controls, but again no significant differences were observed in atrogin-1 protein levels in muscles among the study groups (Figures 2B and S5). No significant differences were observed in either gene expression or protein levels of TRIM32 in any muscles among study groups (Figures 2C and S5). Total protein ubiquitination and C8-20S subunit levels were significantly higher in both muscles of cancer-cachexia rats than in non-cachexia animals (Figures 2D-E and S5). Treatment with formoterol induced a significant decrease in C8-20S protein levels of diaphragm and gastrocnemius muscles in the cancer-cachectic rats, whose levels were similar to those in the controls, while protein ubiquitination was not modified by the $beta_2$ agonist (Figures 2D and S5).

3.5. Effects of formoterol on autophagy markers in muscles

Protein levels of P62 and beclin-1 significantly increased in the diaphragm of cancer-cachexia rats compared to non-cachexia controls (Figures 3A-B and S6). A significant rise in LC3 protein levels was also detected in diaphragm and gastrocnemius muscles of cancer-cachexia

rats compared to non-cachexia controls, and treatment with formoterol of the former animals significantly attenuated such an increase in both muscles (similar to control rats) (Figures 3C and S6). Myostatin protein levels significantly increased in both muscles of cancer-cachectic rats compared to non-cachexia controls and formoterol treatment significantly attenuated such an increase (similar to controls, Figures 3D and S6). Levels of calpain-cleaved α II-spectrin in diaphragm and gastrocnemius did not significantly differ between cancer-cachexia rats and non-cachexia controls (Figures 3E and S6).

3.6. Effects of formoterol on apoptosis markers in muscles

TUNEL positively-stained nuclei were significantly higher in diaphragm and gastrocnemius muscles of cancer-cachexia rats than in non-cachexia controls (Figures 4A and S7). Treatment of cancer-cachexia rats with formoterol significantly reduced TUNEL positively-stained nuclei in the diaphragm (similar to controls), but not in the limb muscle (Figures 4A and S7). In cancer cachectic rats, BAX protein levels were increased only in the diaphragm, and formoterol treatment significantly reduced those levels in the respiratory muscle of the cachectic animals (Figures 4B and S8). Protein levels of BCL-2 did not differ among any of the study muscles (Figures 4C and S8).

3.7. Effects of formoterol on markers of muscle mass maintenance and metabolism

In diaphragm and gastrocnemius muscles of cancer-cachexia rats, levels of mTOR were significantly reduced compared to non-cachexia controls (Figures 5A and S9). Activated AKT levels only significantly decreased in gastrocnemius muscles, but not in diaphragm, of cancer-cachexia rats compared to non-cachexia controls (Figures 5B and S9). Protein levels of MyoD and PGC-1alpha were significantly reduced in diaphragm and gastrocnemius muscles in cancer-cachexia rats compared to non-cachexia controls (Figures 5C-D and S9). No significant differences were observed in levels of PPAR-alpha or PPAR-gamma in either diaphragm or gastrocnemius muscles of cancer-cachectic rats compared to non-cachexia animals (Figures 5E-F and S9). Formoterol treatment did not induce any significant effects in

markers of muscle mass maintenance and metabolism (mTOR, AKT, MyoD, PGC-1alpha, PPAR-alpha, PPAR-gamma, Figures 5A-F and S9).

4. DISCUSSION

In the current study, the hypothesis has been confirmed: administration of the $beta_2$ agonist for seven days attenuated the activation of atrophy signaling pathways and myostatin system, while improving the expression of proteolytic markers and muscle structural alterations in both diaphragm and gastrocnemius muscles of the cancer cachectic rats. Levels of markers involved in muscle repair and maintenance were not affected by formoterol treatment, suggesting that the $beta_2$ agonist rather prevented proteolysis than favored muscle repair and protein anabolism. Interestingly, formoterol treatment did not elicit any significant differences in tumor weights or number of cells in the cancer cachectic rats. The most relevant findings observed in the study are discussed below.

The Yoshida AH-130 ascites hepatoma is a suitable approach to study the underlying biological events of muscle wasting in cancer-induced cachexia, as already demonstrated in several investigations [10-12,26-28,51]. In the study, expression levels of activated NF-κB p65 and FoxO3 were significantly increased in both respiratory and limb muscles. These findings are consistent with previous reports, in which expression of these transcription factors was upregulated in skeletal muscles of tumor-bearing mice [15,18]. Furthermore, levels of total protein ubiquitination and content of the proteasome subunit C8-20S were also significantly greater in both respiratory and limb muscles of the cancer cachectic rats compared to the non-cachectic controls. These results are also in line with previous studies, in which levels of protein ubiquitination and proteasome content were increased in muscles of rodents with cancer cachexia [15,18], with disuse-induced muscle atrophy [17], and in patients with lung cancer cachexia and COPD [46].

The novelty in the present study was the attenuation or even the improvement in the atrophy signaling pathways analyzed in the muscles of the cancer cachectic rats in response to treatment with formoterol for seven days. Importantly, levels of the proteolytic markers total protein ubiquitination and proteasome content were also significantly attenuated, especially the latter, in both respiratory and limb muscles of the cancer cachectic rats treated with the beta$_2$ agonist for one week. These findings suggest that formoterol attenuate ATP-dependent proteolysis through inhibition of the ubiquitin-proteasome proteolytic pathway. In fact, a previous study demonstrated a significant downregulation of the expression of proteasome subunits in limb muscles of cancer cachectic rats treated with formoterol [10]. It should be mentioned that in this model of cancer cachexia, muscle protein levels of MAPK signaling pathway and those of the E3 ligases MuRF-1, atrogin-1, and TRIM32 did not significantly differ between cachectic and non-cachectic conditions, and formoterol treatment did not have any significant effect on those muscles. Nonetheless, gene expression levels of MuRF-1 were upregulated in both diaphragm and gastrocnemius, while those of atrogin-1 were only upregulated in the cachectic limb muscle. Moreover, gene and protein expression levels of TRIM32 did not significantly differ among study groups for any of the muscles. Collectively, these results imply a potential transcriptional regulation of the atrogenes in this model as previously reported [9]. On the other hand, specific characteristics of experimental models, type of tumors, or duration of the study period may account for differences encountered in the levels of expression of the proteolytic markers in the target muscles across studies [15,18]. Indeed, a recent study from our group showed that the ubiquitin-proteasome system was not involved in the process of muscle wasting associated with lung cancer carcinogenesis in mice [50].

As expected, body and muscle weights were significantly reduced in the cancer cachectic rats and formoterol treatment attenuated such alterations. Interestingly, atrophy of both slow- and fast-twitch fibers was also observed in the respiratory and limb muscles of the

cancer cachectic rats, as shown in other models [15,18,50]. Interestingly, formoterol therapy for seven days significantly reversed (to control levels or even better in the limb muscle) the atrophy of the muscle fibers, especially of the slow-twitch fibers in the gastrocnemius, in which the improvement was substantial. Indeed, previous results have already demonstrated that $beta_2$ agonists particularly improved the function and structure of the slow-twitch fibers [38]. These observations also reinforce the validation of the model and the effects of treatment with beta-adrenoceptors, as also previously demonstrated in other experimental conditions [10-12,38].

A significant rise in several structural abnormalities such as inflammatory cell and internal nuclei counts was also observed in the diaphragm and gastrocnemius muscles of the cancer cachectic rats. Importantly, treatment with formoterol significantly attenuated all the structural alterations, especially in the diaphragm, of the cachectic rats. These results also suggest that a damage-repair mechanism may take place similarly in the fibers of both respiratory and limb muscles during the muscle wasting process. As a matter of fact, the rise in inflammatory cells and internal nuclei imply the existence of an underlying repair process following injury of the cachectic muscles during the muscle wasting process.

Moreover, as previously shown in muscles of cachectic animals [7,14] and in patients with COPD [4,46] and LC [46], the number of apoptotic nuclei was also significantly increased in the respiratory and limb muscles of the tumor-bearing rats. A significant rise in protein levels of BAX was only observed in the diaphragm muscle of the cancer cachectic rats, and formoterol significantly attenuated such an increase in the respiratory muscle. It is likely that differences in the activity of each type of muscle may account for the differences in the levels of expression of the apoptotic marker BAX as the diaphragm must maintain continuously its activity in the animals. Levels of the antiaopoptotic BCL-2 did not differ in any muscle among the study groups of rats.

A relevant finding in the study was the specificity of the effects of the $beta_2$ agonist formoterol on skeletal muscles but not on tumor growth (cell numbers) or size of the cancer cachectic rats. Interestingly, it has been suggested that beta-adrenergic signaling regulate tumor growth among other cellular processes and that beta-blockers may be used as adjuvant therapies in the management of patients with cancer [19,20,39]. In the study, formoterol treatment did not induce any significant effects on tumor growth in the cancer cachectic rats, implying that the $beta_2$ agonist exerted selective effects on the muscle fibers of the animals.

A novel finding in the study was the significant rise in the levels of the autophagy markers p62, beclin-1, and LC3 in the limb muscle and diaphragm, especially the latter, of the cancer cachectic rats compared to the non-cachectic control animals. These results are in keeping with a previous study conducted on a mouse model of carcinogenesis induced by urethane administration [50], in which autophagy markers were also increased in both diaphragm and gastrocnemius of mice with lung cancer cachexia. Indeed, in that study [50] autophagy and apoptosis were the most relevant contributors to muscle atrophy in both respiratory and limb muscles.

In the current investigation, beclin-1 and p62 were significantly increased only in the diaphragm of the cancer cachectic rats, while a rise in LC3-II/I ratio levels were seen in both respiratory and limb muscles of these rodents. These observations suggest that autophagy is differentially regulated in the diaphragm in this model of cancer cachexia probably to maintaining the turnover of cell components as previously implied in the pathophysiology of muscular dystrophies, in which autophagy was defective [29]. A novel finding in the investigation was the significant reduction in LC3-II/I ratio levels induced by formoterol therapy in both diaphragm and gastrocnemius muscles. These results were partly in agreement with those previously reported in a model of fast-twitch muscle hypertrophy [30], in which the kinetics of anabolic and catabolic markers and autophagy were studied in response to treatment with formoterol at several time-points. Importantly, formoterol therapy for several

462 days also reduced the expression of autophagy markers in the rats with muscle hypertrophy
463 [30].

464 Interestingly, protein levels of myostatin were significantly greater in both respiratory
465 and limb muscles of cancer cachectic rats. These findings are similar to previous reports, in
466 which myostatin system was also upregulated in models of cancer-induced cachexia [11,15].
467 Furthermore, formoterol also induced a downregulation of the myostatin levels as a result of
468 an upregulation of follistatin expression, thus, improving muscle mass in the cachectic rats
469 [11]. The current observations are also in agreement with the previous findings [11] as to the
470 effects of treatment with the $beta_2$ agonist in the cachectic rats.

471 Levels of mTOR as a marker of protein synthesis were significantly decreased in the
472 diaphragm and gastrocnemius of the cancer cachectic rats. Importantly, in these muscles of
473 tumor-bearing rats treated with formoterol for seven days, mTOR levels did not differ from
474 those seen in the non-treated cachectic animals. Protein levels of activated AKT were also
475 reduced in the gastrocnemius, but not the respiratory muscle, of the cancer cachectic rats, and
476 formoterol did not elicit a significant improvement in its levels in any of the study muscles.
477 These findings are in contrast with previous results in which AKT-mTOR pathway was
478 activated in response to formoterol therapy in the limb muscles of rats, especially in the early
479 phases of the treatment [30]. Differences in experimental design and models may account for
480 the discrepancies among studies.

481 Alterations in the levels of muscle regeneration were previously reported in cancer-
482 cachexia models [58]. Similarly, in the present study, protein levels of myoD were also
483 significantly lower in both respiratory and limb muscles of the cancer cachectic rats than in
484 the controls. Importantly, formoterol treatment prevented a further decline in myoD in the
485 cachectic muscles. PPARs are powerful regulators of skeletal muscle metabolism and
486 mitochondrial biogenesis [31,33]. Moreover, they also play a role in the fiber type shift
487 towards a more resistant phenotype in skeletal muscles [47]. Signaling of muscle atrophy

may also be mediated by PPARs in cachectic conditions [48]. In the present study, levels of PGC-1alpha, but not those of PPAR-alpha or PPAR-gamma, were significantly lower in respiratory and limb muscles of the cachectic rats than control rodents. These findings are in line with previous investigations, in which PPAR levels were altered in muscles of cachectic rats [28] and mice [50,53] and lower limb muscles of patients with respiratory cachexia [48]. Treatment with the $beta_2$ agonist prevented a further decline in PGC-1alpha levels of both study muscles of the cancer cachectic rats, as previously shown to occur in a rat model of hypertrophy [30]. Collectively, these findings imply that important metabolic alterations took place in both respiratory and limb muscles that were partly attenuated by treatment with the $beta_2$ agonist formoterol in this experimental model of cancer-induced cachexia.

4.1. Conclusions

We conclude that muscle wasting in this *in vivo* model of cancer-induced cachexia is characterized by the induction of significant structural alterations, atrophy signaling pathways, proteasome activity, autophagy markers, and myostatin system, along with a significant decline in the expression of muscle regenerative and metabolic markers of both respiratory and limb muscles in a similar fashion. Treatment of the cachectic rats with formoterol for seven days partly attenuated the structural alterations, while improving atrophy signaling pathways and other molecular perturbations in both diaphragm and gastrocnemius. The results reported in this study have relevant therapeutic implications as they showed beneficial effects of the $beta_2$ agonist formoterol in respiratory and limb muscles of the cachectic rats through several key biological pathways in a similar manner.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this study.

AUTHOR CONTRIBUTIONS

Anna Salazar-Degracia: molecular biology, data analyses and interpretation, results preparation including graphical and tabular representation, and manuscript draft writing

Sílvia Busquets: study design, animal experiments, data analyses and interpretation, and manuscript draft writing

Josep M. Argilès: study design, data analyses and interpretation, results preparation, and manuscript draft writing

Núria Bargalló-Gispert: molecular biology experiments and results preparation in the revised manuscript

Francisco J. Lopez-Soriano: study design, data analyses and interpretation, results preparation, and manuscript draft writing

Esther Barreiro: study design, data analyses and interpretation, results preparation, and manuscript writing final version

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FIGURE LEGENDS

Figure 1: Mean values and standard deviation of the following markers: **(A)** activated NF-kB p6, **(B)** activated IkB-alpha, **(C)** activated FoxO-3, **(D)** activated FoxO-1, **(E)** activated ERK1/2, and **(F)** activated p38 activated in diaphragm (black bars) and gastrocnemius (grey bars) muscles as measured by optical densities in arbitrary units (OD, a.u.). **Definition of abbreviations:** NF-kB p65, nuclear factor kappa-light-chain-enhancer of activated B cells p65; IkB-alpha, nuclear factor kappa polypeptide gene enhancer in B-cells inhibitor, alpha; FoxO, forkhead box protein; ERK1/2, extracellular signal-regulated kinase 1/2; F, formoterol. **Statistical significance**: * $p \leq 0.05$ and ** $p \leq 0.01$ between non-cachexia controls and cancer-cachexia rats; § $p \leq 0.05$ between cancer-cachexia rats and cancer-cachexia rats treated with formoterol.

Figure 2: Mean values and standard deviation of the following markers: **(A)** MuRF-1 **(B)** atrogin-1, and **(C)** TRIM32 expressed as a relative mRNA levels (left panel) and a protein content (right panel), **(D)** C8-20S, and **(E)** total ubiquitinated expressed only protein content in diaphragm (black bars) and gastrocnemius (grey bars) muscles. Protein content as measured by optical densities in arbitrary units (OD, a.u.). **Definition of abbreviations:** MuRF-1, muscle ring finger protein-1; TRIM32, tripartite motif-containing protein 32; C8-20S, 20S proteasome alpha subunit C8; F, formoterol. **Statistical significance:** * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ between non-cachexia controls and cancer-cachexia rats; § $p \leq 0.05$ and §§ $p \leq 0.01$ between cancer-cachexia rats and cancer-cachexia rats treated with formoterol.

Figure 3: Mean values and standard deviation of the following markers: **(A)** P62, **(B)** beclin-1, **(C)** ratio of LC3-II/LC3-I, **(D)** myostatin, and **(E)** ratio of calpain-cleaved alpha II-spectrin protein content in diaphragm (black bars) and gastrocnemius (grey bars) muscles as measured by optical densities in arbitrary units (OD, a.u.). **Definition of abbreviations:** P62, nucleoporin p62; LC3, microtube-associated protein 1 light chain 3; F, formoterol. **Statistical**

824 **significance:** * $p \leq 0.05$ and ** $p \leq 0.01$ between non-cachexia controls and cancer-cachexia
825 rats; § $p \leq 0.05$, §§ $p \leq 0.01$, and §§§ $p \leq 0.001$ between cancer-cachexia rats and cancer-
826 cachexia rats treated with formoterol.

827 **Figure 4:** Mean values and standard deviation of **(A)** the percentage of positively stained
828 nuclei for the TUNEL assay and the following markers: **(B)** BAX, and **(C)** BCL-2 protein
829 content in diaphragm (black bars) and gastrocnemius (grey bars) muscles as measured by
830 optical densities in arbitrary units (OD, a.u.). **Definition of abbreviations:** BAX, BCL2
831 associated X protein; BCL-2, B-Cell CLL/Lymphoma 2; F, formoterol. **Statistical**
832 **significance:** * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ between non-cachexia controls and
833 cancer-cachexia rats; § $p \leq 0.05$, and §§§ $p \leq 0.001$ between cancer-cachexia rats and cancer-
834 cachexia rats treated with formoterol.

835 **Figure 5:** Mean values and standard deviation of the following markers: **(A)** mTOR, **(B)**
836 activated AKT, **(C)** myOD, **(D)** PGC-1alpha, **(E)** PPAR-alpha, and **(F)** PPAR-gamma in
837 diaphragm (black bars) and gastrocnemius (grey bars) muscles as measured by optical
838 densities in arbitrary units (OD, a.u.). **Definition of abbreviations**: mTOR, mammalian target
839 of rapamycin; AKT, serine/threonine kinase 1; myoD, myogenic differentiation 1; PGC-
840 1alpha, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR,
841 peroxisome proliferator-activated receptor; F, formoterol. **Statistical significance**: * $p \leq 0.05$,
842 ** $p \leq 0.01$, and *** $p \leq 0.001$ between non-cachexia controls and cancer-cachexia rats.

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844

Table 1. Total body and muscle weights in rats of the study groups.

	Non-cachexia control	Non-cachexia control-formoterol	Cancer-cachexia	Cancer-cachexia-formoterol
Initial body weight (g)	128.1 (6.4)	124.4 (8.1)	126.8 (11.8)	124.4 (7.1)
Tumor weight (g)	-	-	45.3 (5.8)	41.3 (7.7)
Tumor cell content	-	-	3.6×10^{9} ($4\cdot10^{8}$)	3.4×10^{9} ($6\cdot10^{8}$)
Final body weight (g)	164.0 (11.3)	161.3 (8.7)	119.5 (10.0)***	124.8 (8.0)
Body weight gain (%)	+ 27.9 (3.7)	+ 29.7 (2.8)	-5.6 (4.8) ***	+ 0.4 (4.1) §
Diaphragm weight (mg/ 100g IBW)	290.2 (31.9)	319.6 (49.2)	126.7 (13.9)***	128.6 (17.5)
Gastrocnemius weight (mg/ 100g IBW)	693.3 (36.8)	708.0 (13.1)	512.4 (34.7) ***	636.3 (3.1) §§§

Values are expressed as mean (standard deviation). Definition of abbreviations: g, gram; mg, milligram; IBW, initial body weight. **Statistical significance:** *** $p\leq0.001$ between the non-cachexia controls and cancer-cachexia rats; § $p\leq 0.05$ and §§§ $p\leq 0.001$ between cancer-cachexia rats and cancer-cachexia rats treated with formoterol.

Table 2. Muscle fiber type composition and morphometry in diaphragm and gastrocnemius of all study groups.

	Non-cachexia control	Non-cachexia control-formoterol	Cancer-cachexia	Cancer-cachexia-formoterol
Diaphragm				
Type I fibers (%)	34.9 (5.30)	31.5 (3.90)	33.3 (4.45)	32.1 (3.99)
Type II fibers (%)	65.1 (5.30)	68.5 (3.90)	66.7 (4.45)	67.9 (3.99)
Type I fibers area (μm^2)	566.4 (66.2)	570.9 (121.2)	438.0 (69.8) **	519.3 (63.5) §
Type II fibers area (μm^2)	677.4 (55.1)	718.2 (154.4)	461.9 (90.8) ***	599.5 (75.7) §§
Gastrocnemius				
Type I fibers (%)	22.5 (3.84)	21.8 (5.24)	22.9 (4.27)	21.6 (8.08)
Type II fibers (%)	77.5 (3.84)	78.2 (5.24)	77.1 (4.27)	78.4 (8.08)
Type I fibers area (μm^2)	588.2 (90.5)	523.7 (208.1)	424.8 (120.5) **	756.2 (231.1) §§
Type II fibers area (μm^2)	849.2 (156.2)	850.9 (154.4)	669.2 (152.3) *	844.2 (110.2) §§

Values are expressed as mean (standard deviation). Definition of abbreviations: μm, micrometer. **Statistical significance:** * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ between the non-cachexia controls and cancer-cachexia rats; § $p \leq 0.05$ and §§ $p \leq 0.01$ between cancer-cachexia rats and cancer-cachexia rats treated with formoterol.

Table 3. Structural abnormalities in diaphragm and gastrocnemius of all study groups.

	Non-cachexia control	Non-cachexia control-formoterol	Cancer-cachexia	Cancer-cachexia-formoterol
Diaphragm				
Abnormal fraction area (%)	5.29 (0.87)	4.90 (0.78)	10.14 (1.94) ***	5.43 (1.44) §§§
Internal nuclei (%)	3.02 (1.19)	2.93 (1.34)	4.24 (1.56) p=0.100	2.43 (1.20) §
Cellular inflammation (%)	1.64 (1.58)	1.36 (1.42)	4.39 (2.14) *	2.45 (2.15) p=0.067
Other items (%)	0.63 (0.66)	0.61 (0.46)	1.51 (0.93) *	0.55 (0.39) §§
Gastrocnemius				
Abnormal fraction area (%)	2.59 (0.52)	2.51 (0.46)	4.15 (0.81) ***	3.02 (0.91) §§
Internal nuclei (%)	1.32 (0.70)	1.08 (0.15)	2.1 (0.73) *	1.51 (0.82) p=0.075
Cellular inflammation (%)	1.13 (0.25)	1.35 (0.63)	1.89 (0.70) *	1.31 (0.66)
Other items (%)	0.14 (0.12)	0.08 (0.20)	0.16 (0.13)	0.20 (0.23)

Values are expressed as mean (standard deviation). Statistical significance: * $p \leq 0.05$ and *** $p \leq 0.001$ between the non-cachexia controls and cancer-cachexia rats; § $p \leq 0.05$, §§ $p \leq 0.01$ and §§§ $p \leq 0.001$ between cancer-cachexia rats and cancer-cachexia rats treated with formoterol.

Figure 1. Salazar-Degracia A. et al.

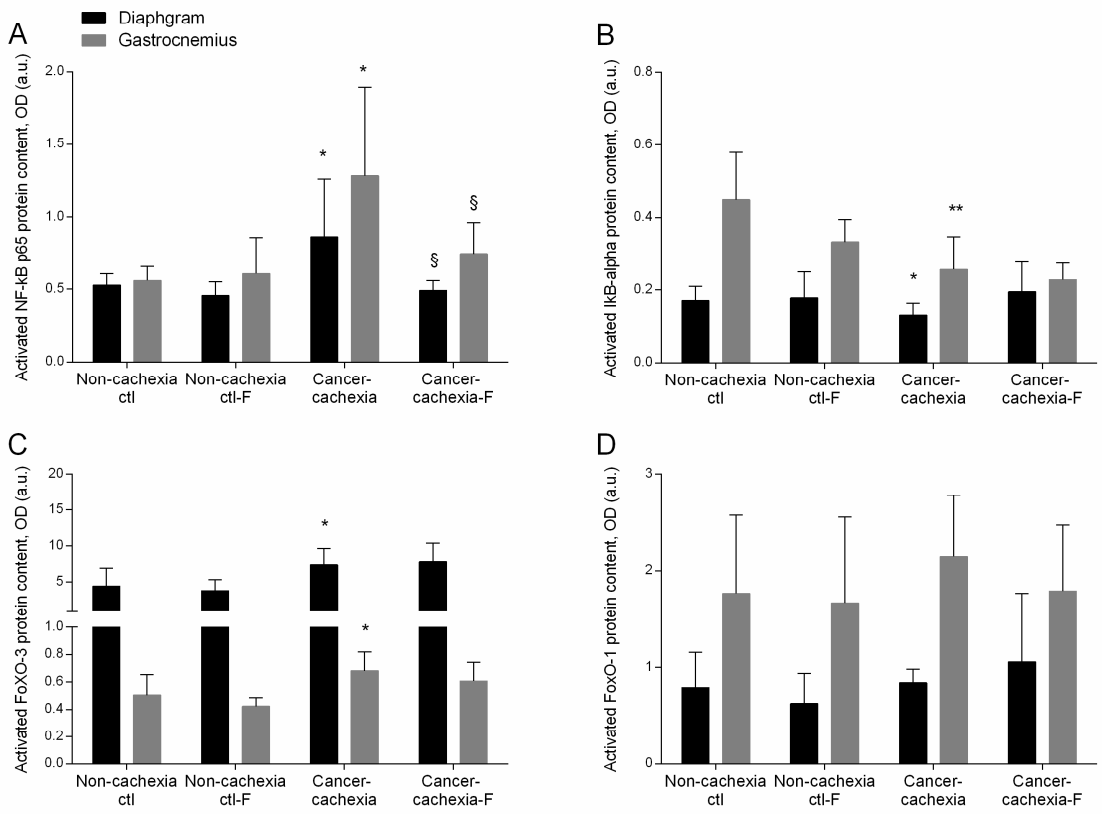
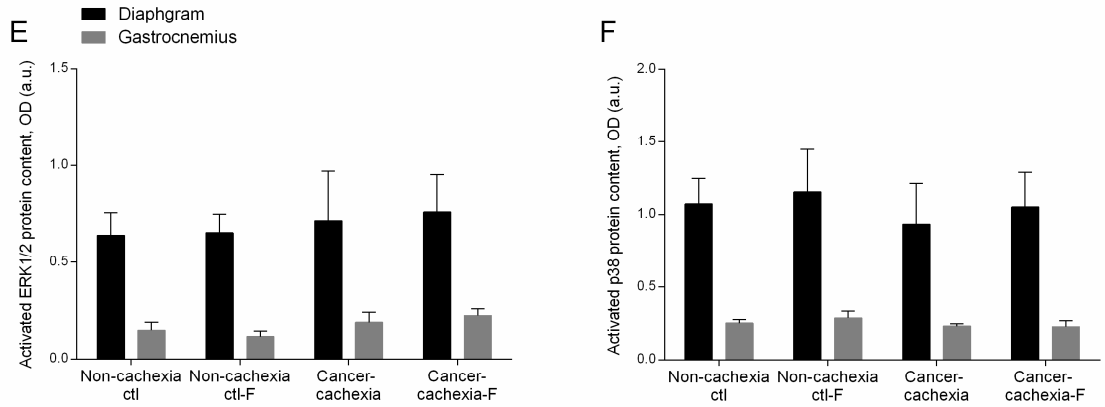


Figure 1. Salazar-Degracia A. et al.



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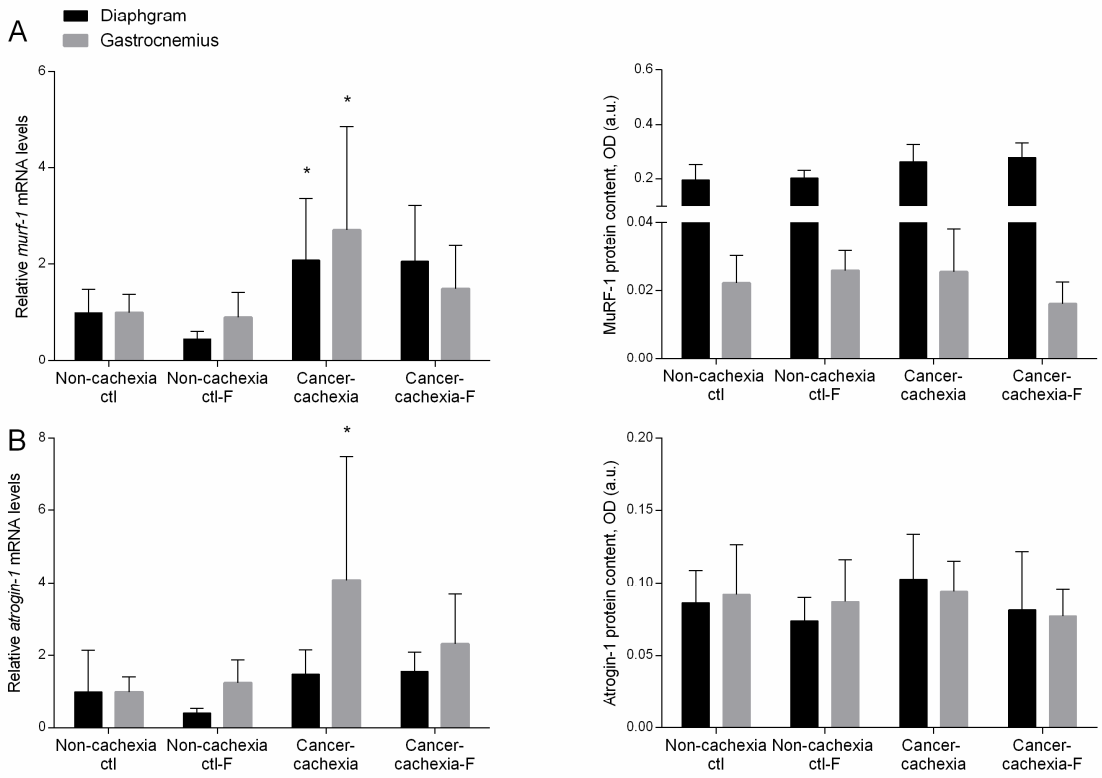
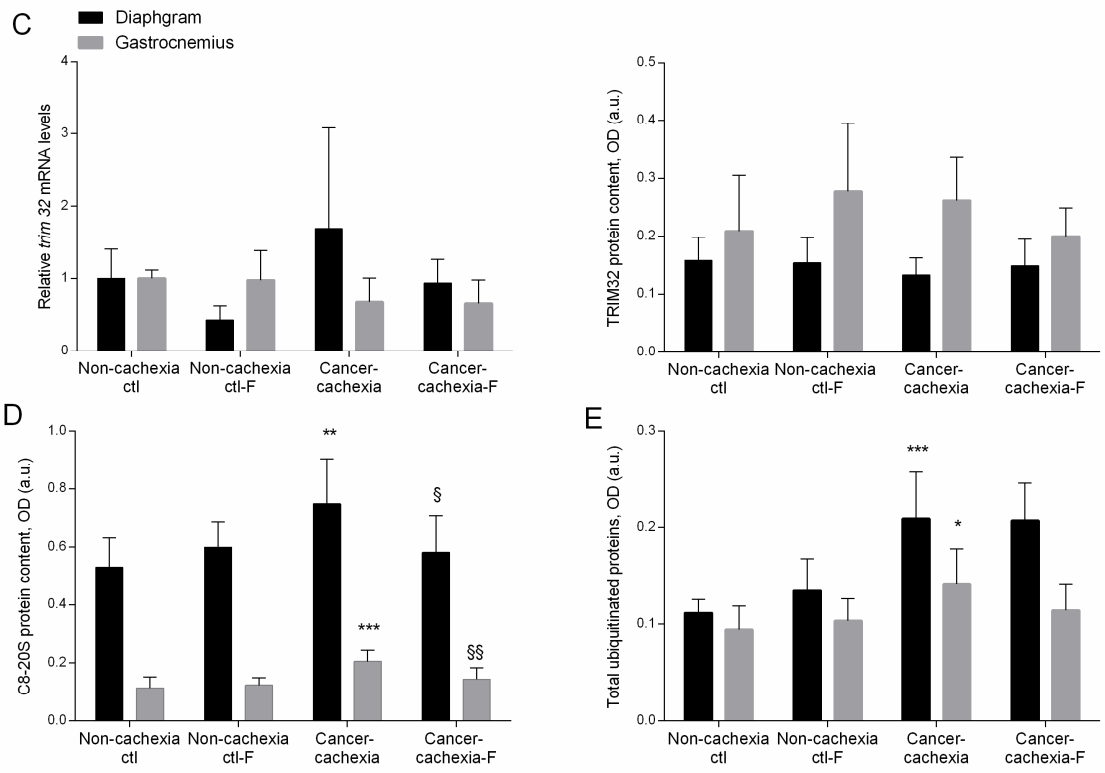


Figure 2. Salazar-Degracia A. et al.



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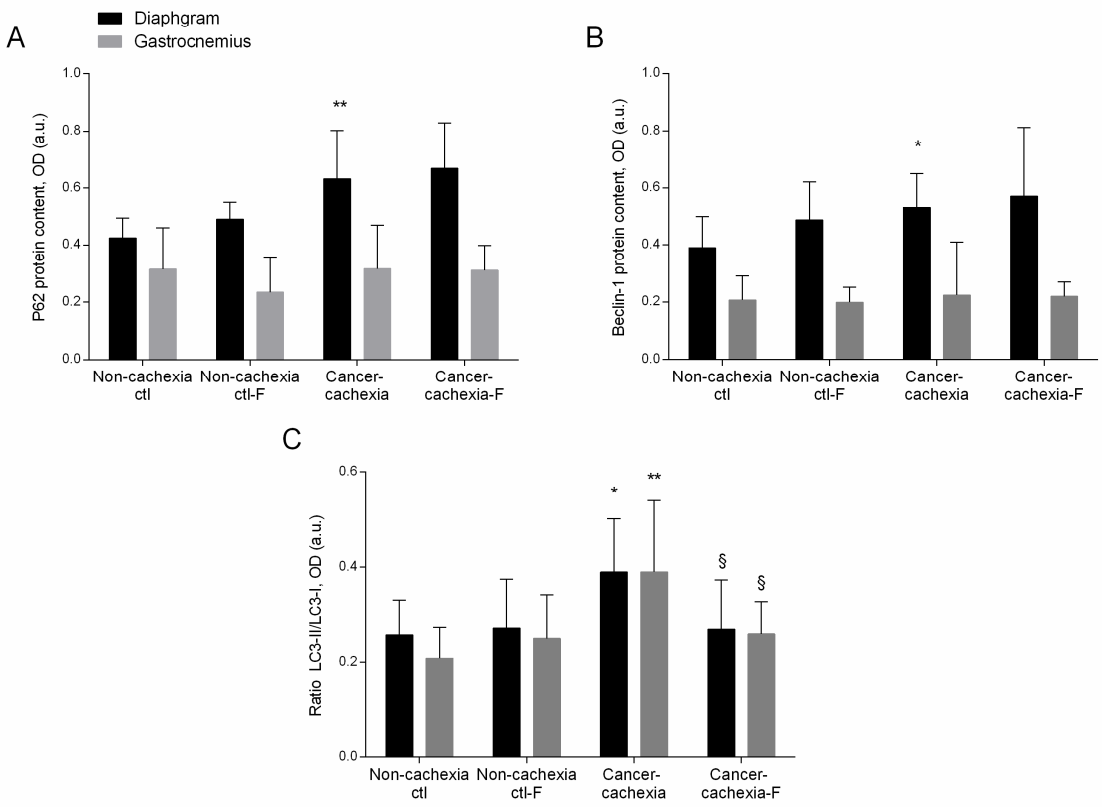

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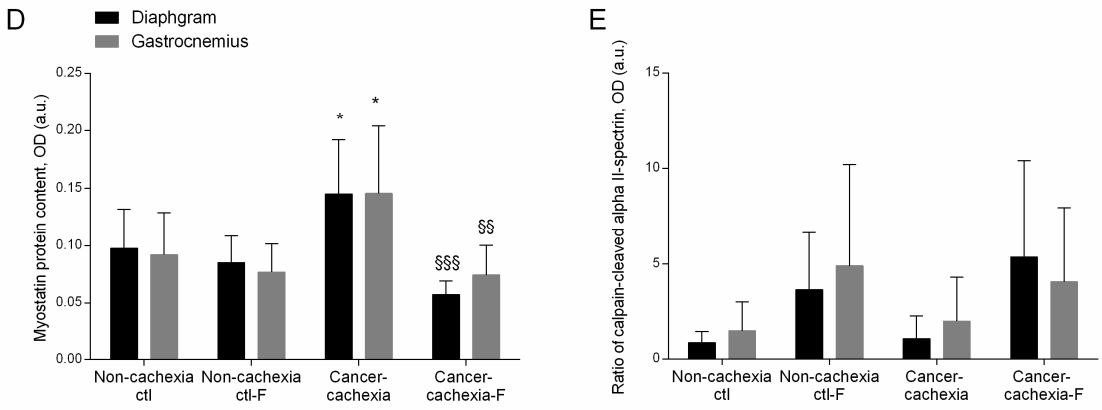

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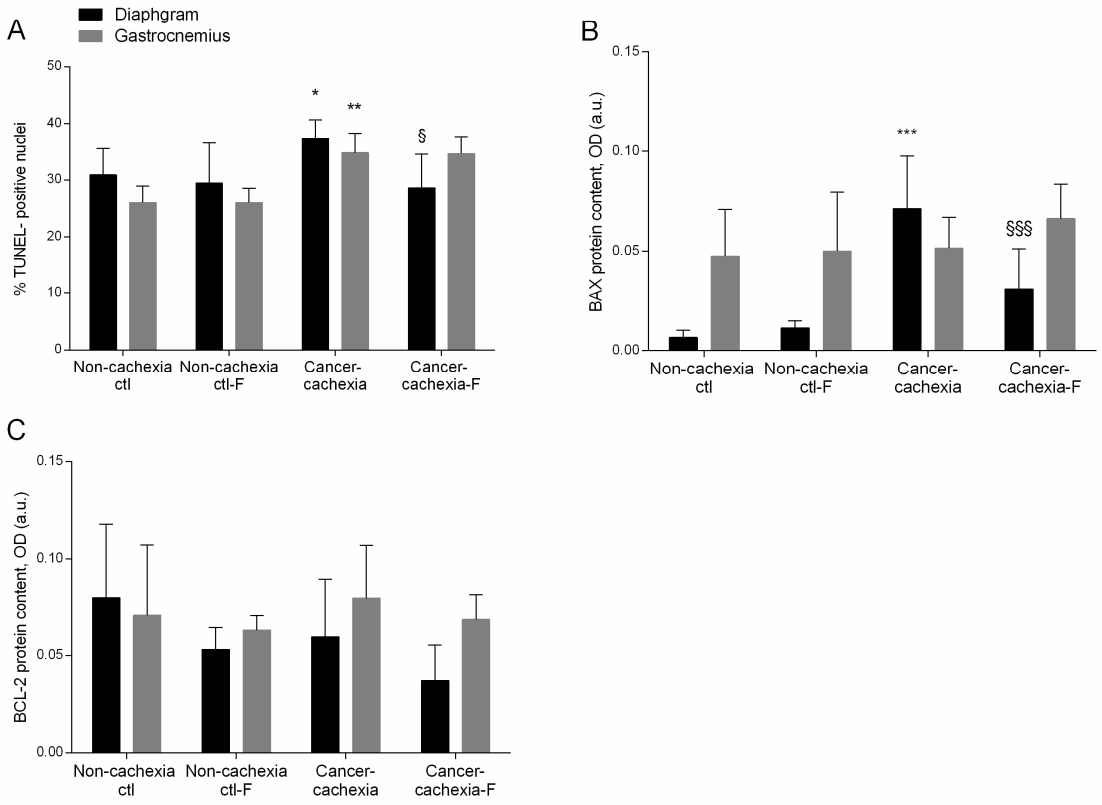
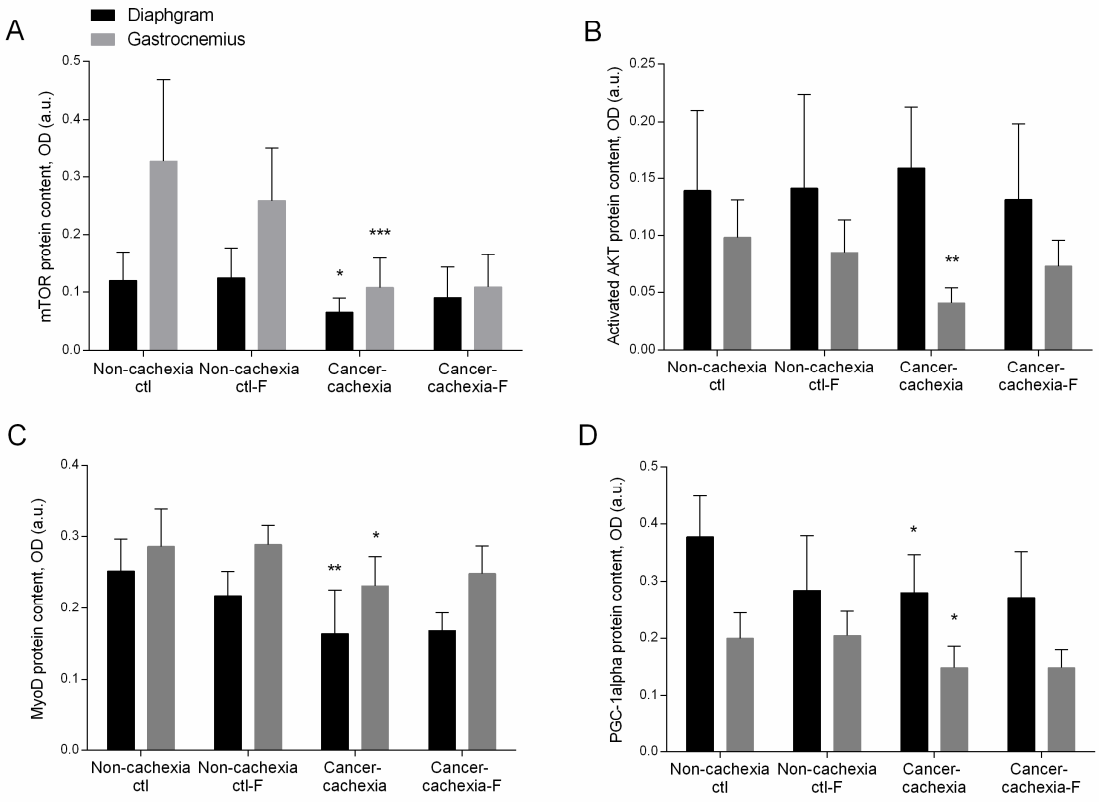


Figure 5. Salazar-Degracia A. et al.



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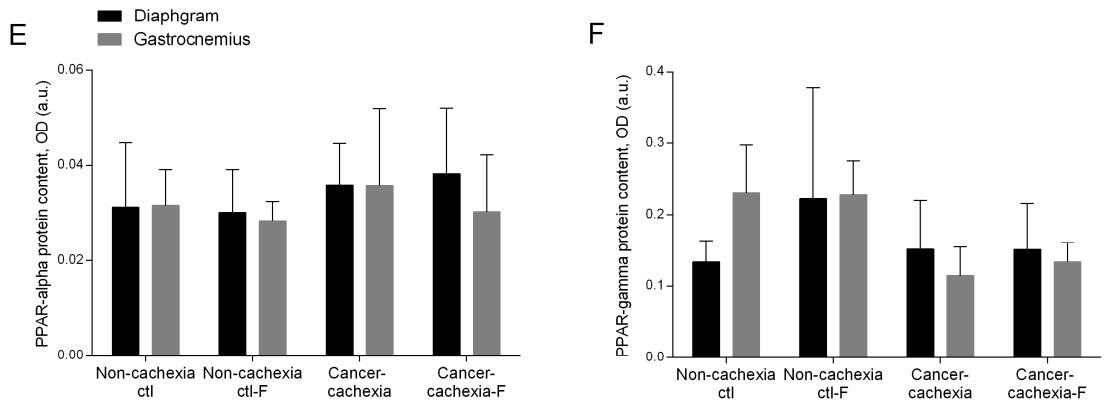

HIGHLIGHTS

EFFECTS OF THE BETA$_2$ AGONIST FORMOTEROL ON ATROPHY SIGNALING, AUTOPHAGY, AND MUSCLE PHENOTYPE IN RESPIRATORY AND LIMB MUSCLES OF RATS WITH CANCER-INDUCED CACHEXIA

Muscle mass loss and wasting are characteristic features of patients with cancer

Beta-adrenoceptors attenuate muscle wasting

Muscle atrophy signaling pathways and altered metabolism attenuated in cancer cachectic animals treated with beta$_2$ agonist formoterol

Treatment of the cachectic rats with formoterol attenuated structural alterations and atrophy signaling and other molecular perturbations

Beneficial effects of formoterol demonstrated in cancer cachectic muscles involved several key biological pathways