BIOLOGICAL MECHANISM-BASED AND PATIENT-CENTERED MANAGEMENT OF CANCER-RELATED SYMPTOMS AND SYNDROMES

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BIOLOGICAL MECHANISM-BASED AND PATIENT-CENTERED MANAGEMENT OF CANCER-RELATED SYMPTOMS AND SYNDROMES

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The lack of recovery prospects in advanced cancer patients has often led to neglect important achievable therapeutic objectives, such as Quality of Life (QL) improvement, aimed at preserving, for as long as possible, patient integration with their family and social environment. In fact, traditional antineoplastic therapy protocols have been for a long time designed to demonstrate an advantage in clinical response and survival but have ignored essential supportive therapies and psychological and social well-being safeguard programs. Recent research of early integrated palliative care, including supportive care, aimed to obtain patient-centered therapeutic objectives. Noteworthy, advanced cancer patients often present a multiplicity of signs and

symptoms responsible for physical impairment and reduction of functional abilities with consequent impossibility of carrying out the common daily activities. Additionally, the psycho-emotional integrity, the maintenance of family and social relationships and the spiritual issues contribute substantially to the optimal patients' QL. Then, in the care of cancer patients their physical, psychological, social and spiritual needs should be globally addressed. In this context, cancer-related symptoms, which often occur in advanced stage cancer patients and can be either improved or worsened by the antineoplastic therapy, should be treated simultaneously with the planning and implementation of the most appropriate antineoplastic therapy. Therefore, any therapeutic approach should ideally be introduced within a context of the "best supportive care", which includes optimal symptom management. To obtain this scope, the knowledge and awareness of the biological specificity of the disease and patient psychosocial interactions can no longer be considered optional by the multidisciplinary medical team in charge. To date, many of the mechanisms at the basis of the pathogenesis of many cancer-related symptoms are far from being fully understood. Consequently, an effective treatment is yet lacking and represent an unmet need in oncology clinical practice. This Research Topic includes articles in the field of biochemical, and molecular investigations, physiological and clinical studies related to the pathogenesis and potential targeted approaches of some important cancer signs and symptoms. We focused on cachexia, anorexia, muscle wasting, osteopenia, cancer-related anemia, physical inactivity and fatigue. The Research Topic includes Original Research, Review and Perspective articles.

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MULTIDIMENSIONAL ASSESSMENT FOR A PATIENT-CENTERED APPROACH

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EDITORIAL
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Editorial: Biological Mechanism-Based and Patient-Centered Management of Cancer-Related Symptoms and Syndromes

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Keywords: cancer cachexia, anorexia, muscle wasting, quality of life, supportive care, inflammation, interleukin 6, cancer-related anemia

Editorial on the Research Topic

Biological Mechanism-Based and Patient-Centered Management of Cancer-Related Symptoms and Syndromes

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In recent years, full recovery rates for cancer patients significantly increased and mean survival improved. Moreover, chronicization of cancer disease and concerns about aggressive care close to the end-of-life raised the awareness of better risk-benefit balancing. As a result, patients are increasingly aware of quality of life (QoL) issues, and physicians need to find the right balance between cancer treatment and patient well-being. Notably, for most tumors, especially at advanced stages, the concept of cancer as a multidimensional systemic disease replaced the idea of cancer as an organ-confined disease. A new way of design of antineoplastic treatments aiming to patient-centered outcomes including clinical benefit, symptom control, and psycho-social well-being is needed. For this scope, the notion of simultaneous care, i.e., early integration of palliative care into the cancer disease trajectory, must be pursued in clinical practice.

Cancer-associated symptoms, such as anorexia, fatigue, depression, and syndromes such as cachexia, appear more frequently in advanced stages but they actually start with the onset of cancer. Therefore, any antineoplastic therapeutic approach should ideally be planned within the context of "best supportive care," including optimal symptom management and careful psychosocial and spiritual counseling (Madeddu et al., 2015). However, to date the exact biological mechanisms of cancer-associated symptoms and their specific treatment have not been well defined yet.

This Research Topic aimed to gain the knowledge of the pathophysiology of cancer symptoms and syndromes to implement a proactive structured evaluation and targeted interventions. We included just some significant conditions, namely anorexia, weight loss/cachexia, and anemia that are very important for patient suffering, although yet unmeet concerns in clinical practice. The final aim is to develop a model, which could be applied also to other symptoms or syndromes.

This Topic provides significant contributions in the issue of cancer-related muscle wasting and cachexia pathogenesis and treatment. Interestingly, Barreto et al. analyzed the proteomic signature of muscle wasting induced by different conditions (i.e., cancer cachexia and chemotherapy). They

showed a significant activation mainly of the pathways that regulate nucleotide and fatty acid metabolism, ATP synthesis, muscle and heart function, and ROS scavenging. This evidence has important translational implications and supports a combination strategy as the potentially most effective treatment for cachexia. This evidence is further supported by Pin et al. which suggested that inhibiting a single proteolytic pathway is not a good strategy to contrast cancer-induced muscle wasting. In fact, they showed that the inhibition of the Ca^{2+}-dependent proteases did not change body weight loss and muscle wasting in some animal experimental models of cancer cachexia.

As a consequence of changes in body composition and energy metabolism, as well as of treatment-related toxicities, advanced cancer patients very often show a decreased tolerance to exercise and reduced levels of physical activity. Marmonti et al. present a model of bed-rest induced muscle wasting, demonstrating how inactivity may contribute to muscle atrophy associated with a significant decline in muscle mass and force, as well as bone mass loss. Similarly, Bonetto et al. demonstrated that bone loss is associated with tumor growth in two experimental models of colorectal cancer cachexia (HT-29, and $Apc^{Min/+}$).

A fundamental step in establishing an effective targeted supportive care of cancer symptoms is represented by their structured multidimensional evaluation. Argilés et al. presents the validation of the new tool Cachexia Score (CASCO) and its shorten version miniCASCO, for the diagnosis and numerical staging of cancer cachexia in different level of severity.

One of the most frequent and clinically relevant signs of advanced stage cancer patients is cancer-related anemia (CRA). The review by Madeddu et al. is focused on the main and novel mechanisms involved in the multifactorial pathogenesis of CRA and provides useful insights for the development of an effective mechanism-based approach, which may be able to promote a relevant amelioration of patients' quality of life.

During the last years, it has become very clear that a combination of nutrition, nutraceuticals, and drugs is a much-preferred therapeutic approach to fight against cancer cachexia. In the present Research Topic different therapeutic approaches have been proposed. Blauwhoff-Buskermolen et al. showed in a population of advanced non-small-cell lung cancer patients that those with anorexia (one of the main features of cachexia) had significantly higher ghrelin levels compared to those without anorexia. Indeed, ghrelin plays an important orexigenic role by stimulating the production of orexigenic neurons such as neuropeptide Y. Then, anorectic/cachectic cancer patients can benefit from a treatment with a ghrelin receptor agonist (Garcia et al., 2013; Temel et al., 2016). Interleukin-6 (IL-6) is the main proinflammatory cytokines involved in the etiopathogenenesis of the main symptoms and syndromes of advanced cancer, including CRA (Macciò et al., 2005) and cachexia (Zimmers et al., 2016). Consistently, IL-6 inhibitors obtained promising results in the treatment of cachexia and related symptoms (Bayliss et al., 2011; Bekaii-Saab et al., 2011; Prado et al., 2012). In our topic, Au et al. investigated the potential mechanisms of Selumetinib (mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor) in Lewis lung carcinoma, an experimental animal model of cancer cachexia. Selumetinib reduced tumor mass and circulating and tumor IL-6, but did not preserve muscle mass. Another strategy is discussed by Molfino et al. they showed that a dietary supplementation with docosahexaenoic acid (DHA) was associated with increased DHA levels and omega-3 index in red blood cells membranes of breast cancer patients.

We hope that the present Research Topic could contribute a greater insight to enable earlier and more effective supportive and palliative care for cancer patients. In fact, although traditional cornerstones, such as diagnosis, staging, and anticancer treatment will continue to play a crucial role, the knowledge of the biological specificity of cancer-associated symptoms should be considered a central issue by the multidisciplinary team to optimize a patient-focused care. We are awareness that such multidimensional personalized approach should include also the assessment of the psychosocial and spiritual domains, which can help patients to develop personal priorities regarding relationships, religious and spiritual beliefs, deal with the urgency of resolving conflicts, and achieve personally meaningful goals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Cancer and Chemotherapy Contribute to Muscle Loss by Activating Common Signaling Pathways

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Cachexia represents one of the primary complications of colorectal cancer due to its effects on depletion of muscle and fat. Evidence suggests that chemotherapeutic regimens, such as Folfiri, contribute to cachexia-related symptoms. The purpose of the present study was to investigate the cachexia signature in different conditions associated with severe muscle wasting, namely Colon-26 (C26) and Folfiri-associated cachexia. Using a quantitative LC-MS/MS approach, we identified significant changes in 386 proteins in the quadriceps muscle of Folfiri-treated mice, and 269 proteins differentially expressed in the C26 hosts ($p < 0.05$; $-1.5 \geq$ fold change $\geq +1.5$). Comparative analysis isolated 240 proteins that were modulated in common, with a large majority (218) that were down-regulated in both experimental settings. Interestingly, metabolic (47.08%) and structural (21.25%) proteins were the most represented. Pathway analysis revealed mitochondrial dysfunctions in both experimental conditions, also consistent with reduced expression of mediators of mitochondrial fusion (OPA-1, mitofusin-2), fission (DRP-1) and biogenesis (Cytochrome C, PGC-1α). Alterations of oxidative phosphorylation within the TCA cycle, fatty acid metabolism, and Ca^{2+} signaling were also detected. Overall, the proteomic signature in the presence of both chemotherapy and cancer suggests the activation of mechanisms associated with movement disorders, necrosis, muscle cell death, muscle weakness and muscle damage. Conversely, this is consistent with the inhibition of pathways that regulate nucleotide and fatty acid metabolism, synthesis of ATP, muscle and heart function, as well as ROS scavenging. Interestingly, strong up-regulation of pro-inflammatory acute-phase proteins and a more coordinated modulation of mitochondrial and lipidic metabolisms were observed in the muscle of the C26 hosts that were different from the Folfiri-treated animals. In conclusion, our results suggest that both cancer and chemotherapy contribute to muscle loss by activating common signaling pathways. These data support the undertaking of combination strategies that aim to both counteract tumor growth and reduce chemotherapy side effects.

Keywords: Folfiri, C26, proteomics, muscle, inflammation, cachexia, mitochondria, mitochondrial fusion and fission

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INTRODUCTION

According to the American Cancer Society, colorectal cancer represents the third leading cause of cancer-related deaths in the United States (American Cancer Society, 2015). Each year, about 150,000 Americans are diagnosed with colorectal cancer, and one third of those individuals die from the disease (Siegel et al., 2015). Colorectal cancer therapy frequently includes treatment with 5-fluorouracil (5-FU), Leucovorin (LV) and CPT-11, a combination also known as Folfiri. Among the several side effects frequently associated with the administration of Folfiri, increased fatigue represents one of the most common (Montagnani et al., 2011). Notably, cachexia poses a serious problem for patients' quality of life and survival.

Cachexia is a devastating condition associated with several types of malignant cancers and is comorbid in 22–55% of all colorectal cancer cases (Thoresen et al., 2013). A major contributor of colorectal cancer morbidity and mortality, cachexia is primarily responsible for body and muscle weight loss and correlates with tumor burden, increased pro-inflammatory cytokine levels, fatigue, and reduced response to chemo- and radio-therapy (Ravasco et al., 2007; Bapuji and Sawatzky, 2010; Fearon et al., 2012). Studies suggest that cachexia may result from tumor-host interactions or activation of an inflammatory response. We reported that blocking muscle wasting can prolong life even in the absence of effects on tumor growth (Benny Klimek et al., 2010). Hence, targeting cachexia *per se* could improve outcomes and enhance tumor-free survival.

Notably, although the molecular mechanisms responsible for the development of cancer cachexia have been studied for quite some time, it is not completely clear whether cancer treatments also play a causative role in the development of cachexia. Along this line, the use of cytotoxic and anti-proliferative drugs for the treatment of cancer is frequently accompanied by several pronounced side effects, including nausea, diarrhea, anorexia and fatigue, all of which are responsible for significantly decreasing the quality of life of cancer patients and increasing morbidity and mortality. Interestingly, findings show that chemotherapy can promote cachexia development regardless of its effects on tumor growth (Damrauer et al., 2008; Garcia et al., 2008).

Furthermore, it has also been reported that cancer patients affected with muscle depletion (regardless of body weight) are more susceptible to developing severe drug-associated toxicity and show a poorer prognosis. Conversely, subjects with higher muscle mass or not showing sarcopenia are generally more resistant and may tolerate higher doses of chemotherapy (Antoun et al., 2010; Prado et al., 2011; Thoresen et al., 2013; Jung et al., 2015; Stene et al., 2015). We recently reported that Folfox and Folfiri, which are chemotherapeutics utilized for the treatment of solid tumors, may contribute to the development of cachexia and muscle weakness by promoting oxidative stress-associated MAPK activation and by affecting the muscle mitochondrial pool (Barreto et al., 2016). Despite this, it is still partially unknown whether chemotherapy directly promotes cachexia and whether this occurs by activating the same molecular mechanisms associated with muscle wasting in the presence of a tumor.

The purpose of this study was to identify and compare signaling pathways associated with cancer- and chemotherapy-induced muscle wasting based on differences in protein expression. We performed LC-MS/MS-based proteomic profiling of quadriceps muscle from mice bearing the Colon-26 (C26) adenocarcinoma and from mice administered Folfiri for 5 weeks (Bonetto et al., 2012; Barreto et al., 2016). We then employed a software-based analysis to identify upstream regulators and causal networks associated with known diseases and functions. Together, this study represents one of the first attempts to perform a proteomic-based investigative approach in the skeletal muscle of mice that are affected with cachexia with potential translational implications for tumor-derived cachexia, as well as muscle depletion due to chemotherapy.

Abbreviations: C26, Colon-26 adenocarcinoma; LC-MS/MS, liquid chromatography tandem mass spectrometry; 5-FU, 5-fluorouracil; LV, leucovorin; ROS, reactive oxygen species; TCA, tricarboxylic acid; ATP, adenosine triphosphate; MAPK, mitogen-activated protein kinase; APR, acute phase response.

MATERIALS AND METHODS

Animals

All experiments were conducted with the approval of the Institutional Animal Care and Use Committee at Indiana University School of Medicine and were in compliance with the National Institutes of Health Guidelines for Use and care of Laboratory Animals. In order to investigate the effect of chemotherapy on muscle mass, 8-week old CD2F1 male mice ($n = 8$; Harlan, Indianapolis, IN) were administered Folfiri, a combination of 5-fluorouracil (50 mg/kg), leucovorin (90 mg/kg) and CPT-11 (24 mg/kg), intraperitoneally (i.p.), twice a week for five consecutive weeks (Barreto et al., 2016). Based on previous findings, the amounts of drugs that were delivered to the experimental animals were not exceeding clinically relevant concentrations (Barreto et al., 2016). Control mice received an equal volume of vehicle. All drugs were purchased from Sigma Aldrich (St. Louis, MO). For the cancer cachexia model, Colon26 cells were cultured in DMEM medium supplied with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained in a 5% CO_2, 37°C humidified incubator. Cells were passaged when sub-confluent, and 1×10^6 cells were injected subcutaneously in 8-week old CD2F1 male mice ($n = 8$). Non-tumor bearing normal mice were used as controls. Mice were weighed daily then euthanized under light isoflurane anesthesia. Tissues were collected and weighed, then snap frozen in liquid nitrogen and stored at −80°C for further studies.

Sample Preparation

DL-Dithiothreitol (DTT), urea, triethylphosphine, iodoethanol, and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade 0.1% formic acid in acetonitrile (ACN) and 0.1% formic acid in water (H_2O) were purchased from Burdick and Jackson (Muskegon, MI, USA). Modified sequencing grade porcine trypsin was obtained from Princeton Separations (Freehold, NJ, USA). To 70 mg of each of the liquid N_2-pulverized quadriceps muscle

samples, 400 μL of 8 M urea/10 mM DTT was added. Each tissue sample was treated by light sonication and mixed for 1 h at room temperature, followed by centrifugation at 13,000 rpm for 10 min. The protein concentration was measured by Bradford assay (Bradford, 1976). 20 μL was removed and 20 μL of 100 mM ammonium carbonate, pH 10.8, was added to the samples. 40 μL of reduction/alkylation cocktail (97.5% acetonitrile, 2% iodoethanol, and 0.5% triethylphosphine) was added to each sample, and samples were incubated in a 37°C incubator for 1.5 h. The samples were speed vacuumed to dryness overnight, and the dry pellets were resuspended in 50 μL ammonium bicarbonate. 2.5 μg trypsin in 100 μL ammonium bicarbonate was added to each sample, and they were incubated at 37°C for 4 h. 2.5 μg trypsin in 50 μL ammonium bicarbonate was then added to each sample, and they were incubated at 37°C overnight.

LC-MS/MS

The digested samples were analyzed using a Thermo Scientific Orbitrap Velos Pro mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo Scientific). Tryptic peptides were injected onto a C18 reversed phase column (TSKgel ODS-100V, 3 μm, 1.0 × 150 mm) at a flow rate of 50 μL/min. The mobile phases A and B were LC-MS grade H_2O with 0.1% formic acid and ACN with 0.1% formic acid, respectively. The gradient elution profile was as follows: 5% B for 5 min, 10–35% B for 150 min, 35–80% B for 10 min, 80% B for 10 min, and 5% B for 5 min. The data were collected in the "Data dependent MS/MS" mode of FT-IT with the ESI interface using normalized collision energy of 35% (CID). Dynamic exclusion settings were set to repeat count 1, repeat duration 30 s, exclusion duration 120 s, and exclusion mass width 10 ppm (low) and 10 ppm (high).

Peptide/Protein Identification and Quantification

The acquired data were searched against the UniProt protein sequence database of MOUSE (released on 06/24/2015) using X1 Tandem algorithms in the Trans-Proteomic Pipeline (TPP, v. 4.6.3) (http://tools.proteomecenter.org/software.php). General parameters were: parent monoisotopic mass error set as 10 ppm, cleavage semi set as yes, missed cleavage sites set at 2, and static modification set as + 44.026215 Da on Cysteine. The searched peptides and proteins were validated by PeptideProphet (Ma et al., 2012) and ProteinProphet (Nesvizhskii et al., 2003) in the TPP. Only proteins and peptides with protein probability ≥0.9000 and peptide probability ≥0.8000 were reported. False discovery rate (FDR) was estimated by a non-parametric concatenated randomized target-decoy database search (Elias and Gygi, 2007). For this experiment and those TPP settings, protein identification FDR was <0.2%. Protein quantity was determined using an in-house label-free quantification software package, IdentiQuant^{XL}, developed to individually and accurately align the retention time of each peptide and to apply multiple filters for exclusion of unqualified peptides to enhance label-free protein quantification. As previously described in detail (Lai et al., 2011), peptide retention time determination using clustering, extraction of peptide intensity using MASIC (Monroe et al., 2008), peptide coefficient of variation calculation, and peptides correlation were all conducted within the software platform to "filter out" unqualified peptides. Using only qualified peptides, protein intensity was calculated using the formula: Protein Intensity = (intensity of peptide 1)/(peptide 1 sharing times) + (intensity of peptide n)/(peptide n sharing times). For a peptide shared by different proteins, the intensity of this peptide was divided by the number of times the peptide was shared. Raw data were deposited in the PeptideAtlas database and are available through identifier PASS00863 (http://www.peptideatlas.org/PASS/PASS00863).

Western Blotting

Total protein extracts were obtained by homogenizing 100 mg quadriceps muscle tissue in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) completed with protease (Roche, Indianapolis, IN) and phosphatase (Thermo Scientific, Rockford, IL) inhibitor cocktails. Cell debris were removed by centrifugation (15 min, 14000 g) and the supernatant collected and stored at −80°C. Protein concentration was determined using the BCA protein assay method (Thermo Scientific, Rockford, IL). Protein extracts (30 μg) were then electrophoresed in 4–15% gradient SDS Criterion TGX precast gels (Bio-Rad, Hercules, CA). Gels were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked with SEA BLOCK blocking reagent (Thermo Scientific, Rockford, IL) at room temperature for 1 h, followed by an overnight incubation with diluted antibody in SEA BLOCK buffer containing 0.2% Tween-20 at 4°C with gentle shaking. After washing with PBS containing 0.2% Tween-20 (PBST), the membrane was incubated at room temperature for 1 h with either Anti-rabbit IgG (H+L) DyLight 800 or Anti-mouse IgG (H+L) DyLight 600 (Cell Signaling Technologies, Danvers, MA). Blots were then visualized with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Optical density measurements were taken using the Gel-Pro Analyzer software. Antibodies used were OPA-1 (#80471), Mitofusin-2 (#9482), DRP-1 (#8570), Cytochrome C (#11940) from Cell Signaling Technologies (Danvers, MA), PGC-1α (#ab3242) from Abcam (Cambridge, MA) and α-Tubulin (#12G10) from Developmental Studies Hybridoma Bank (Iowa City, IA).

Statistics

Comparisons among tissue weights reported in **Table 1** were carried out using Student's *t*-test. A value of $p \leq 0.05$ was considered statistically significant. As for the LC-MS/MS proteomic study, only the proteins identified with at least two peptides and with $-1.5 \geq$ fold change (FC) $\geq +1.5$ were included in the analysis. Comparative analysis between the two datasets was carried over by means of Correlation Engine (Illumina, San Diego, CA). Finally, statistically significant and differentially expressed proteins (FDR < 5%) from both datasets were imported into Ingenuity Pathway Analysis (IPA; Qiagen, Valencia, CA) to identify significant pathways, upstream regulators and causal networks associated with known diseases and functions.

TABLE 1 | Body and tissue weights in tumor-bearing mice and chemotherapy-treated animals.

	Cancer		Chemotherapy	
	Control ($n = 8$)	C26 ($n = 8$)	Vehicle ($n = 8$)	Folfiri ($n = 8$)
IBW	25.35 ± 1.61	26.60 ± 1.12	26.70 ± 1.70	24.6 ± 2.70
FBW	25.62 ± 1.80	22.4 ± 2.63^{a}	28.80 ± 1.70	24.6 ± 3.10^{bb}
GSN	0.57 ± 0.02	0.44 ± 0.03^{aaa}	0.55 ± 0.04	0.49 ± 0.01^{bb}
Quadriceps	0.74 ± 0.05	0.56 ± 0.08^{aaa}	0.77 ± 0.05	0.62 ± 0.02^{bbb}
Heart	0.53 ± 0.02	0.45 ± 0.06^{aa}	0.57 ± 0.04	0.61 ± 0.02
Liver	4.52 ± 0.23	4.65 ± 0.64	4.88 ± 0.52	4.95 ± 0.34
Spleen	0.28 ± 0.03	1.09 ± 0.81^{aaa}	0.27 ± 0.03	0.81 ± 0.12^{bbb}
Fat	2.25 ± 0.35	0.89 ± 0.45^{aaa}	2.89 ± 0.54	1.08 ± 0.32^{bbb}

Data are expressed as means ± SD. Initial body weight (IBW) and Final body weight (FBW) are reported in grams (g). Gastrocnemius (GSN), quadriceps, liver, spleen and fat are reported as weight/100 mg IBW. Significance of the differences: $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ vs. Control; $^{bb}p < 0.01$, $^{bbb}p < 0.001$ vs. Vehicle.

RESULTS

Tumor Growth and Chemotherapy Administration Promote the Occurrence of Severe Cachexia

In order to limit the variability across the different animal models, both tumor hosts and animals treated with chemotherapy were sacrificed when muscle loss was comparable (about 15%) and resembling a condition of severe cachexia, as previously shown (Bonetto et al., 2011). In particular, CD2F1 male mice ($n = 8$) were injected s.c. with C26 adenocarcinoma cells and weighed daily. After 14 days from tumor injection, tumor hosts were sacrificed when their final body weight reached about 87% of the control animals ($p < 0.01$) (**Table 1**) (Bonetto et al., 2011). In this setting, marked muscle wasting was observed, both at the gastrocnemius and quadriceps level (−23 and −25% vs. control, respectively; $p < 0.001$). A condition associated with cardiac atrophy was also displayed and is associated with tumor growth (−15% vs. control; $p < 0.01$). Similar to that previously described in the same experimental model of cancer cachexia (Bonetto et al., 2011, 2012), severe depletion of adipose tissue (−61% vs. control; $p < 0.001$), as well as splenomegaly (+289% vs. control; $p < 0.001$), were observed (**Table 1**). In separate set of experiments, CD2F1 normal mice were administered Folfiri (twice/week) for 5 weeks, and body weight was monitored daily (Barreto et al., 2016). At the time of sacrifice, the chemotherapy-treated mice showed significant loss of body weight (−15%, $p < 0.01$), consistent with depletion of gastrocnemius (−11%, $p < 0.01$), quadriceps (−20%, $p < 0.001$), and fat (−63%, $p < 0.001$) mass. Interestingly, the heart mass was not affected by chemotherapy administration. Similar to the tumor-bearing animals, a dramatic increase in spleen size (+200% vs. vehicle, $p < 0.001$) was also observed (**Table 1**).

C26 Tumor and Folfiri Influence the Skeletal Muscle Proteome

In order to elucidate the mechanisms responsible for muscle wasting in cancer-associated cachexia and chemotherapy-induced cachexia, we investigated the muscle proteome in C26 hosts and in mice treated with Folfiri. By taking advantage of a LC-MS/MS quantitative approach, we detected 422 proteins in the muscle of animals carrying the C26 tumor and 511 proteins in the muscle of mice exposed to chemotherapy (**Tables S1–S3**). Of note, 269 proteins, among the ones identified with at least two peptides and with $-1.5 \geq$ fold change (FC) $\geq +1.5$, were differently expressed in the C26 hosts, while 386 were significantly ($p < 0.05$) modulated in the muscle of animals treated with Folfiri (**Table S4**).

In particular, among the 269 proteins modulated in the cancer setting, 235 were down-regulated, while 34 were up-regulated (**Figure 1A**, **Table S4**). Analogously, following chemotherapy administration, a large majority of proteins (345) were down-regulated, while only a small subset of proteins (41) was up-regulated or expressed exclusively in the muscle of animals receiving Folfiri (**Figure 1A**, **Table S4**). Comparative analysis performed by means of Illumina Correlation Engine identified 240 proteins that were modulated in both experimental conditions ($p = 3.3E\text{-}244$), with a significant positive correlation (218 proteins; $p < 1.0E\text{-}323$) for the proteins that were down-regulated in both experimental models (**Figure 1B**). Quite interestingly, members of metabolic pathways (39.1% in Folfiri, 44.5% in C26) and structural proteins (24.4% in Folfiri, 21.9% in C26) were the most represented in both subsets (**Figure 1C**, left and middle panels). A similar situation was also observed among the 240 proteins modulated in common, with metabolic and structural proteins representing the large majority and totaling about 68% (**Figure 1C**, right).

Muscle Mitochondrial Dysfunctions Are the Main Event Associated with Tumor Growth or Chemotherapy Treatment

The IPA-based analysis performed on the proteins detected in both datasets identified a series of pathways that were similarly affected by both cancer and chemotherapy (**Figure 2**). In particular, the most represented among the Top-20 pathways influenced by either tumor growth or chemotherapy treatment were associated with mitochondrial dysfunctions, but also alterations of oxidative phosphorylation, TCA cycle, epithelial and tight junction signaling, glycolysis, fatty acid β-oxidation and

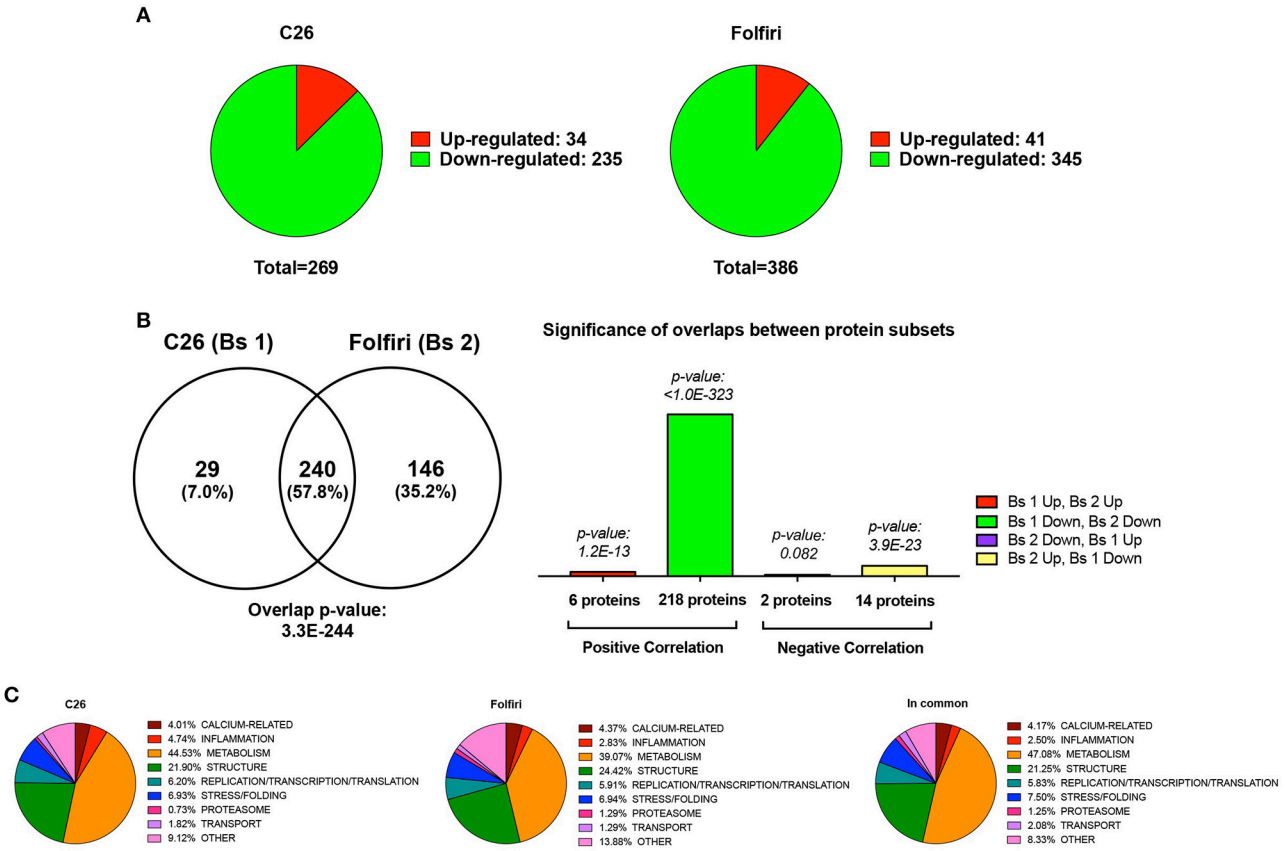

FIGURE 1 | Cancer and chemotherapy promote the down-regulation of 235 and 345 muscle proteins, respectively. (A) Pie charts showing the number of proteins that are down-regulated (green) or up-regulated (red) following tumor growth (C26, left) or chemotherapy (Folfiri, right). **(B)** Comparative analysis between C26 and Folfiri biosets (Bs 1 and Bs 2, respectively). The Venn diagram (left) shows the number of proteins that are modulated in common or in the presence of either C26 or Folfiri. The overlap *p*-value, indicating the statistically significant overlap between the two datasets, is also reported. Analogously, the significance of the overlap between different protein subsets within the group of proteins modulated in both subsets is also presented (right). **(C)** The proteins detected in the C26 (left) and Folfiri (middle) datasets, or modulated in common (right) were classified based on their function and/or pathway and distributed as shown in the pie charts. The percentage is expressed over the total number in proteins in each dataset.

protein kinase A (**Figures 2**, **3A**; **Table S4**). Here we show that proteins taking part to the β-oxidation were markedly reduced in the C26-bearing animals, while regulators of the synthesis of fatty acids, such as FAS, TKT and PDK4, were significantly up-regulated (**Figures 3A,C**; **Table S4**). Similarly, members of the respiratory chain, such as NDUS6, NDU5, and CISD1, were not detected in the muscle of tumor hosts, suggesting that the energetic metabolism was severely compromised (**Figures 3A,C**; **Table S4**). Interestingly, all proteins modulated in the Folfiri dataset were drastically down-regulated with few exceptions, namely several enzymes involved in the metabolism of lipids (PLIN1, HSD17B10, FASN, and ACOT2) or amino acids, such as leucine and valine (IVD, HIBCH, ALDH6A1), the GMP reductase 1 (GMPR) involved in the synthesis and conversion of nucleotides, two regulators of the Krebs cycle (MCP2 and PCCB), and two members of the mitochondrial respiratory chain (NDUFB8 and UQCR10) (**Figures 3A,C**). In line with these and previous observations (Pin et al., 2015; Barreto et al., 2016), alterations of muscle mitochondrial homeostasis, as suggested by the levels of markers of mitochondrial fusion (OPA-1, mitofusin-2), fission (DRP-1) and biogenesis (Cytochrome-C, PGC-1α), were displayed in the muscle of both C26 hosts and animals exposed to chemotherapy (**Figure 4**).

In line with previous observations (Costelli and Baccino, 2003; Fearon et al., 2012), we also show that the levels of PSMA6 and UBA1, major proteasomal components, were increased in the muscle of Folfiri-treated animals. Similarly, UBA1 and UB2L3, enzymes associated with the proteasome system, were also up-regulated in the muscle of C26 hosts (**Table S4**).

Of note, epithelial and tight junction signaling, as well as actin cytoskeleton and calcium signaling, were modulated following either C26 growth or Folfiri treatment (**Figures 2**, **3B,C**; **Table S4**). In particular, 15 calcium-binding proteins were markedly down-regulated by Folfiri administration, thus suggesting a deregulation of these pathways (**Figures 3B,C**; **Table S4**). Interestingly, structural proteins, such as KERA and LAMA2, were up-regulated in the muscle of both Folfiri-treated animals and tumor-bearing mice (**Figures 3B,C**; **Table S4**),

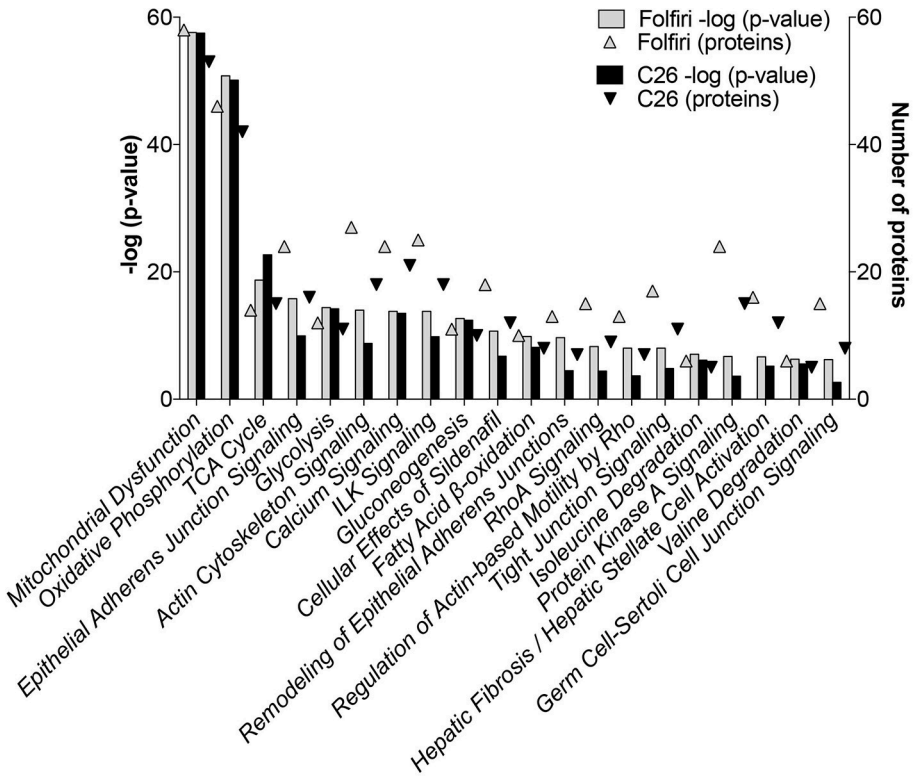

FIGURE 2 | Pathway analysis of muscle proteomic profiling in cancer or chemotherapy-induced cachexia. By utilizing the IPA software, the C26 and Folfiri datasets were subjected to pathway analysis. The pathways were ranked based on their overlap *p*-value (bars). Top-20 pathways are reported in the diagram, along with the number of proteins modulated within each pathway (triangles).

unlike other proteins, such as MYOZ2, overexpressed in the muscle of C26 hosts and, conversely, down-regulated in the muscle of chemotherapy-treated animals (**Table S4**). Interestingly, proteins of the 14-3-3 family were down-regulated both in tumor hosts and Folfiri-treated animals (**Table S4**).

Furthermore, in line with our previous findings (Bonetto et al., 2011), the expression of the majority of the identified positive acute phase response (APR) proteins (CO3, FIBA, FIBB, FIBG, and HPT) was more elevated in the muscle of C26 hosts with respect to the controls, while all negative APRs (TTHY, TRFE and ALBU) were down-regulated (**Figure 3C**; **Table S4**). Also, in the presence of chemotherapy, the expression of a number of proteins associated with inflammatory pathways was affected, although the large majority of these mediators were generally down-regulated (**Figure 3C**; **Table S4**).

Similar Mechanisms Are Likely Responsible for Muscle Wasting in Both C26- and Folfiri-Associated Cachexia

The "Upstream Regulator Analysis" predicted which transcriptional regulators are involved upstream of the changes observed and whether they are likely activated (z-score > 2) or inhibited (z-score < -2). In particular, among the Top-20 upstream regulators expected to be activated in both datasets, the histone lysine demethylase KDM5A, the mTORC2 subunit RICTOR, the mitogenic-activated protein kinase isoform 4 (MAP4K4), and the contraction regulator Smoothelin-like 1 (SMTNL1) showed the highest p-value, providing evidence of a statistically significant overlap between our data and the pathways generally associated with these transcription factors (**Figures 5A–C**). Conversely, among the Top-20 upstream regulators characterized by a z-score < -2 (i.e., likely inhibited) in the skeletal muscle of mice either carrying the C26 tumor or treated with Folfiri, the insulin receptor (INSR), the Peroxisome Proliferator-Activated Receptor Gamma Co-activator 1 Alpha (PPARGC1A, also known as PGC1α) and the tumor suppressor gene RB1 were the highest ranked, whereas the insulin-like growth factor-1 receptor (IGF1R), the regulators of muscle differentiation MYOD1 and MEF2C, as well as other members of the Peroxisome Proliferator-Activated Receptor Gamma family were also identified with lower p-values (**Figures 5B–D**). Of note, no major difference between the two datasets were reported, thus further supporting the idea that similar mechanisms contribute to muscle wasting in both experimental conditions.

Muscle Disorders and Alterations of Energy Production and Nucleotide Metabolism Are Associated with Cachexia Due to Cancer or Chemotherapy

The "Disease and Function Analysis" anticipated which alterations were likely associated with the protein changes

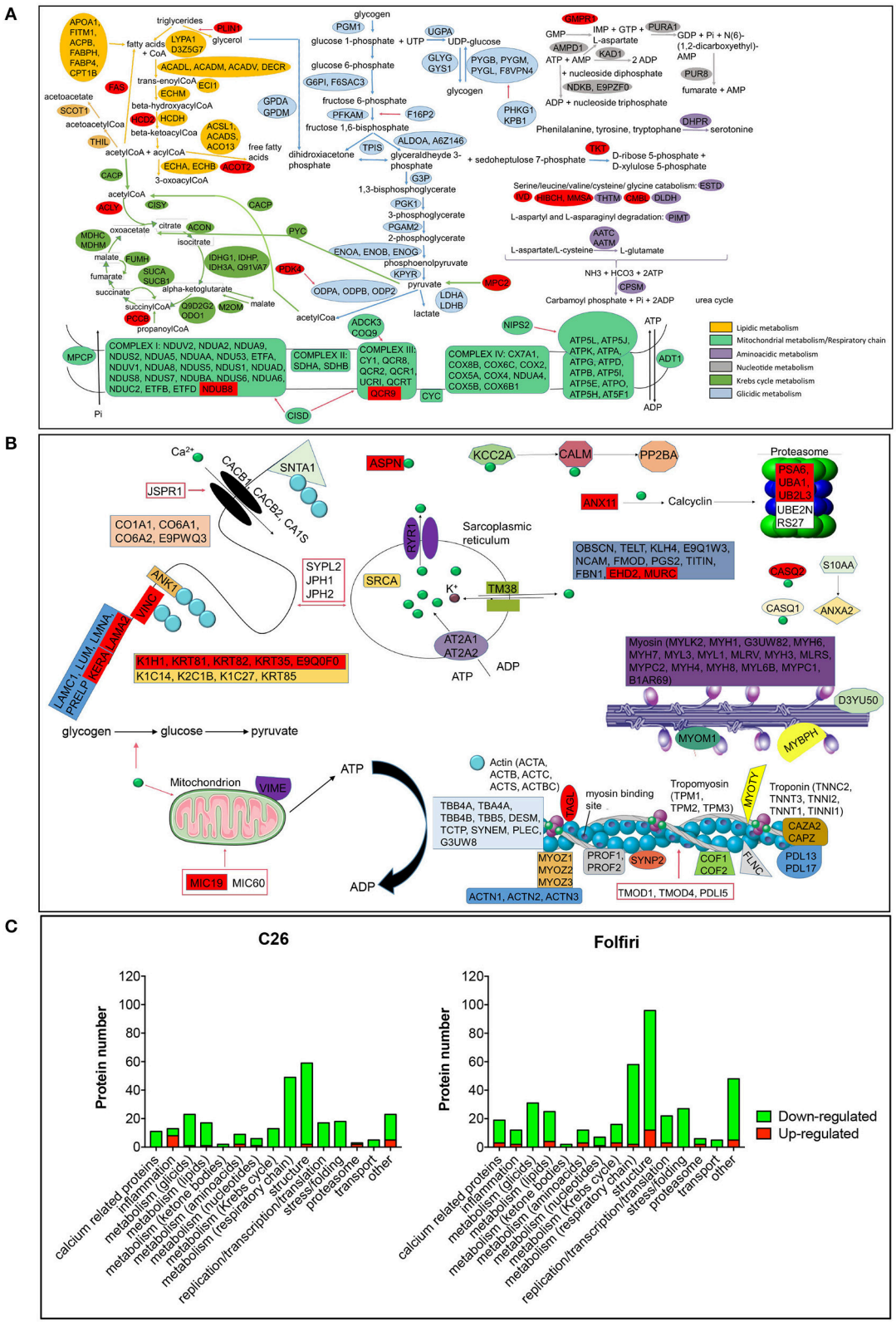

FIGURE 3 | Major pathways affected in cancer- and drug-induced cachexia. (A) Proteins belonging to any metabolic pathway are indicated and classified as shown in color legend (right). Proteins up-regulated in almost one comparison (C26 vs. control or Folfiri vs. vehicle) are shown in red. All other proteins reported are

(Continued)

FIGURE 3 | Continued
down-regulated. **(B)** Structural proteins, calcium- and proteasome-associated proteins affected by either cancer or chemotherapy. **(C)** Number of proteins taking part to any of the major pathways affected in cancer- and chemotherapy-induced cachexia. Up-regulated proteins are reported in red, down-regulated proteins are shown in green.

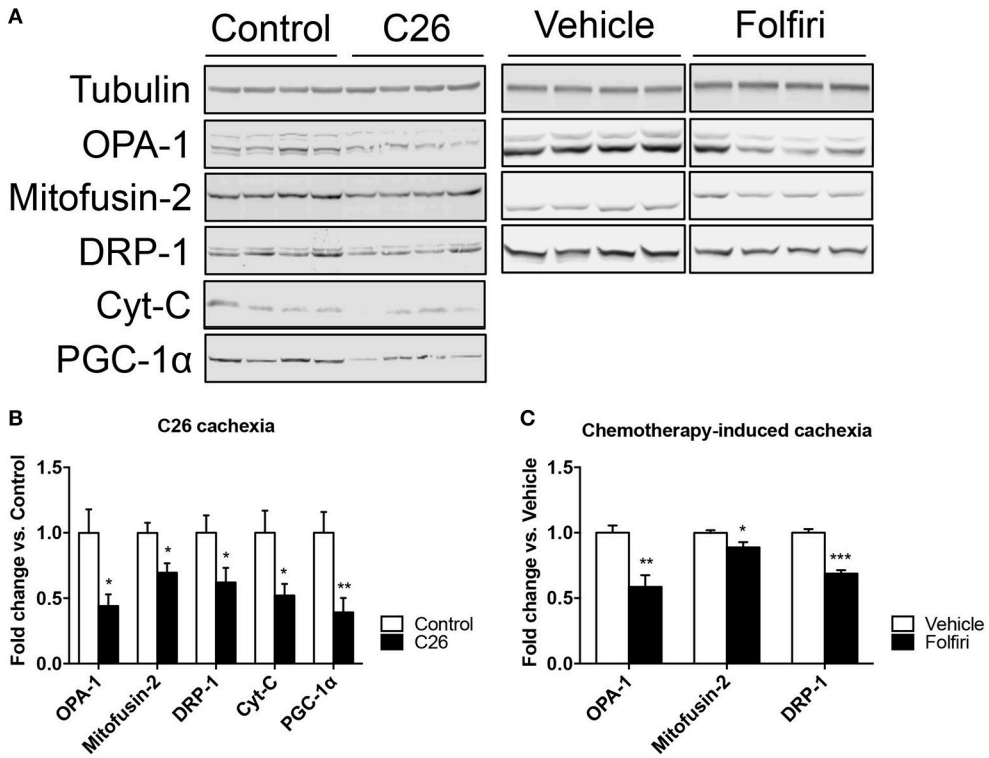

FIGURE 4 | The expression of markers of mitochondrial fusion, fission and biogenesis is affected by tumor and drug-induced cachexia. (A) Representative western blotting for OPA-1, Mitofusin-2, DRP-1, Cytochrome-C (Cyt-C), and PGC-1α in the muscle of C26 hosts or mice exposed to Folfiri. **(B,C)** Quantification of the bands ($n = 4$). Significance of the differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control or Vehicle.

reported in the muscle of tumor hosts or in mice exposed to chemotherapy. Interestingly, in both datasets, movement disorders, damage or death of muscle cells and muscle weakness/fatigue were activated (z-score > 2), (**Figures 6A–C**). Further, cardiac dysfunctions seemed to be associated exclusively with tumor growth (**Figure 6A**), consistent with the decrease in heart mass shown in **Table 1**. Similarly, the evidence of drug-related neurotoxicity was reported only in the Folfiri dataset (**Figure 6B**). Conversely, inhibition of nucleotide metabolism and synthesis of ATP, as well as reduced muscle function and modification of ROS, were predicted in both experimental conditions (**Figures 6B–D**), while alterations of fatty acid metabolism and lipid oxidation were only associated with the chemotherapy treatment (**Figure 6D**).

DISCUSSION

Cachexia is a devastating syndrome associated with many disease states, such as cancer, congestive heart failure, diabetes, kidney failure, and HIV/AIDS (Bonetto et al., 2012; Fearon et al., 2012; Dutt et al., 2015). Cancer cachexia is characterized by systemic inflammation, negative protein and energy balance, and an involuntary loss of lean body mass, with or without wasting of adipose tissue (Aoyagi et al., 2015). Muscle weakness has been postulated to occur due to a combination of muscle breakdown, dysfunction, and a decrease in the ability to repair (Isaac et al., 2016). Effective therapies are presently limited, whereas the removal of the primary tumor remains the only definitive treatment strategy. The idea that anticancer treatments may also result in muscle atrophy is currently being debated. Along this line, we recently reported that chemotherapy regimens utilized for the therapy of colorectal cancer, such as Folfox and Folfiri, drive alterations consistent with muscle wasting and muscle weakness (Barreto et al., 2016). Despite this, the mechanisms responsible for muscle loss in the presence of anticancer treatments are not completely known. Furthermore, it is not clear whether similar mechanisms are activated in the presence of either cancer or chemotherapy, thus leading to muscle wasting.

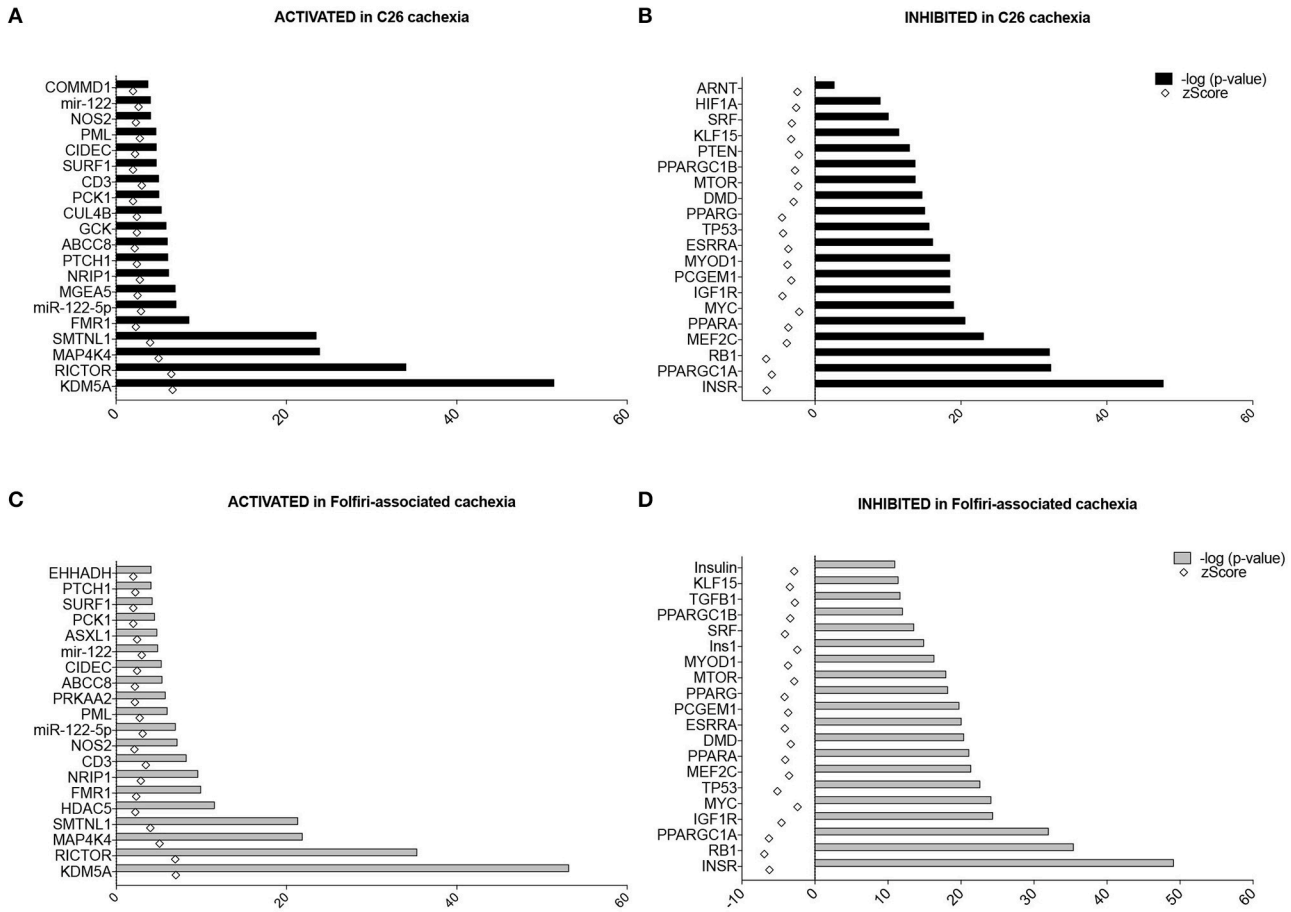

FIGURE 5 | Upstream regulators in C26- and Folfiri-induced cachexia. The IPA-mediated analysis identified several upstream regulators ranked based on their overlap *p*-value, whose activation (*z*-score > 2) or inhibition (*z*-score < −2) is associated with the phenotype observed. Top panel: Top-20 upstream regulators activated **(A)** or inhibited **(B)** in C26 cachexia. Bottom panel: Top-20 upstream regulators activated **(C)** or inhibited **(D)** in Folfiri-associated cachexia.

It has been suggested that anorexia, i.e., the reduced or loss of the desire to eat, may represent one of the major causes associated with body and muscle weight loss in oncologic patients (Molfino et al., 2010). Despite the fact that anorexia has been shown to play a role in the development of cachexia in several experimental models, in the present research work we assessed the proteomic profiling in animals only based on the amount of muscle wasting. Moreover, based on available studies, pair-feeding was not performed, particularly because previous results have shown that muscle protein waste is mainly associated with acceleration of protein breakdown rates, regardless of food intake, whereas in pair-fed animals the decrease of skeletal muscle protein content is mainly due to impaired protein synthesis (Tessitore et al., 1993). Therefore, reduced food intake and metabolic competition by the tumor do not seem to justify the hypercatabolic state in the tumor hosts. This view also is shared by the ground-breaking report from Lecker et al. (2004). Similarly, Garcia et al. (2013) showed that cisplatin administration normally affects food intake. However, pair feeding experiments carried out in the same report showed that chronic administration of cisplatin did not induce anorexia and that animals receiving chemotherapy were showing exacerbated body weight loss, regardless of their food intake (Garcia et al., 2013). More recently, in another model we showed that chronic administration of Folfiri to normal animals does not cause anorexia and is only responsible for acute toxicity associated with sudden and temporary drops in food intake, while, on the contrary, the cumulative food intake does not differ from the vehicle-treated animals (Barreto et al., 2016).

In the present experimental work the proteomic analysis performed in skeletal muscle revealed a similar impairment of several metabolic pathways and muscle structures, also consistent with previously published observations (Fontes-Oliveira et al., 2013; Pin et al., 2015; Barreto et al., 2016). Along this line, the large majority of all metabolic enzymes, particularly those associated with the maintenance of the aerobic catabolism (i.e., Krebs cycle and respiratory chain), were drastically down-regulated in both datasets, further supporting the idea that muscle wasting due to chemotherapy administration can be defined as real cachexia. Indeed, oxidative pathways and mitochondrial abnormalities with consequent decreased

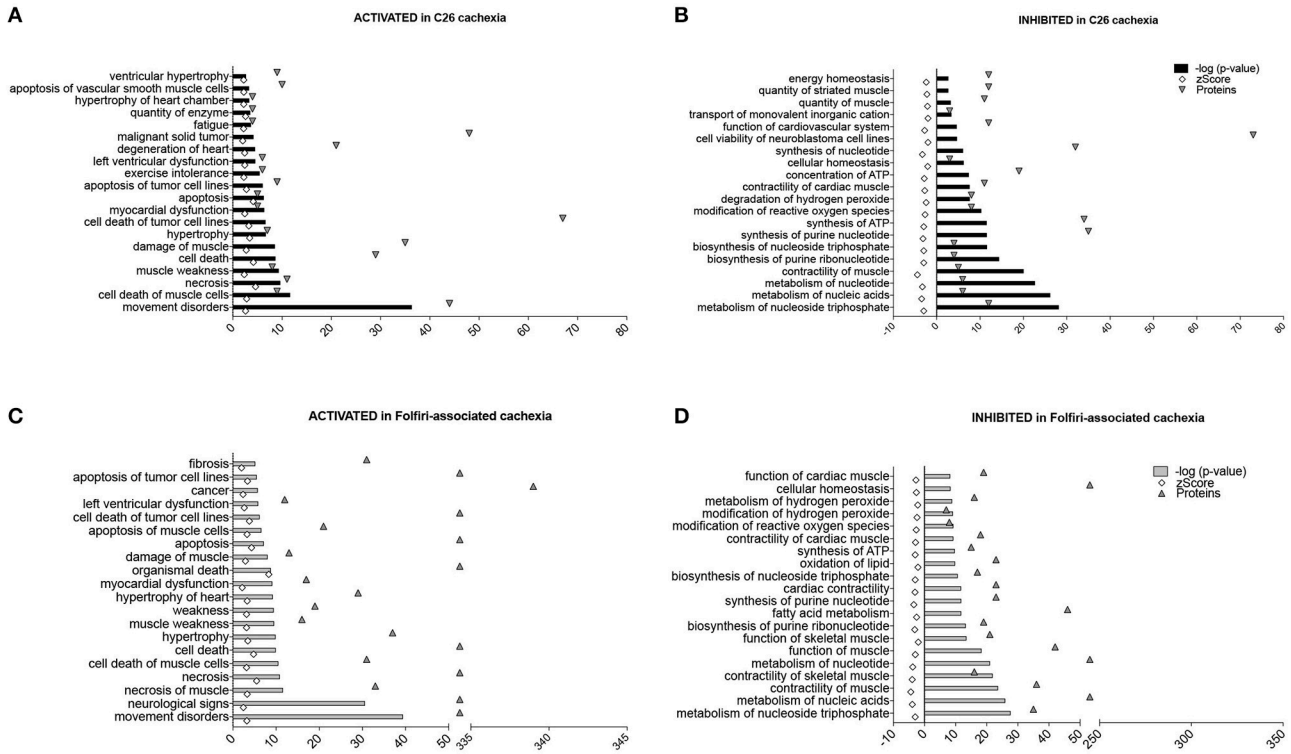

FIGURE 6 | Diseases and functions associated with C26- and Folfiri-induced cachexia. The IPA software identified diseases and functions, ranked based on their overlap *p*-value, expected to be activated (*z*-score > 2) or inhibited (*z*-score < −2) in the C26 and Folfiri datasets. Top panel: diseases and functions activated **(A)** or inhibited **(B)** in C26 cachexia. Bottom panel: diseases and functions activated **(C)** or inhibited **(D)** in Folfiri-associated cachexia. Only the Top-20 diseases and functions are reported in the diagram, along with the number of correlated proteins (triangles).

production of ATP are already well documented features of cachexia and suggest that changes in these pathways might also contribute to muscle weakness, as frequently observed in association with chemotherapy (Fontes-Oliveira et al., 2013; Argilés et al., 2015; Barreto et al., 2016; Carson et al., 2016). In addition, mitochondrial alterations associated with decreased expression of markers of mitochondrial fusion and fission that are normally involved in the maintenance of the integrity and plasticity of the mitochondrial network (Pernas and Scorrano, 2016) were also reported in the muscle of both C26 bearers and mice treated with chemotherapy. This is also consistent with previous reports from our group and others that suggest that cachexia is generally associated with severe alterations of the muscle mitochondria, which may contribute to the occurrence of muscle atrophy, muscle weakness, as well as the transition to more glycolytic muscle fibers (Pin et al., 2015; Barreto et al., 2016). Interestingly, mitochondrial dysfunctions and increased oxidative stress have been shown to play a role in causing disruptions of the neuromuscular junctions, thus possibly explaining the occurrence of muscle weakness and fatigue following cancer development or chemotherapy treatment (Ibebunjo et al., 2013). This is also consistent with our data showing abnormal junction signaling in the muscle of tumor hosts and animals treated with Folfiri.

Notably, our data also show that a few significant differences exist between the two experimental conditions. In particular, all the enzymes of the aerobic metabolism contributing to the Krebs cycle or the respiratory chain are markedly down-regulated in the muscle of tumor hosts, while the same response is not observed following chemotherapy administration, where a considerable number of proteins appear up-regulated. Similarly, pathways associated with the lipidic metabolism were enhanced by the presence of the C26 adenocarcinoma and substantially inhibited following Folfiri administration. In particular, here we showed that tumor growth is associated with decreased activation of the β-oxidation, generally associated with the breakdown of lipids and fatty acids. Interestingly, the synthesis of FAS and TKT, normally associated with the synthesis of fatty acids, were significantly up-regulated. This apparent discrepancy with the phenotype observed in tumor hosts, characterized by severe depletion of fat tissues, may actually result from a survival mechanism that attempts to restore the fat stores, which are essential in a conditions associated with reduced energy metabolism. To further support this point, we also showed that members of the respiratory chain were not detectable in the muscle of tumor hosts, suggesting that the energetic metabolism was impaired.

Our analysis also provides evidence of a concerted down-regulation of structural proteins and calcium-related proteins

in the muscle of cachectic mice. In particular, alterations of calcium homeostasis have been reported in clinical and experimental cachexia and other inflammation-driven muscle diseases (Isaac et al., 2016), analogously to the impairment of sarcoplasmic structure (Fontes-Oliveira et al., 2013). A large number of calcium-related proteins and almost all structural proteins that were detected were also shown to be down-regulated in tumor-bearing mice, coherently with the loss of skeletal muscle mass and the occurrence of muscle weakness (Bonetto et al., 2009; Waning et al., 2015). Furthermore, proteins of the 14-3-3 family were also shown to be decreased both in tumor hosts and Folfiri-treated animals. Interestingly, these proteins were recently identified as novel myokines required for maintaining myosin content in skeletal muscle (McLean et al., 2015).

A number of other factors in cancer patients are known to increase the catabolic response, leading to unsustainable levels of fat and muscle mobilization and levels of muscle depletion that cause significant morbidity and mortality (Aoyagi et al., 2015). The up-regulation of proteasomal components observed in association with the occurrence of cachexia is consistent with the well-known activation of skeletal muscle degradative systems, such as the ATP-ubiquitin-dependent one (Bossola et al., 2001; Onesti and Guttridge, 2014). This has also been suggested by the overexpression of muscle-specific ubiquitin ligases, as previously reported in conditions associated with cancer cachexia (Lecker et al., 2004). Conversely, we recently showed that mechanisms other than the ones associated with the activation of proteasome-dependent muscle catabolism are responsible for muscle wasting after Folfiri treatment (Barreto et al., 2016). Regardless, here we show that the levels of major proteasomal components were increased in the muscle of Folfiri-treated animals. Similarly, enzymes associated with the proteasome system were also up-regulated in the muscle of C26 hosts. The discrepancy with our previous data may also suggest that the proteasome-dependent systems might have been involved in promoting chemotherapy-dependent muscle depletion at earlier time points, consistent with findings associated with cachexia (Lecker et al., 2004).

In line with previous reports, a robust skeletal muscle APRs transcriptomic response in association with the activation of muscle catabolism was confirmed in the muscle of C26-bearing mice (Bonetto et al., 2011). Also in this case, the response associated with tumor growth was more coordinated than that following administration of Folfiri. Inflammation and high APR levels are considered a hallmark of cancer cachexia, and an integrated physiological response of substrate mobilization driven by inflammation was proposed as mainly responsible for the development of cachexia (Aoyagi et al., 2015). Despite this, the specific mechanisms by which these cytokines produce skeletal muscle dysfunction remain partially undefined (Isaac et al., 2016). It has been hypothesized that hepatic synthesis of positive acute phase response proteins using amino acids liberated from skeletal muscle proteins is a major driver of skeletal muscle proteolysis (Bonetto et al., 2011). In particular, the levels of fibrinogen expressed in liver vs. muscle in this experimental model suggest that muscle might be a greater source of APR proteins than liver (Bonetto et al., 2011).

In the present work, we show that most of the APR proteins are evenly increased in the muscle of mice carrying a tumor or chronically administered chemotherapy, thus supporting the idea that amino acids freed from skeletal muscle structural proteins through proteolysis would be re-synthesized into these secreted proteins and exported from the cell, possibly contributing to muscle wasting (Bonetto et al., 2011). Altogether, this might suggest that the mechanisms responsible for muscle depletion in the presence of a tumor are also playing a role in promoting muscle wasting upon administration of chemotherapy. In particular, and coherent with our previous findings (Bonetto et al., 2011), a large number of proteins associated with inflammatory pathways was affected both in the presence of cancer or chemotherapy, although in the latter the large majority of these mediators were generally down-regulated.

In conclusion, in the present study we aimed at investigating whether *in vivo* chemotherapy administration could drive the development of cachexia similarly to cancer alone. In particular, in order to unravel the direct modulatory effects of either cancer or chemotherapy on muscle proteome we analyzed the proteomic profiling in the skeletal muscle of C26 tumor hosts or animals exposed to Folfiri. Our study design did not take into consideration the complexity of the interactions between tumor- and chemotherapy-driven mediators, thus apparently representing a limitation. Despite recognizing the importance of future investigations particularly designed to fill this gap of information, we believe this approach was required to assess the effects that are exclusively dependent on the use of anticancer drugs and to definitively include the derangements associated with chemotherapy treatment among the conditions characterized by the occurrence of a cachectic phenotype. Along this line, the data in the present study showed remarkable similarities to the proteomic signatures of cachectic muscles from mice carrying tumors or exposed to chemotherapy, thus further validating the idea that anticancer therapies play a substantial role in causing muscle wasting and muscle weakness, similar to cancer. Analogously, the expected disease pattern associated with the described phenotypes was similar in both experimental conditions, which is consistent with the state of activation of the putative upstream regulators. Of note, signs of neurotoxicity were expected exclusively after Folfiri administration, which is consistent with previous findings that report chemotherapy-related neurotoxicity and muscle weakness (Cordier et al., 2011; Barreto et al., 2016; Taillibert et al., 2016). Ultimately, we showed that dysfunctions of the mitochondrial metabolism represent the main consequence associated with the development of cachexia, thereby corroborating the idea that strategies aimed at protecting the muscle mitochondrial pool may, at the same time, contribute to preserve muscle mass and muscle function in the occurrence of cancer or in association with chemotherapy. Based on our results, future studies will warrant the combination of strategies aimed to both counteract tumor growth and reduce the side effects of chemotherapy.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RB and AB; Performed the experiments: RB, GM, and AB; Analyzed the data: GM, FW, and AB; Contributed reagents/materials/analysis tools: FW, FN, and TZ; Wrote the paper: GM, FW, and AB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00472

Table S1 | Detected proteins in the quadriceps muscle of C26-bearing mice.

Table S2 | Detected proteins in the quadriceps muscle of mice treated with Folfiri.

Table S3 | Peptide identification and protein coverage.

Table S4 | Proteomic profiling and pathway analysis of differentially expressed proteins in the skeletal muscle of tumor-bearing mice or mice treated with chemotherapy.

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Plasma Ghrelin Levels Are Associated with Anorexia but Not Cachexia in Patients with NSCLC

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Background and Aims: The ghrelin receptor is one of the new therapeutic targets in the cancer anorexia-cachexia syndrome. Previous studies revealed that plasma ghrelin levels were high in patients with anorexia nervosa and low in obese subjects. We studied to what extent ghrelin levels are related with anorexia and cachexia in patients with cancer.

Materials and Methods: Fasted ghrelin levels were determined as well as anorexia and cachexia in patients with stage III/IV non-small cell lung cancer before chemotherapy. Total plasma ghrelin was measured by radioimmunoassay. Anorexia was measured with the FAACT-A/CS questionnaire (cut-off value $\leq$ 37). Cachexia was determined as >5% weight loss (WL) in 6 months or >2% WL in 6 months in combination with low BMI or low muscle mass. The Kruskal-Wallis test was performed to assess differences in plasma ghrelin levels between four groups: patients with (+) or without (−) anorexia (A) or cachexia (C). Multiple regression analyses were performed to assess differences in plasma ghrelin levels between patients C+ and C− and patients with A+ and A− (adjusted for age and sex).

Results: Forty patients with stage III (33%) or stage IV (68%) were recruited, of which 50% was male. Mean age was 59.6 ± 10.3 years. Sixteen patients had no anorexia or cachexia (A−C−), seven patients had both anorexia and cachexia (A+C+), ten patients had anorexia without cachexia (A+C−) and seven patients had cachexia without anorexia (A−C+). The levels of total plasma ghrelin were significantly different between the four groups of patients with or without anorexia or cachexia ($p = 0.032$): the A+C− patients had significantly higher ghrelin levels [median (IQR): 1,754 (1,404–2,142) compared to the A−C+ patients 1,026 (952–1,357), $p = 0.003$]. A+ patients had significantly higher ghrelin levels compared A− patients (C+ and C− combined, β: 304, $p = 0.020$). Plasma ghrelin levels were not significantly different in C+ patients compared to C− patients (A+ and A− combined, β: −99, $p = 0.450$).

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Conclusions: Patients with anorexia had significantly higher ghrelin levels compared to patients without anorexia. We therefore hypothesize that patients with cancer anorexia might benefit from treatment with a ghrelin receptor agonist to prevent WL and deterioration in physical functioning.

Keywords: ghrelin, anorexia, cachexia, NSCLC, appetite, weight loss

INTRODUCTION

One of the promising new therapeutic targets for cancer-associated weight loss (cachexia) is the ghrelin receptor. Ghrelin is a 28-amino acid peptide that is the natural ligand for the growth hormone secretagogue receptor-1a (Kojima et al., 1999). Ghrelin plays an important role in several physiological processes including increasing appetite by stimulating the production of orexigenic neurons such as neuropeptide Y and agouti-related protein-expressing neuron (Zhang and Garcia, 2015). Ghrelin is produced by endocrine cells of the antrum during periods of fasting (Kojima et al., 1999). Ghrelin levels are high in patients with anorexia nervosa, low postprandial, and low in obesity (Zhang and Garcia, 2015). Ghrelin levels increase in patients who develop anorexia during chemotherapy but remain stable in patients without anorexia during chemotherapy (Shimizu et al., 2003). The evidence on changed ghrelin levels in cancer cachexia however, appears to be inconclusive. Some studies showed increased levels of ghrelin in patients with cachexia compared to patients without cachexia (Shimizu et al., 2003; Karapanagiotou et al., 2009), whereas other studies did not show these differences (D'Onghia et al., 2007; Huang et al., 2007).

Randomized trials with ghrelin and ghrelin agonists have shown promising results regarding improvements in appetite, food intake, lean body mass, and quality of life of patients with cancer cachexia (Garcia et al., 2013; Temel et al., 2016). Still, no significant effect on physical functioning and survival was demonstrated in two recent large randomized double blind phase III trials (Temel et al., 2016).

As cachexia may be accompanied by a loss of appetite (anorexia) and ghrelin is directly involved in the regulation of hunger and appetite, it is hypothesized that the presence or absence of anorexia may intervene in the association between cancer cachexia and ghrelin (Shimizu et al., 2003). To test this hypothesis, we studied associations between plasma ghrelin levels and anorexia and cachexia in patients with advanced NSCLC.

MATERIALS AND METHODS

In this prospective study, patients with stage III or IV NSCLC and starting with chemotherapy were recruited at the department of Pulmonology of the VU University Medical Center in Amsterdam, The Netherlands.

Exclusion criteria: systemic anticancer treatment in the past month, clinically overt ascites or serious pitting edema, Diabetes Mellitus, current use of high dose of corticosteroids, presence of other active inflammatory disease (for example HIV or active colitis) and insufficient command of the Dutch language. The research protocol was approved by the Medical Ethics Committee of the VU University Medical Center Amsterdam and the study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki of 1975 as revised in 1983. Written informed consent was obtained from all participants.

Measurements

Weight Loss and BMI

Body weight was measured (with patients wearing light indoor clothes without shoes) within 0.2 kg on a calibrated scale (Seca type 888). Self-reported body weight 6 months before inclusion was assessed. A correction factor for clothes or clothes and shoes (1.3 kg for females, 1.6 kg for males, 1.6 kg for females, and 2.0 kg for males, respectively) was applied when necessary (Frank and Dunlop, 2000). Relative weight change in 6 months was calculated. Body height was measured using a stadiometer; the patient was standing barefoot and height was determined to the nearest cm. BMI was calculated as the ratio of body weight (kg)/height $(m)^2$.

SMI

Skeletal muscle area (cm^2) was measured with SliceOmatic Software V 5.0 (Tomovision, Magog, Canada) using routine CT scans conducted for diagnostic purposes. The fourth thoracic vertebra (T4) was used for the assessment of the skeletal muscle area and in patients without evaluable T4 images, L3 images were used. The structures of muscles were quantified based on pre-established thresholds of Hounsfield Units (HU) (−29 to +150) of skeletal muscle tissue (Mitsiopoulos et al., 1998). Cross-sectional areas (cm^2) of the sum of all these muscles were computed by summing tissue pixels and multiplying by the pixel surface area for each patient. Skeletal Muscle Index (SMI) was calculated as the ratio of skeletal muscle area (cm^2)/height $(m)^2$.

Cachexia

Cachexia was defined as:

- Weight loss >5% in 6 months or
- Weight loss >2% in 6 months in combination with BMI <20 or
- Weight loss >2% in 6 months in combination with low skeletal muscle index (SMI): L3: <55 cm^2/m^2 for males, <39 cm^2/m^2 for females (Fearon et al., 2011), T4: <66.0 cm^2/m^2 for males, <51.9 cm^2/m^2 for females (cut-off value p50 for T4, comparable to L3 cut-off values in patients with SMI data on both levels, data not published).

Anorexia

- The 12 items of the Anorexia/Cachexia subscale (A/CS) of the Functional Assessment of Anorexia/Cachexia Therapy

(FAACT) questionnaire (4th version, Dutch) (Ribaudo et al., 2000) were scored on a five-point Likert scale (0 = not at all, 1 = a little bit, 2 = somewhat, 3 = quite a bit, and 4 = very much). For scoring the FAACT − A/CS, the FACIT manual was applied (Cella, 1997). A lower score indicates less appetite. A decreased appetite was defined as having a score of ≤37 (Blauwhoff-Buskermolen et al., 2016).

Ghrelin

Venous blood samples were collected between 7:30 and 9:00 a.m. after an overnight fast. Blood samples were collected using EDTA tubes containing 250 KIU of aprotinin (BD Diagnostics, Plymouth, UK), immediately placed on ice, and centrifuged. Plasma was stored at −80°C until assayed. Total plasma ghrelin was determined by radioimmunoassay (RIA) (EMDMillipore Corporation, Merck Life Sciences, KGaA, Darmstadt, Germany). The intra-assay variation was below 4% and the inter-assay variation was below 5%. The lower limit of quantitation (LOQ) was 240 pg/mL. Analyses were performed at the Endocrine Laboratory of the Department of Clinical Chemistry of the VU University Medical Center.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows v. 23.0 (IBM Corporation, Armonk, NY, USA). Descriptive statistics (count (%) and means ± *SD* or median (IQR), as appropriate) were used to describe the study sample. An independent samples Kruskal-Wallis Test was performed to assess differences in ghrelin levels between four groups: patients with (+) or without (−) anorexia (A) or cachexia (C) because of rather small subgroups. Mann-Whitney *U*-tests were performed to assess the largest difference between subgroups.

Linear regression analyses were performed with logtransformed ghrelin levels to assess differences in ghrelin levels for patients with and without anorexia and with and without cachexia. In multiple regression analyses, adjustments for age and sex were performed. A $p \leq 0.05$ was considered significant for all analyses.

RESULTS

Forty patients with stage III (33%) or stage IV (68%) were recruited, of which 50% was male. Mean age was 59.6 ± 10.3 years (**Table 1**).

Sixteen patients had no anorexia or cachexia (A−C−), seven patients had both anorexia and cachexia (A+C+), ten patients had anorexia without cachexia (A+C−) and seven patients had cachexia without anorexia (A−C+). Of the 14 cachectic patients, 11 patients were diagnosed as such based on >5% weight loss. Two patients were found to be cachectic based on 2–5% weight loss in combination with low SMI and one patient was found to be cachectic based on 2–5% weight loss in combination with low BMI.

Figure 1 shows boxplots with median ghrelin levels for the four groups. Ghrelin levels were significantly different between the four groups ($p = 0.032$). In *post-hoc* analyses, the A+C− patients had significantly higher ghrelin levels (median: 1,754 pg/mL, IQR 1,404–2,142) compared to the A−C+ patients (median: 1,026 pg/mL, IQR 952–1,357, $p = 0.003$).

TABLE 1 | Patient characteristics ($n = 40$).

	n (%)
Gender (males)	20 (50)
Age in years†	59.6 ± 10.3
Cancer stage	
III	13 (33)
IV	27 (68)
BMI in kg/m^2†	23.9 ± 4.0
FAACT-A/CS‡	38 (35–42)

†*Mean ± sd,* ‡*Median (IQR).*

When cachexia and anorexia were analyzed separately, ghrelin levels were not significantly different between patients with (1,230 pg/mL; IQR 1,026–1,678, $n = 14$) or without cachexia (1,462 pg/mL; IQR 1,212–2,092, $n = 26$; $\beta = -99$, $p = 0.450$ adjusted for age and sex). Patients with anorexia ($n = 17$) had significantly higher ghrelin levels (1,624 pg/mL; IQR 1,391–2,102) compared to patients without anorexia (1,212 pg/mL; IQR 957–1,686), $n = 23$; $\beta = 304$, $p = 0.020$ adjusted for age and sex).

DISCUSSION

In NSCLC, we found that patients with anorexia had significantly higher plasma ghrelin levels compared to patients without anorexia. In contrast to previous literature, we did not find associations between ghrelin levels and cachexia.

We hypothesize that anorexia, and accompanying decreased food intake, may lead to increased ghrelin levels in patients with cancer, as endocrine cells of the antrum react to an empty stomach with production of ghrelin (Kojima et al., 1999). This is also supported by the fact that patients with anorexia nervosa also have high ghrelin levels (Zhang and Garcia, 2015).

In patients with cancer, partial resistance to the orexigenic effects of increased ghrelin levels has been hypothesized by Garcia et al. (2005). They compare ghrelin resistance to insulin resistance in type 2 diabetes mellitus, which is overcome by using high doses of insulin. Further elevation in ghrelin levels (three- to four-fold from baseline) may be able to increase appetite and food intake (Garcia et al., 2005). Randomized trials with ghrelin and ghrelin agonists have shown positive results regarding improvements in appetite, food intake, lean body mass and quality of life of patients with cancer cachexia (Garcia et al., 2013; Temel et al., 2016). However, no significant effect on physical functioning and survival was demonstrated in two well-designed large phase III double blind randomized controlled trials (Temel et al., 2016). As anorexia may be present in the pre-cachectic stage and precede significant weight loss and deterioration in physical functioning, future studies should consider treatment with ghrelin in patients with cancer anorexia (for example, in the stage of pre-cachexia) rather than patients with cachexia, in order to study whether significant weight loss and deterioration in

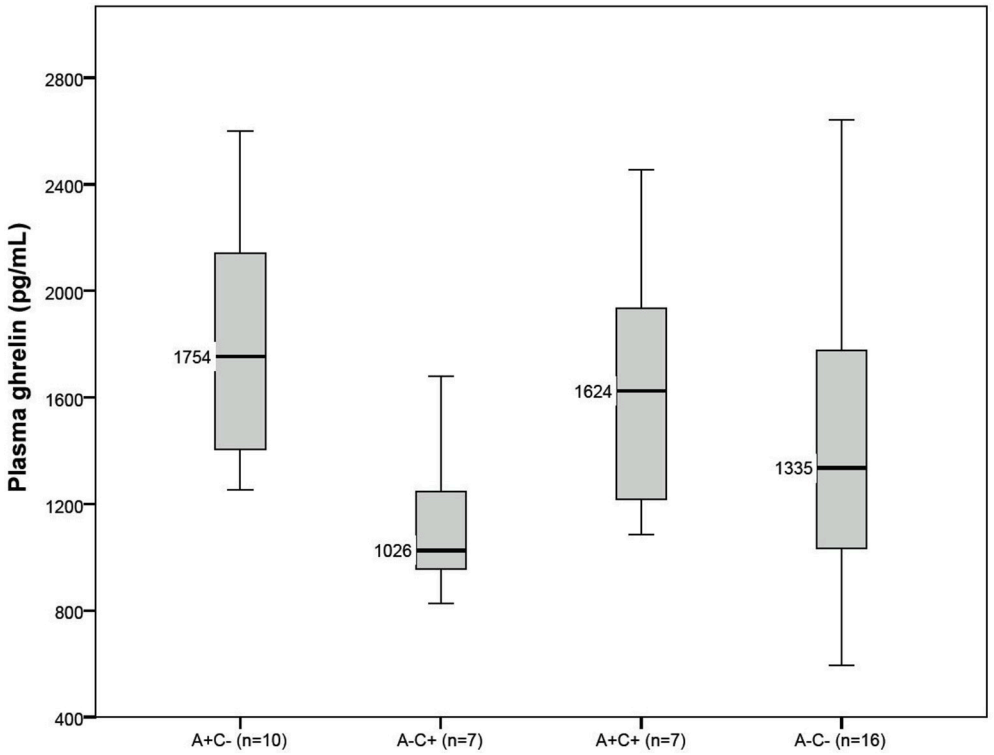

FIGURE 1 | Boxplots for fasting plasma ghrelin levels for patients with (+) or without (–) anorexia (A) and cachexia (C). The thick black horizontal line in each boxplot represents the median value.

physical functioning can be prevented. Literature search resulted in only one small study in seven patients with severe cancer anorexia and this study had promising result: ghrelin infusion resulted in a marked increase in energy intake of 31% and higher meal appreciation scores (Neary et al., 2004). Future studies with larger sample sizes with important end points such as quality of life are of interest, which preferably also include measurement of plasma ghrelin levels in order to learn more about the physiology of ghrelin in patients with cancer cachexia.

In our study we measured only total ghrelin and not acylated ("active") ghrelin and deacylated ("inactive") ghrelin separately. The effects of ghrelin on appetite have been assigned to active ghrelin, however the major form of ghrelin in serum is deacylated ghrelin. In a study of Garcia and colleagues, active ghrelin levels and the active to total ghrelin ratio were significantly increased in subjects with cancer-induced cachexia, compared with cancer patients without cachexia and non-cancer controls (Garcia et al., 2005). Nevertheless, there is growing evidence supporting that deacylated ghrelin is also closely linked with food intake and gut motility (Chen et al., 2009). In future studies, acylated and deacylated ghrelin should be analyzed separately in order to learn more about the differences between the two subforms.

We need to remark that absolute reported concentrations of plasma ghrelin are dependent on the analytical method used, therefore we advise to be careful when comparing our reported absolute values with literature on ghrelin levels in humans measured with other methods. Also, when comparing our results to the results of other studies, attention should be paid to the used definitions of anorexia and cachexia. We have used the most recent published classifications of anorexia and cachexia, however this makes comparison to other studies (which used other definitions of anorexia and cachexia) difficult.

This is the first study on plasma ghrelin levels and associations with anorexia and cachexia in patients with cancer. In conclusion, patients with anorexia had significantly higher ghrelin levels compared to patients without anorexia, whereas no differences were found between patients with and without cachexia. In future studies, the effect of ghrelin (agonists) in the treatment of cancer anorexia should be evaluated to prevent severe weight loss and improve clinical outcomes such as physical functioning, quality of life, and survival.

AUTHOR CONTRIBUTIONS

Conception and design: SB, JL, HV, Md. Collection and assembly of data: SB. Data analysis and interpretation, manuscript writing, and final approval of manuscript: All authors.

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Interference with Ca^{2+}-Dependent Proteolysis Does Not Alter the Course of Muscle Wasting in Experimental Cancer Cachexia

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Protein hypercatabolism significantly contributes to the onset and progression of muscle wasting in cancer cachexia. In this regard, a major role is played by the ATP-ubiquitin-proteasome-dependent pathway and by autophagy. However, little is known about the relevance of the Ca^{2+}-dependent proteolytic system. Since previous results suggested that this pathway is activated in the skeletal muscle of tumor hosts, the present study was aimed to investigate whether inhibition of Ca^{2+}-dependent proteases (calpains) may improve cancer-induced muscle wasting. Two experimental models of cancer cachexia were used, namely the AH-130 Yoshida hepatoma and the C26 colon carcinoma. The Ca^{2+}-dependent proteolytic system was inhibited by treating the animals with dantrolene or by overexpressing in the muscle calpastatin, the physiologic inhibitor of Ca^{2+}-dependent proteases. The results confirm that calpain-1 is overexpressed and calpastatin is reduced in the muscle of rats implanted with the AH-130 hepatoma, and show for the first time that the Ca^{2+}-dependent proteolytic system is overactivated also in the C26-bearing mice. Yet, administration of dantrolene, an inhibitor of the Ca^{2+}-dependent proteases, did not modify tumor-induced body weight loss and muscle wasting in the AH-130 hosts. Dantrolene was also unable to reduce the enhancement of protein degradation rates occurring in rats bearing the AH-130 hepatoma. Similarly, overexpression of calpastatin in the tibialis muscle of the C26 hosts did not improve muscle wasting at all. These observations suggest that inhibiting a single proteolytic system is not a good strategy to contrast cancer-induced muscle wasting. In this regard, a more general and integrated approach aimed at targeting the catabolic stimuli rather than the proteolytic activity of a single pathway would likely be the most appropriate therapeutic intervention.

Keywords: muscle protein turnover, calpains, calpastatin, muscle atrophy, proteostasis

INTRODUCTION

Cachexia is a wasting syndrome that frequently occurs in cancer patients, worsening both their prognosis and quality of life and significantly reducing survival rate. Although at different degrees, virtually all body compartments are affected by cachexia, the skeletal muscle being among the most compromized. Previous studies showed that cancer-induced muscle wasting mainly derives from acceleration of protein breakdown rates. The main proteolytic systems operating in the muscle were all found hyperactivated in tumor hosts, suggesting that they could be considered as potential therapeutic targets (reviewed in Argilés et al., 2014). Recent reports however, showed that inhibition of proteasome-dependent proteolysis or autophagy was not able to improve muscle wasting in tumor-bearing animals (Penna et al., 2013, 2016).

The contribution of proteasomes and autophagy to the onset and progression of cancer-induced muscle wasting was deeply investigated, however quite little is known about the Ca^{2+}-dependent proteolytic system.

Calpains (EC 3.4.22.17) are a family of Ca^{2+}-dependent cysteine proteases. Three members of this family are mainly expressed in the skeletal muscle, namely the ubiquitous μ- and m-calpains and the muscle-specific p94 calpain (also known as calpains-1, -2, and -3). Both μ- and m- calpains consist of two subunits of 80 and 30 kDa, respectively (reviewed in Ono et al., 2016). The former (constituted by four domains) contains the catalytic cleft, whereas the latter (two domains) is endowed with regulatory functions. The p94 calpain also includes three insertions that are not shared with other calpains. When intracellular Ca^{2+} concentrations increase, inactive calpains, normally localized in the cytosolic compartment, translocate to the cell membrane and the 80 kDa subunit is converted by autoproteolysis to a 75 kDa form. A number of cytoskeletal proteins were proposed as substrates of calpains, among which desmin, dystrophin, fodrin, myosin, troponins I, and T (reviewed in Ono et al., 2016). Other reports showed that also calpastatin, the physiologic inhibitor of calpains, and the plasmamembrane 130 kDa Ca^{2+}-ATPase are cleaved by Ca^{2+}-dependent proteases (Pontremoli et al., 1991; Salamino et al., 1992; Ono et al., 2016).

The physiological relevance of calpains is demonstrated by their involvement in several processes such as cell proliferation, differentiation, migration, apoptosis, and aging. Ca^{2+}-dependent proteases were also proposed to contribute to different pathologies. A cause-effect relationship was established among calpain deficiencies and diseases, for this reason defined calpainopathies (Ono et al., 2016). As for cancer cachexia, increased calpain mRNA levels were reported in the skeletal muscle of animals bearing the AH-130 hepatoma (Busquets et al., 2000). The contribution of calpain activity to muscle wasting induced by tumor growth in rats bearing the Yoshida AH-130 hepatoma was suggested by the progressive reduction of both calpastatin and the 130 kDa Ca^{2+}-ATPase levels (Costelli et al., 2001), as well as by the increased cleavage *in vitro* of specific fluorogenic substrates (Costelli et al., 2002; Borges et al., 2014). More recently increased calpain expression was reported in the muscle of tumor-bearing rats treated with sorafenib (Toledo et al., 2016). In patients with cancer, however, both increased or unchanged muscle calpain levels were observed (Smith et al., 2011; Tardif et al., 2013). Differencies in tumor type and disease severity likely account for such discrepancies.

Although a number of data support the involvement of Ca^{2+}-dependent proteolysis in cancer cachexia, protein hypercatabolism was not suppressed in muscles isolated from tumor-bearing animals and exposed to calpain inhibitors (Temparis et al., 1994; Baracos et al., 1995; Llovera et al., 1995). Such studies, however, were performed on preparations of isolated muscles incubated in the presence of calpain inhibitors, which is a rather non-physiological setting. Previous studies from our laboratory showed that leupeptin, an inhibitor of cysteine proteases including calpains and many lysosomal cathepsins, improved muscle wasting in rats bearing the AH-130 hepatoma (Tessitore et al., 1994).

On the whole, while some evidences do not support a role of calpain system in cancer-associated muscle wasting, other findings suggest the opposite. Thus, the working hypothesis in the present study is that specific inhibition of calpain activity could be a means to prevent, or at least delay, muscle wasting that occurs in cancer cachexia. Along this line, the calpain system was modulated in tumor-bearing animals either by pharmacological or genetic means.

MATERIALS AND METHODS

Reagents

All reagents supplied by Sigma-Aldrich (St. Louis, MO, USA), unless differently specified.

Animals and Treatments

Male Wistar rats (5 weeks old/150 g) or male Balb/c mice (5 weeks old/20 g) were provided by Charles River, Calco, Italy. They were housed on a regular dark-light cycle (light from 08:00 to 20:00), with free access to food and water, and cared for in compliance with the Italian Ministry of Health Guidelines and the Policy on Humane Care and Use of Laboratory Animals (NIH, 1996). The experimental protocol was approved by the Bioethical Committee of the University of Turin. Both rats and mice were randomized into two groups, namely controls and tumor hosts. As for rats, ($n = 8$) they were injected intraperitoneally (i.p.) with 10^8 AH-130 Yoshida ascites hepatoma cells, whereas tumor-bearing mice ($n = 6$) were subcutaneously (s.c.) inoculated between the shoulder blades with 5×10^5 Colon 26 (C26) carcinoma cells. Control animals (rats: $n = 8$; mice: $n = 6$) received saline, i.p. or s.c. In a second experiment rats were divided into four groups ($n = 8$): Controls, dantrolene treated controls, AH-130 hosts and dantrolene-treated AH-130 hosts. Dantrolene (10 mg/kg b.w.) was daily administered s.c., starting on the day of tumor transplantation. Finally, another experiment using the C26 tumor was performed. the animals were divided into two groups, namely controls (C) and tumor hosts (C26). Each group was further divided into transfected ($n = 6$ for both C and C26) and untransfected

($n = 6$ for both C and C26). Transfection procedure is described below.

AH-130 hosts and C26-bearing mice were euthanized under isoflurane anesthesia. The former were sacrificed at days 4 or 7 after tumor transplantation, while sacrifice time for the latter was day 14 of tumor growth. Several muscles and organs were rapidly excised, weighed, frozen in isopentane cooled with liquid N_2, and stored at −80°C for subsequent analysis.

Histological Analysis

Serial 10 μm-thick frozen sections were cut from cryopreserved tissue blocks, adhered to Superfrost Plus microscopy slides and stained with hematoxylin and eosin. All sections were examined by light microscopy (Nikon Eclipse TS100) and digital images were obtained with a Nikon COOLPIX 4500 camera. To assess myofiber cross sectional area (CSA) ∼300–400 fibers of *tibialis anterior* muscle sections were counted and measured using the Image J software (http://rsb.info.nih.gov/ij/; NIH, Bethesda, MD).

For immunofluorescence, transverse sections were fixed in 4% paraformaldehyde and probed with the primary anti-calpastatin mAb 35,23 (1:200 Melloni et al., 2006). Detection was performed using a FITC-conjugated mouse IgG secondary antibody (1:2,000; Bio-Rad Laboratories, Hercules, CA). Nuclei were stained with the DAPI fluorochrome and the images captured with a Nikon COOLPIX 4500 camera in an epiilluminated fluorescence microscope (Axiovert 35, Zeiss, Germany).

Protein Turnover

Muscle protein turnover rates were determined as previously described (Costelli et al., 1993). Briefly, rats received an i.p. dose of $NaH^{14}CO3$ (250 μCi/kg b.w.) 24 h before tumor transplantation. They were then sacrificed at days 0 and 7 after tumor inoculation and total protein radioactivity determined. Briefly, gastrocnemius was rapidly weighed and homogenized to 10% (wt/vol) in chilled water. Total protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as working standard. Trichloroacetic acid-insoluble proteins were processed for lipid extraction, extensively hydrolyzed, and counted in a liquid scintillation spectrometer to obtain total protein radioactivity. Fractional rates of protein degradation (k_d) were calculated as follows and expressed as %/day:

$$k_d = \ln(\text{total protein radioactivity})/t.$$

Calpastatin Transfection

RNCAST600 cloned in pEYFP-C1 (De Tullio et al., 2009) was used to express a calpastatin form containing the regulatory L-domain and a single inhibitory unit. The left *tibialis anterior* muscle was injected with 25 μl of 0.5 U/μl hyaluronidase (to improve transfection efficiency) and 2 h later injected with 50 μg of plasmidic DNA, while the contralateral muscle served as control. One minute following DNA injection transcutaneous pulses were applied by two stainless steel plate electrodes (gap between plates: 4 mm). Electrical contact with the leg skin

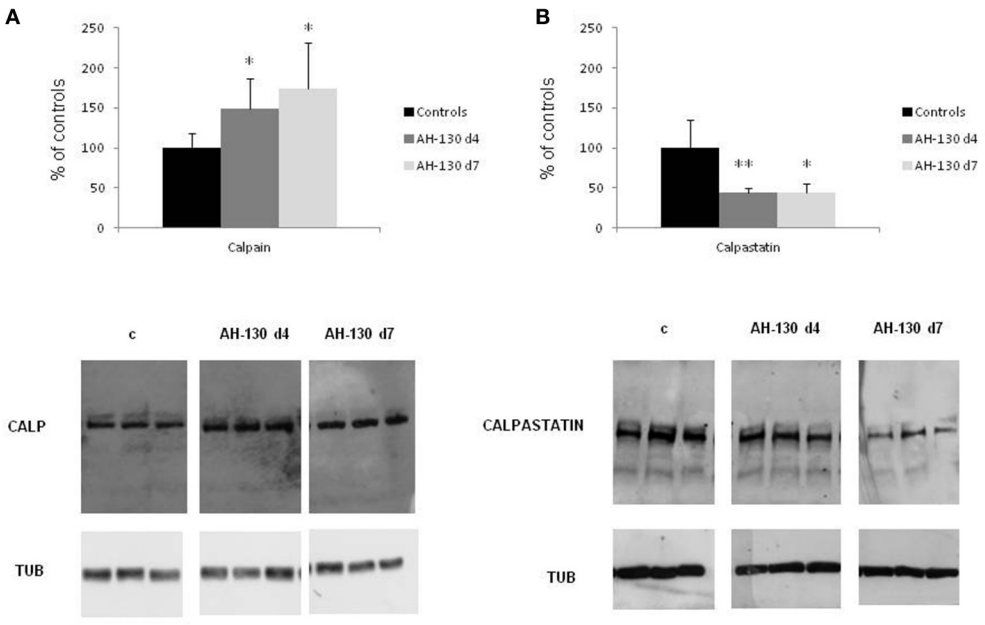

FIGURE 1 | Calpain and calpastatin expression in controls and rats bearing the AH-130 hepatoma. (A) Calpain protein expression in tibialis muscles of controls and AH-130 tumor-bearing rats after 4 and 7 days from tumor inoculation. **(B)** Calpastatin protein expression in tibialis muscles of controls and AH-130 tumor-bearing rats after 4 and 7 days from tumor inoculation. Densitometric quantifications (histograms) were normalized according to tubulin levels. Blot representative patterns are shown. Data (mean ± *SD*, $n = 8$) are expressed as % of controls. Significance of the differences (ANOVA): $^*p < 0.05$; $^{**}p < 0.01$ vs. controls.

was assured by shaving each leg and applying conductive gel. Electric pulses with a standard square wave were delivered by an electroporator (ECM-830, BTX-Harvard Apparatus, Holliston, MA, USA). Three pulses (20 ms each) of 75 V/cm were administered to the muscle with a delivery rate of 1 pulse/s. The polarity was then reversed and a further three pulses were delivered to the muscle. Electroporation was performed 10 days before animal sacrifice. With the transfection procedure described, no sign of muscle damage and inflammatory infiltrate could be seen by histological analysis (Penna et al., 2010). The expression of YFP-RNCAST600 was confirmed by western blotting (see below), using an anti-GFP antibody (Figure S1; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western Blotting

Approximately 50 mg of gastrocnemius or 25 mg of tibialis muscle were homogenized in 80 mmol/L Tris-HCl, pH 6.8, containing 100 mmol/L dithiothreitol, 70 mmol/L SDS, and 1 mmol/L glycerol, with freshly added protease and phosphatase inhibitor cocktails; kept on ice for 30 min; centrifuged at 15,000 × g for 10 min at 4°C and the supernatant collected. Protein concentration was assayed according to Bradford, using bovine serum albumin as working standard. Equal amounts of protein (30 μg) were heat-denaturated in sample-loading buffer (50 mmol/L Tris-HCl, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The filters were blocked with Tris-buffered saline containing 0.05% Tween and 5% non-fat dry milk and then were incubated overnight with a mouse monoclonal anti-calpain-1 antibody (1:1000; Chemicon, Temecula, CA, USA, Milan, Italy), recognizing the catalytic subunit (∼80 kDa) of calpain-1; a commercial anti-calpastatin antibody recognizing the whole protein (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the monoclonal anti-calpastatin antibody mAb 35,23 recognizing also the partially digested forms of calpastatin (1:1,000; provided by R. De Tullio, characterization described in Melloni et al., 2006); an antibody against tubulin (1:8,000, mouse monoclonal antibody, clone T5168); or GAPDH (1: 5,000, goat polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA).

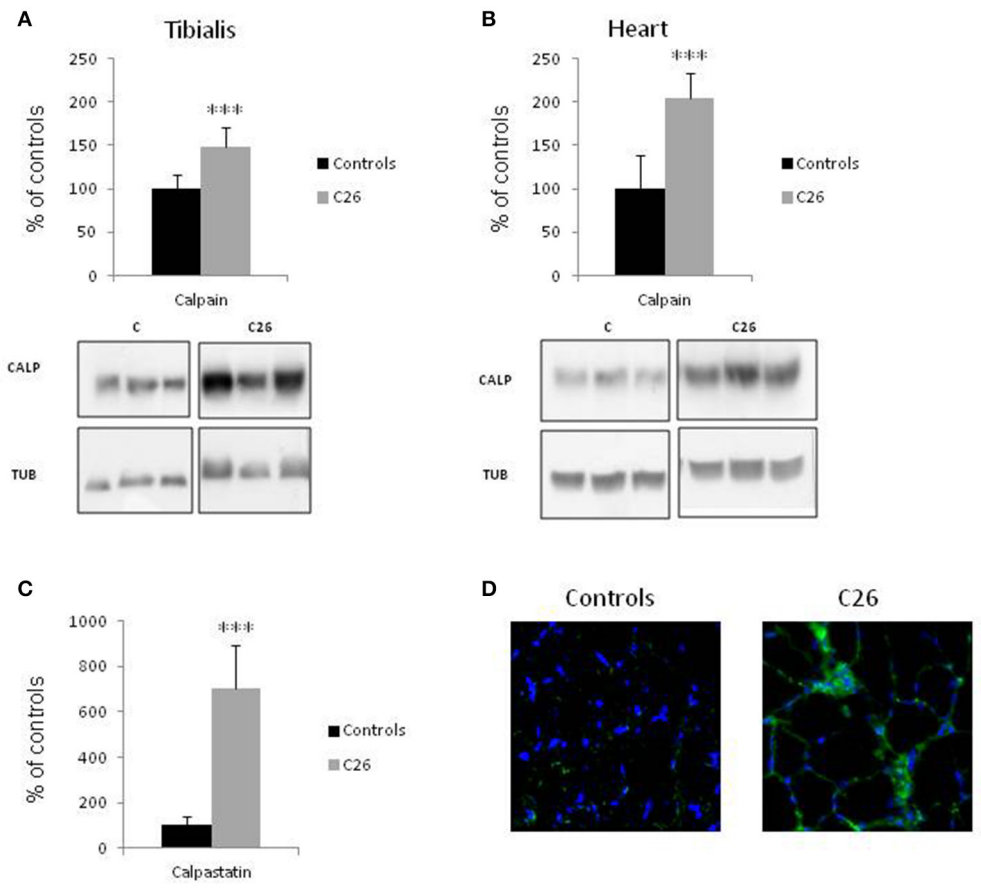

FIGURE 2 | Calpain and calpastatin expression in control animals and in mice implanted with the C26 tumor. Calpain protein levels in the tibialis muscle **(A)** and in the heart **(B)** of controls and C26 hosts. **(C)** Calpastatin digestion products were detected in the tibialis muscle of controls and C26 tumor-bearing mice by the mAb 35,23. **(D)** Immunofluorescence images of tibialis muscle sections in controls and C26-bearing mice stained for calpastatin (green) and nuclei (blue). Densitometric quantifications (histograms) were normalized according to tubulin levels. Blot representative patterns are shown. Data (mean ± *SD*, $n = 6$) are expressed as % of controls. Significance of the differences (*t*-test): ***$p < 0.001$ *vs.* controls.

Peroxidase-conjugated IgGs (Bio-Rad Laboratories, Hercules, CA) were used as secondary antibody. For quantification, performed by densitometric analysis (TotalLab; Non-linear Dynamics, Newcastle on Tyne, UK), samples were run on a single gel (representative patterns shown in Figures) and normalized against tubulin or GAPDH levels.

Data Analysis and Presentation

Results are expressed as means ± *SD*. The significance of the differences was evaluated by analysis of variance (ANOVA) followed by Tukey's test or by Student's "*t*"-test (indicated in Figure legend). As for the fractional rates of protein turnover, the significance of the differences was calculated by comparing linear regression by ANOVA (Lee and Lee, 1982).

RESULTS

Rats bearing the Yoshida AH-130 hepatoma and mice implanted with the C26 colon carcinoma show a marked loss of both body weight and muscle mass (Costelli et al., 1993; Bonetto et al., 2009).

Skeletal muscle wasting is associated with enhanced activity of the Ca^{2+}-dependent proteolytic system. In the muscle of rats bearing the AH-130 hepatoma, the levels of calpain-1 are increased while those of the endogenous specific inhibitor calpastatin are reduced (**Figures 1A,B**; Costelli et al., 2001). Similarly, calpain-1 levels are increased in both the *tibialis anterior* (**Figure 2A**) and heart (**Figure 2B**) of mice implanted with the C26 tumor. In parallel, digestion products of calpastatin, markers of calpain activity (De Tullio et al., 2000), accumulate in the muscle of C26 hosts (**Figure 2C** and Figure S1). Finally, in these latter calpastatin is diffused in the cytosol (**Figure 2D**), a localization that is associated with calpain activation and Ca^{2+} increase (De Tullio et al., 1999).

To understand if interfering with the activity of the Ca^{2+}-dependent proteolytic system can antagonize cancer-induced muscle wasting, rats bearing the AH-130 hepatoma were treated with dantrolene, a molecule that inhibits Ca^{2+} release from the sarcoplasmic reticulum, thus reducing calpain activation (Perry et al., 2010; Malvestio et al., 2016). The results show that dantrolene administration did not prevent either the loss of body and muscle weight (**Figure 3A** and Table S1) or the acceleration of protein breakdown rates (**Figure 3B**). No appreciable effects of dantrolene could be observed on tumor mass (total cell number: $1{,}198 \times 10^6 \pm 239$ and $1{,}480 \times 10^6 \pm 359$ in untreated and treated AH-130 hosts, respectively) and on food intake (C = 136 ± 17 g, C + dantrolene = 129 ± 22 g, AH-130 = 94 ± 10 g, AH-130 + dantrolene = 85 ± 16 g, $p < 0.01$ tumor hosts vs. controls, irrespective of treatment). In addition, dantrolene did not exert any effect on control rats (**Figure 3**). Similar data were obtained using a cell-permeable calpain inhibitor (Figure S2).

The use of systemic inhibitors is limited by pharmacokinetics, biological effective dose and potential toxicity. For this reason, as

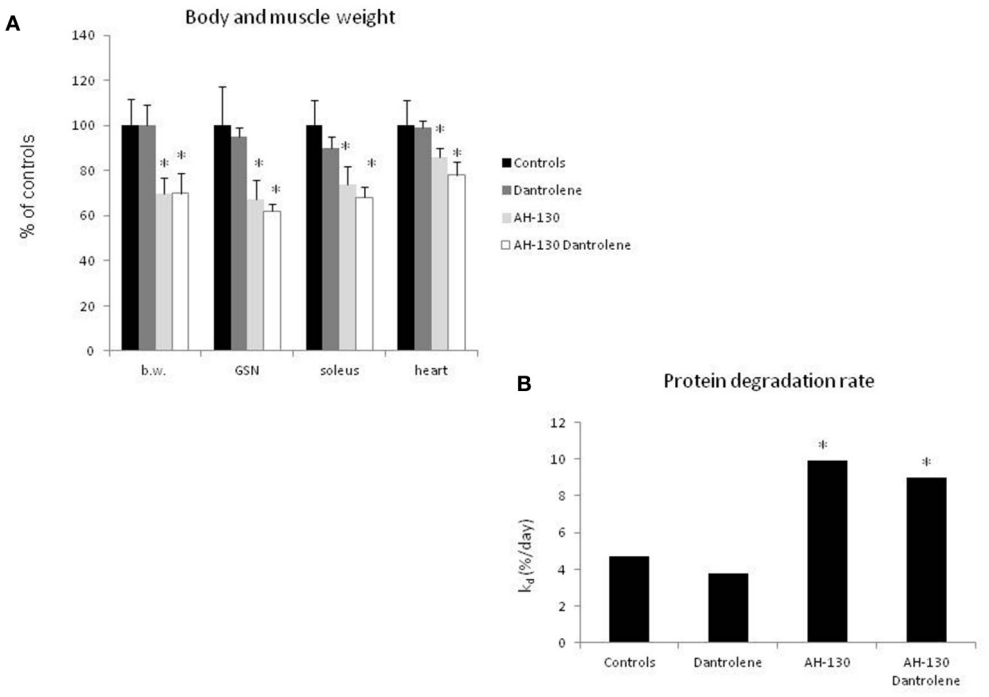

FIGURE 3 | Effects on treatment with dantrolene on muscle weight and protein degradation rates in rats bearing the AH-130 hepatoma. (A) Body weight (b.w.), gastrocnemius (GSN), soleus, and heart weight in controls and AH-130 tumor-bearing rats. The data are reported as % of controls (mean ± *SD*, $n = 8$). Significance of the differences (ANOVA): $^*p < 0.05$ vs. controls. **(B)** Fractional rates of protein degradation in the gastrocnemius muscle, expressed as % day (mean ± *SD*, $n = 8$), in controls and AH-130 hosts, treated or not with dantrolene. Significance of the differences (linear regression comparison; see Materials and Methods): $^*p < 0.05$ vs. controls.

a second strategy to specifically target calpain activity locally in the skeletal muscle, a gene approach aimed at hyperexpressing calpastatin was used. The left *tibialis anterior* muscle of mice bearing the C26 tumor was transfected with an expression vector encoding a calpastatin species containing, in addition to a single inhibitory unit, also the regulatory L-domain (RNCAST600; transfection efficiency: 30%). The transfected sequence, even if produced as a fusion protein with the YFP tag at the C-terminus, maintains the ability to inhibit calpain activity (Figure S3; De Tullio et al., 2009). Being muscle transfection localized unilaterally to the *tibialis anterior*, no effects on body weight were observed, as expected (**Figure 4A**). RNCAST600 overexpression, however, was also unable to interfere with both loss of muscle mass (**Figure 4B**, Figure S4; Table S2) and changes of myofiber cross sectional area (CSA; **Figure 5**, Figure S4). A tendency to reduce muscle mass that does not reach significance, could be observed in control mice receiving electroporation. Despite not significant, such reduction in size could suggest that the transfection procedure might have produced a systemic reaction, likely of inflammatory nature, that could slightly affect the whole muscle compartment and that could have been partially antagonized in tumor hosts. However, the observation that no differences were observed in terms of CSA (**Figure 5B**, Figure S4) supports the lack of effectiveness of RNCAST600 overexpression in preventing C26-induced muscle atrophy. RNCAST600 overexpression did not affect tumor mass (C26 = 355 ± 61 mg, C26-RNCAST600 = 326 ± 58 mg).

DISCUSSION

The results shown in the present study demonstrate that the expression of molecules pertaining to the calpain-dependent proteolytic pathway is altered in the muscle of tumor hosts, indicating an enhanced activity of this system and confirming previous reports (Costelli et al., 2005). Most importantly, however, inhibiting this proteolytic system does not contrast cancer-induced muscle wasting.

By all available evidence, the calpain system does not contribute significantly to bulk protein degradation, but only performs a limited proteolysis of substrates. Along this line, in normal conditions of calcium homeostasis calpains basically

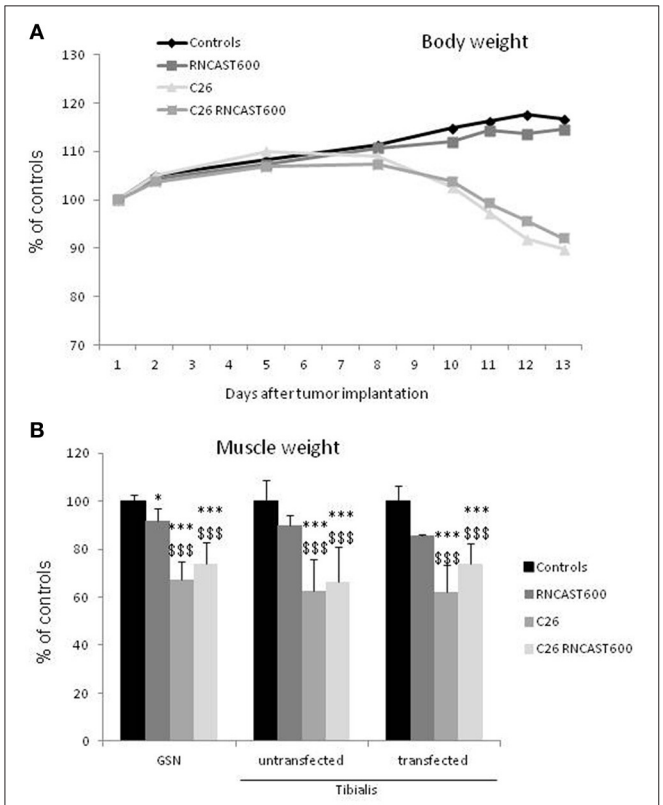

FIGURE 4 | Calpastatin overexpression in the tibialis muscle of controls and C26 hosts. (A) Body weight of controls and C26-bearing mice transfected (RNCAST600) and untransfected. The data are reported as % of controls (means ± *SD*; d_0 C = 18.72 ± 0.373 g). **(B)** Gastrocnemius (GSN) and tibialis muscle weight. Data are expressed as % of controls (mean ± *SD*, $n = 8$). Significance of the differences (ANOVA): $^{*}p < 0.05$; $^{***}p < 0.001$ vs. controls, $^{\$\$\$}p < 0.001$ vs. RNCAST600.

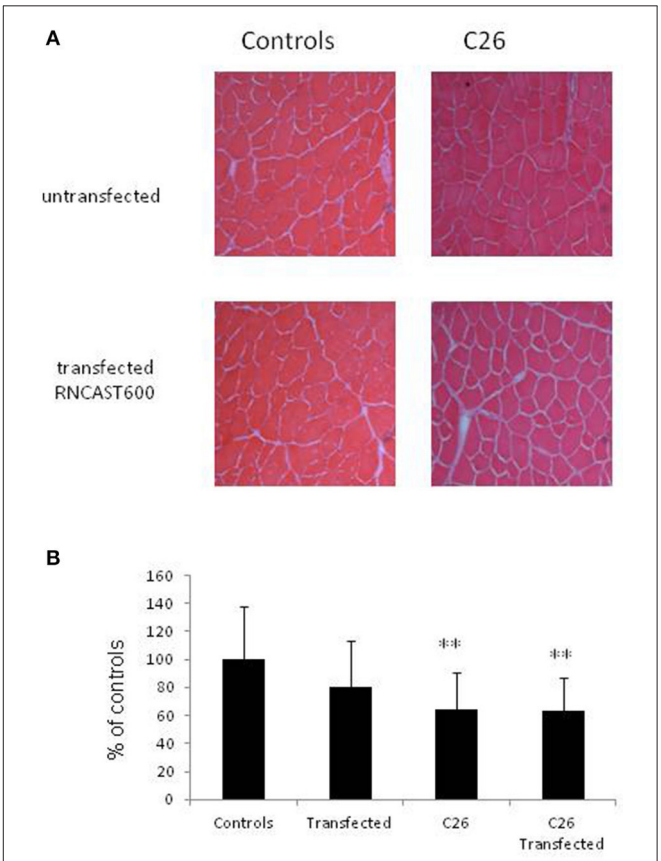

FIGURE 5 | Myofiber cross-sectional area. (A) Representative histological pattern (hematoxylin and eosin staining) of transfected and untransfected muscles. **(B)** Morphometric analysis performed on hematoxylin and eosin stained sections of tibialis muscle derived from controls and C26 tumor-bearing mice untransfected or transfected with RNCAST600. Data (mean ± *SD*, $n = 8$) are expressed as percentages of controls. Significance of the differences (ANOVA): $^{**}p < 0.01$ vs. controls.

exert a processing rather than a degradative action aimed to induce functional modifications in the target proteins (Goll et al., 2003). Calpains directly recognize substrates, differently from the lysosomal and the proteasomal systems that need ubiquitylation or sequestration into autophagosomes to tag their substrates (Goll et al., 2003). It is now well-accepted, in this regard, that while calpains are required to initiate the degradation of myofibrillar proteins, they are not involved in the bulk degradation of sarcoplasmic or sarcolemmal proteins (Huang and Zhu, 2016). Calpains appear to sever thick and thin filaments from myofibrils, thereby permitting a subsequent step in which suitable protein substrates are allowed to gain access to the degradative machinery (proteasome and lysosomal-dependent systems: Reviewed in Huang and Zhu, 2016). The activity of such a physiologically operating pathway can be enhanced by homeostasis alterations. As an example, oxidative stress was shown to increase calpain expression in cultured myocytes (Dargelos et al., 2010) and to induce the degradation of myofibrillar proteins (Smuder et al., 2010). Of interest, oxidative stress was shown to occur in the skeletal muscle of cachectic tumor-bearing animals (Mastrocola et al., 2008; Assi et al., 2016).

The calpastatin construct (RNCAST600) used in the present study encodes a single inhibitory unit and a complete regulatory L-domain. Previous data show that YFP-tagged RNCAST600 retains its ability to inhibit calpain activity (Stifanese et al., 2008; De Tullio et al., 2009) and since it contains the L domain, it can bind calpain also in the inactive form (Melloni et al., 2006). In addition, we have here observed that 1 week after transfection RNCAST600 is still expressed, not degraded, and effectively inhibits calpain activity (Figure S1). The lack of effect of RNCAST600 in preventing cancer-induced muscle wasting could depend on a sort of "calpastatin resistance". A loss of inhibitory efficiency, that allows calpain to be active even at high concentrations of calpastatin, was previously observed in those calpastatin forms containing also the regulatory region (XL-L and L-domains; De Tullio et al., 2014). Following $[Ca^{2+}]_i$ increase, calpastatin, that is normally localized in perinuclear aggregates, diffuses in the cytosol and interacts with active calpain. Such diffusion can be reversed, even in the presence of Ca^{2+}, if exon 6-containing calpastatins are phosphorylated by PKA (De Tullio et al., 1999). This process ultimately leaves active calpain free to operate on its targets even in the presence of high amounts of inhibitor. The possibility that these regulations contribute to the lack of effect of calpastatin overexpression in the muscle of the C26 hosts cannot be discarded. Future challenges will be to explore whether the transfection of different forms of calpastatin (i.e., without L-domain or with an L-domain lacking exon 6) could affect cancer-induced muscle wasting.

In addition to myofibrillar protein degradation, calpain activation also positively modulates endoplasmic reticulum (ER) stress (Muruganandan and Cribb, 2006; Langou et al., 2010). Muscle wasting induced by cancer was enhanced by inhibiting both ER stress and unfolding protein response (UPR) (Bohnert et al., 2016). An intriguing speculation, in this regard, is that calpain inhibition could result in reduced myofilament release from sarcomeres, but also in ER stress and UPR down-regulation (Isaac et al., 2016), still shifting protein turnover toward degradation.

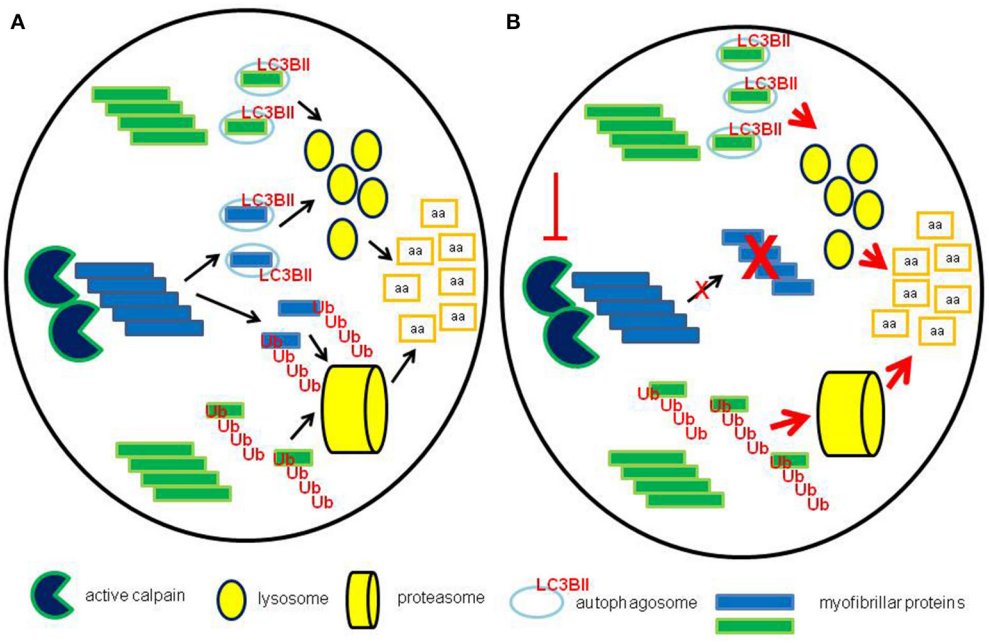

FIGURE 6 | Maintenance of proteostasis after calpain inhibition. (A) In normal conditions calpains contribute, together with other systems such as caspases, to the degradation of myofibrillar proteins by severing thick and thin filaments from myofibrils, providing substrates for both proteasome and lysosomal-dependent systems. **(B)** When calpains are inhibited, pharmacologically or by genetic manipulation, the activity of both lysosomes and proteasomes is likely enhanced, in the attempt to maintain the physiological rates of protein degradation.

The results shown in the present study support the consolidating notion that modulating the activity of a single proteolytic system would not be an adequate strategy to defeat cachexia. Indeed, previous reports showed that proteasome specific inhibition was unable as well to counteract muscle wasting in animals bearing experimental tumors (Fermoselle et al., 2013; Chacon-Cabrera et al., 2014; Penna et al., 2016). This is not totally unexpected, taking into consideration that muscle protein homeostasis (proteostasis) results from the convergent regulation of protein synthesis and degradation rates. These latter depend on the activity of the whole protein catabolic machinery, with the proteolytic pathways acting in concert, thereby providing substrates for *de novo* protein synthesis and energy production.

The interplay among the different proteolytic systems likely allows the myofiber to preserve proteostasis. If a single proteolytic pathway is failing, the others might well-increase their activity, aiming to maintain the fractional degradation rates at physiological levels (**Figure 6**). As an example, inhibition of the ubiquitin-proteasome system led to increased autophagy, while proteasome-dependent proteolysis was activated when autophagy was blocked (Yamaguchi et al., 2012). Similarly, calpain inhibition induced autophagy by reducing the levels of the Atg12-Atg5 conjugate and/or by degrading beclin-1 (Yamaguchi et al., 2012). The exogenous inhibition of a single proteolytic pathway could be associated with inefficient disposal of altered proteins/organelles by the other systems, overcoming the ability to maintain myofiber homeostasis, thus contributing to the wasting phenotype. Such a hypothesis appears particularly intriguing in cancer cachexia, where, in face of enhanced bulk proteolysis, the autophagic-lysosomal system is not able to efficiently get rid of accumulated proteins (Penna et al., 2013; Pigna et al., 2016).

On the whole, accumulating evidence indicate that most of the components of the muscle proteolytic machinery (calpains, proteasomes, lysosomes) are overexpressed in cancer cachexia, while systems involved in the endogenous control of protein turnover, such as calpastatin and deubiquitylating enzymes (Goll et al., 2003; Wing, 2016) are down-regulated. These changes are often considered as controlling events, however they might well-reflect an adaptive response to sustained hypercatabolic stimuli. The above reported observation that calpains appear to act upstream of both proteasome and lysosomal proteolysis supports the existence of a hierarchy in the hypercatabolic response in muscle wasting. In this regard, (upstream) therapeutic strategies should be focused on the real trigger(s) and on controlling events rather than on the single (downstream) proteolytic pathways.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: PC, MM, and RD; Performed the experiments: FPin, VM, and FPenna; Analyzed the data: RD and PC; Contributed reagents/materials/analysis tools: RD; Wrote the paper: FB and PC.

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SUPPLEMENTARY MATERIAL

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Differential Bone Loss in Mouse Models of Colon Cancer Cachexia

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Cachexia is a distinctive feature of colorectal cancer associated with body weight loss and progressive muscle wasting. Several mechanisms responsible for muscle and fat wasting have been identified, however it is not known whether the physiologic and molecular crosstalk between muscle and bone tissue may also contribute to the cachectic phenotype in cancer patients. The purpose of this study was to clarify whether tumor growth associates with bone loss using several experimental models of colorectal cancer cachexia, namely C26, HT-29, and $Apc^{Min/+}$. The effects of cachexia on bone structure and strength were evaluated with dual energy X-ray absorptiometry (DXA), micro computed tomography (μCT), and three-point bending test. We found that all models showed tumor growth consistent with severe cachexia. While muscle wasting in C26 hosts was accompanied by moderate bone depletion, no loss of bone strength was observed. However, HT-29 tumor bearing mice showed bone abnormalities including significant reductions in whole-body bone mineral density (BMD), bone mineral content (BMC), femoral trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th), but no declines in strength. Similarly, cachexia in the $Apc^{Min/+}$ mice was associated with significant decreases in BMD, BMC, BV/TV, Tb.N, and Tb.Th as well as decreased strength. Our data suggest that colorectal cancer is associated with muscle wasting and may be accompanied by bone loss dependent upon tumor type, burden, stage and duration of the disease. It is clear that preserving muscle mass promotes survival in cancer cachexia. Future studies will determine whether strategies aimed at preventing bone loss can also improve outcomes and survival in colorectal cancer cachexia.

Keywords: muscle wasting, cachexia, bone loss, osteopenia, osteoporosis, colon cancer

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INTRODUCTION

Colorectal cancer represents the third most common cancer in the United States and worldwide (Siegel et al., 2016) and is associated with the development of cachexia in up to 30% of the cases. Cachexia, defined as loss of body weight and depletion of muscle mass (i.e., sarcopenia), with or without loss of fat tissue (Fearon et al., 2011), represents a devastating complication of cancer

(Costelli and Baccino, 2003; Tisdale, 2009; Fearon et al., 2012). It has been estimated that up to 80% of cancer patients will develop cachexia over the course of their disease (Haehling and Anker, 2010). The development of cachexia often results in worsened quality of life, decreased tolerance to radio- and chemotherapy, and overall reduced survival. Indeed, it is estimated that cachexia is responsible for 25–30% of all cancer-related deaths (Tisdale, 2009; Muscaritoli et al., 2010). In cancer patients, cachexia is generally diagnosed in association with unintentional weight loss of at least 5% of initial weight and is normally accompanied by muscle weakness, fatigue, anorexia, changes in body composition (including lean and fat mass), increased inflammatory state, anemia and low levels of serum albumin. Of note, it has been shown that body and muscle weight loss positively correlate with enhanced mortality (Evans et al., 2008; Fearon et al., 2011).

Interestingly, while cancer patients have an increased risk of bone loss and osteoporosis, as often shown in patients affected with lung cancer (Fearon, 1992) or in those undergoing radio- or chemotherapy treatments (Mcdonald et al., 2016; Monroy-Cisneros et al., 2016), this feature represents a largely unexplored aspect of cachexia research. A growing body of evidence has led to the identification of molecular mechanisms and signaling pathways associated with muscle and fat loss in cancer. Whether the same pathways also interfere with the homeostasis of bone tissue is not completely clear. Along this line, it has been proposed that similar mechanisms associated with muscle wasting may also play a fundamental role in promoting cancer-associated bone loss, thus leading to the hypothesis that muscle and bone are regulated in tandem in cachexia (Kandarian, 2008). In more recent years, several reports suggested that osteoporosis as well as bone metabolic dysfunction and the decay of bone tissue may represent one of the peculiarities of cachexia and may participate directly in cachexia development and sustainment (Verschueren et al., 2013; Huo et al., 2015). Further, bone and muscle tissues, besides playing a fundamental role in body growth and movement, have been recently described as endocrine organs (Karsenty and Ferron, 2012; Pedersen and Febbraio, 2012; Laurent et al., 2016). Interestingly, muscle and bone loss have been correlated in human and animal models during exercise, aging, disuse, and inflammatory conditions such as arthritis and cancer (Digirolamo et al., 2013). Moreover, with increasing recognition of the physiologic and molecular crosstalk between muscle and bone (Cianferotti and Brandi, 2014), mediators shown to be associated with the pathogenesis of skeletal muscle and fat loss were reported to affect bone tissue in a similar manner (Choi et al., 2013; Waning et al., 2015).

The goal of this study was to clarify whether tumor growth is associated with the occurrence of bone loss. For this purpose, cachexia was induced in mice using colorectal cancer xenografts (murine C26 and human HT-29) or genetically induced tumors (Apc^{Min}) and the effects in terms of tumor-associated loss of bone mass were assessed. In addition, we examined the effects of colorectal cancer growth on bone structure, and mechanical properties were determined by utilizing dual energy X-ray absorptiometry (DXA), micro computed tomography (μCT) and three-point bending test. Ultimately, our findings provide evidence that the development of bone effects might depend upon tumor type, burden, stage and duration of the disease.

MATERIALS AND METHODS

Animals

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee at Indiana University School of Medicine and were in compliance with the National Institutes of Health Guidelines for Use and care of Laboratory Animals and with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Eight-week old CD2F1 male mice (Harlan, Indianapolis, IN) were injected intrascapularly (s.c.) with 1×10^6 C26 (Colon-26) adenocarcinoma cells in sterile saline and sacrificed after 14 days, when body weight loss was about 15% of the initial body weight, a condition referred to as severe cachexia (Bonetto et al., 2011). Control mice received an equal volume of saline. Eight-week old athymic nude (Nu/Nu) male mice (Harlan, Indianapolis, IN) were injected subcutaneously between the scapulae with 2×10^6 HT-29 cells and sacrificed after 47 days from tumor inoculation. Control mice received an equal volume of saline. Twelve-week old C57BL6/J-Apc^{Min}/J ($Apc^{Min/+}$) male mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in our colony for up to 27 weeks of age. Animals were genotyped upon delivery, according to the protocol provided by The Jackson Laboratory. Mice were sacrificed when muscle weight loss was about 25% of the initial body weight (i.e., the weight recorded at time of delivery at our facility). Age-matched C57BL6/J mice served as controls (The Jackson Laboratory, Bar Harbor, ME). Animals were monitored and weighed daily until the day of sacrifice. At time of sacrifice, all mice displayed evident tumor growth and no animals were excluded from the study. Several tissues were collected, weighed, snap frozen in liquid nitrogen and stored at −80°C for further studies. The tibialis anterior muscle was frozen in liquid nitrogen-cooled isopentane, mounted in OCT and stored for morphological analyses.

Cell Culture

Murine C26 cells were kindly provided by Donna McCarthy (Ohio State University) and cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) supplied with 10% fetal bovine serum, 1% glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin. Human HT-29 cells (ATCC, Manassas, VA) were cultured in McCoy's 5a Modified Medium supplied with 10% fetal bovine serum, 1% glutamine, 1% sodium pyruvate, and 1% penicillin/streptomycin. Both cell lines were maintained in a 5% CO_2, 37°C humidified incubator.

Dual-Energy X-Ray Absorptiometry (DXA)

Assessment of lean tissue, as well as whole body bone mineral density (BMD) and bone mineral content (BMC) were assessed by means of DXA scanning of frozen carcasses. According to the manufacturer's guidelines, in order to calibrate and validate the apparatus for its performance, a spine phantom was scanned

using the Lunar PIXImus densitometer (PIXImus, Fitchburg, WI) before scanning the first carcass. Animal carcasses were placed in a prone position with the limbs outstretched. From the whole-body scans, areal BMD and BMC were calculated for the entire body minus head ROI, and regionally for humerus, femur, and lumbar spine (L5) using the Lunar ROI tools.

Micro Computed Tomography (MCT)

After euthanasia, the right femur was dissected from each mouse, fixed for 2 days in 10% neutral buffered formalin, and then transferred into 70% ethanol for μCT scanning on a high-throughput μCT specimen scanner (μCT-35; Scanco Medical AG). The distal 33% of each bone was scanned using the following conditions: 50 kV, 120 mA, 151-ms integration time, 0.5 mm Al filter, and 10-μm voxel resolution (Bouxsein et al., 2010). Three-dimensional morphometric properties of the distal femur cancellous bone were measured as previously described (Niziolek et al., 2011). Briefly, trabecular bone volume fraction (BV/TV; %), trabecular number (Tb.N; 1/mm), and trabecular thickness (Tb.Th; mm) were determined on a 1.5 mm region of the distal femur secondary spongiosa, using an ROI beginning 0.6 mm proximal to the distal growth plate (identified by radiolucency and morphology) and extending proximally for 1.5 mm. The trabecular bone was digitally isolated from the cortical compartment by manually lassoing the trabecular bone every 15 slices, then interpolating the trabecular compartment in intervening slices using the contouring function in the Scanco software. All measurements were calculated automatically using the Scanco software (μCT v6.1).

Three-Point Bending Test

In order to define the bone mechanical properties in the presence of colorectal cancer, the bones were loaded to failure by three-point bending. Briefly, the left femurs were removed from the carcasses, wrapped in saline soaked gauze, and stored at −20°C. Prior to testing they were rehydrated overnight in 0.9% NaCl at room temperature. Testing was performed on a miniature materials testing machine (Vitrodyne V1000; Liveco, Inc., Burlington, VT, USA), which has a force resolution of 0.05 n. The lower supports were set at the maximal allowable distance for each bone without compromising the test (10.0 mm for the femur). The crosshead speed during testing was 0.2 mm/s, and force-displacement data was collected every 0.01 s. From the data, a force vs. displacement graph was created, and the ultimate force (F_U; N), stiffness (S; N/mm) and post yield energy to failure (U_{PY}; mJ) were calculated as shown in Mcateer et al. (2010).

Statistical Analysis

All results were expressed as means ± SEM. In particular, changes in muscle and fat mass (**Figure 1**) are presented as percentage (%) of the tissue weights normalized to the initial body weight (IBW). Significance of the differences was evaluated by Student's *t*-test. Difference was considered significant when $p < 0.05$.

RESULTS

Colorectal Cancer Causes Body Weight Loss Associated with Muscle and Fat Wasting

In order to investigate whether the growth of colorectal cancer associates with the development of cachexia and bone loss, we took advantage of three different *in vivo* models. In particular, CD2F1 mice injected with the well-characterized C26 murine colorectal cancer cells showed progressive loss of body weight (−14%, $p < 0.01$), accompanied by marked muscle depletion, as suggested by the reduction in skeletal muscle mass (GSN: −16%; Quadriceps: −26% vs. Control) and by the decrease in lean tissue content ($C = 2.49 \pm 0.38$ g, C26 $= 2.05 \pm 0.39$ g; −18%, $p < 0.05$), assessed by means of DXA. Consistently, the epididymal fat was also severely depleted in the tumor hosts (−62%, $p < 0.05$) (**Figure 1A**), while tumor size (0.67 ± 0.29 g) was in line with previous studies using the same experimental model (Bonetto et al., 2011). Of note, tumor growth also caused cardiac muscle atrophy (−17%, $p < 0.01$) (**Figure 1A**). To the extent of establishing and characterizing new preclinical mouse models of colorectal cancer, we injected the HT-29 human colorectal adenocarcinoma in athymic nude mice. After 47 days from tumor inoculation, the animals showed significantly reduced body weight (−14%; $p < 0.05$ vs. Control) along with depletion of skeletal muscle (GSN: −18%; Quadriceps: −20% vs. Control), overall reduction in lean tissue content ($C = 6.80 \pm 0.63$ g, HT-29 $= 5.82 \pm 0.38$ g; −14%, $p < 0.05$) and severe decrease in fat mass (−78%, $p < 0.05$) (**Figure 1B**). This was also consistent with remarkable tumor growth (2.71 ± 1.37 g). Similarly, at around 27 weeks of age, the $Apc^{Min/+}$ mouse, an extensively studied genetic model of colorectal cancer development, displayed severe body weight loss (−27%, $p < 0.001$), consistent with overall loss of skeletal muscle mass (GSN: −32%; Quadriceps: −43% vs. Control), lean tissue ($C = 9.67 \pm 0.72$ g, $Apc^{Min/+}$ =: 5.37 ± 1.11 g; −44%, $p < 0.001$) and adipose tissue (−69%, $p < 0.001$), but no change in heart weight (**Figure 1C**).

Bone Tissue Is Differentially Affected by Colorectal Cancer

Bone loss in the presence of colorectal cancer was assessed by means of DXA (**Figure 2**) or μCT scans (**Figure 3**). Based on the DXA scan quantification, while a moderate loss of whole body bone mineral density (BMD) was observed in the C26 hosts (**Figure 2E**), the HT-29 hosts showed decreased BMD (−5%, $p < 0.05$), along with depletion of bone tissue at the level of vertebrae (−11%, $p < 0.01$) and femur (−15%, $p < 0.01$) (**Figure 2F**). Similarly, the $Apc^{Min/+}$ mice displayed an overall severe depletion of bone tissue (−18%, $p < 0.001$), even more exacerbated in the L5 vertebrae (−22%, $p < 0.001$), femur (−31%, $p < 0.001$) and humerus (−25%, $p < 0.001$) (**Figure 2G**). Interestingly, no significant change in bone mineral content (BMC) was detected in the C26 or HT-29 hosts (**Figures 2E,F**), while an overall marked bone tissue loss was detected in the $Apc^{Min/+}$ animals (−28%, $p < 0.01$), and more specifically in femur (−18%, $p < 0.05$) and humerus (−16%, $p <$

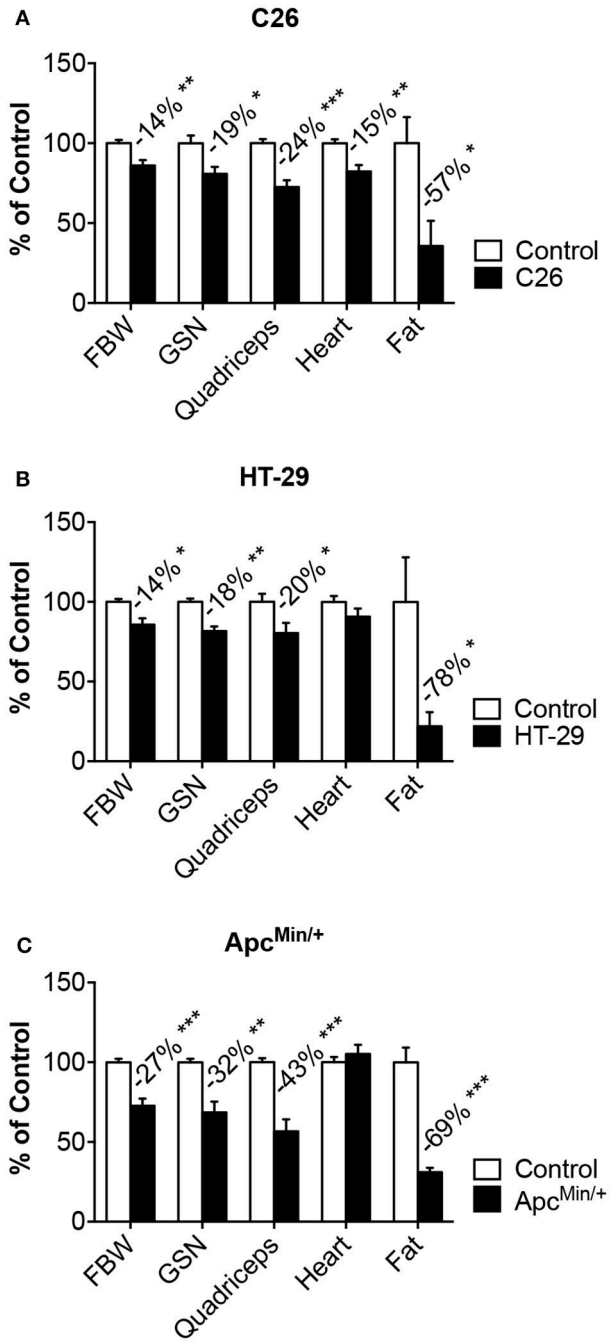

FIGURE 1 | Models of colorectal cancer show muscle and fat wasting. Tumor growth was associated with changes in body weight, muscle (gastrocnemius, quadriceps, heart) and fat tissue in mice bearing colorectal cancers. C26 **(A)**, $Apc^{Min/+}$ **(B)**, HT-29 **(C)**. $n = 4$–8. Data (means ± SEM) are expressed as percentage (%) of the respective Control group. Significance of the differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control. FBW, tumor-free final body weight; GSN, gastrocnemius.

0.05) (**Figure 2G**). Consistently, the μCT analysis revealed varying levels of bone loss across our models. In particular, while no significant changes were observed in the C26 model (**Figures 3A–D**), decreased BV/TV (−14%, $p < 0.05$), Tb.N (−35%, $p < 0.05$) and Tb.Th (−15%, $p < 0.01$) were detected in the bone of mice bearing the HT-29 tumors (**Figures 3B–E**). Analogously, the $Apc^{Min/+}$ showed reduced BV/TV (−37%, $p < 0.001$), Tb.N (−19%, $p < 0.01$) and Tb.Th (−28%, $p < 0.001$) (**Figures 3C–F**).

The $Apc^{Min/+}$ Mouse Shows Reduced Bone Strength

In order to investigate whether changes in bone strength were associated with the development of colorectal cancer *in vivo*, femurs from HT-29 bearers or $Apc^{Min/+}$ mice were subjected to three-point bending mechanical testing. Of note, despite a moderate loss of bone tissue, as shown in **Figure 3**, no change in bone strength, measured by three-point bending test (**Figures 4A,B**), was detected in the HT-29 tumor-bearing mice (**Figure 4C**). Conversely, the $Apc^{Min/+}$ mice showed significant decrease in ultimate force (F_U; −44%, $p < 0.001$), stiffness (S; −43%, $p < 0.001$), and energy to failure (U_{PY}; −57%, $p < 0.01$) when compared to the wild type controls (**Figure 4D**).

DISCUSSION

Extensive skeletal muscle wasting, with or without fat depletion, is one of the hallmarks of cancer cachexia (Fearon et al., 2011). Indeed, skeletal muscle loss and weakness are debilitating consequences of several advanced malignancies, which often associate with bone metastases (Waning et al., 2015). While the mechanisms associated with the development of bone metastases have been investigated for quite some time, it is not clear whether the occurrence of tumor-derived muscle wasting also directly affects bone tissue and its mechanical properties. In the present study, we aimed to investigate whether the occurrence of colorectal cancer was also associated with abnormalities in bone structure and mechanical properties. A better understanding of how cancer cachexia impacts the musculoskeletal system requires the generation of proper pre-clinical models for use in mechanistic studies. Indeed, only a handful of mouse models, only partially characterized, are currently in use for the study of cancer cachexia (Mori et al., 1991; Aulino et al., 2010; Benny Klimek et al., 2010). Therefore, we examined new and well-characterized experimental models of colorectal cancer cachexia to determine whether tumor growth is associated with the occurrence of bone pathology.

Here, we show that bone loss, accompanied by aberrations in bone structure and function, is associated with colorectal cancer cachexia by utilizing well-known and new *in vivo* models of colorectal cancer. We examined mice bearing the murine C26 tumor (Bonetto et al., 2009, 2011) or the human HT-29 colorectal adenocarcinoma. We also studied the $Apc^{Min/+}$ mouse (Mehl et al., 2005; Bonetto et al., 2012; White et al., 2012), which carries a heterozygous germ line mutation at codon 850 of the Apc gene responsible for the development of spontaneous colorectal adenomas. We found that all models showed tumor growth consistent with severe cachexia, consistently with muscle loss and fat depletion, although at a different extent across the

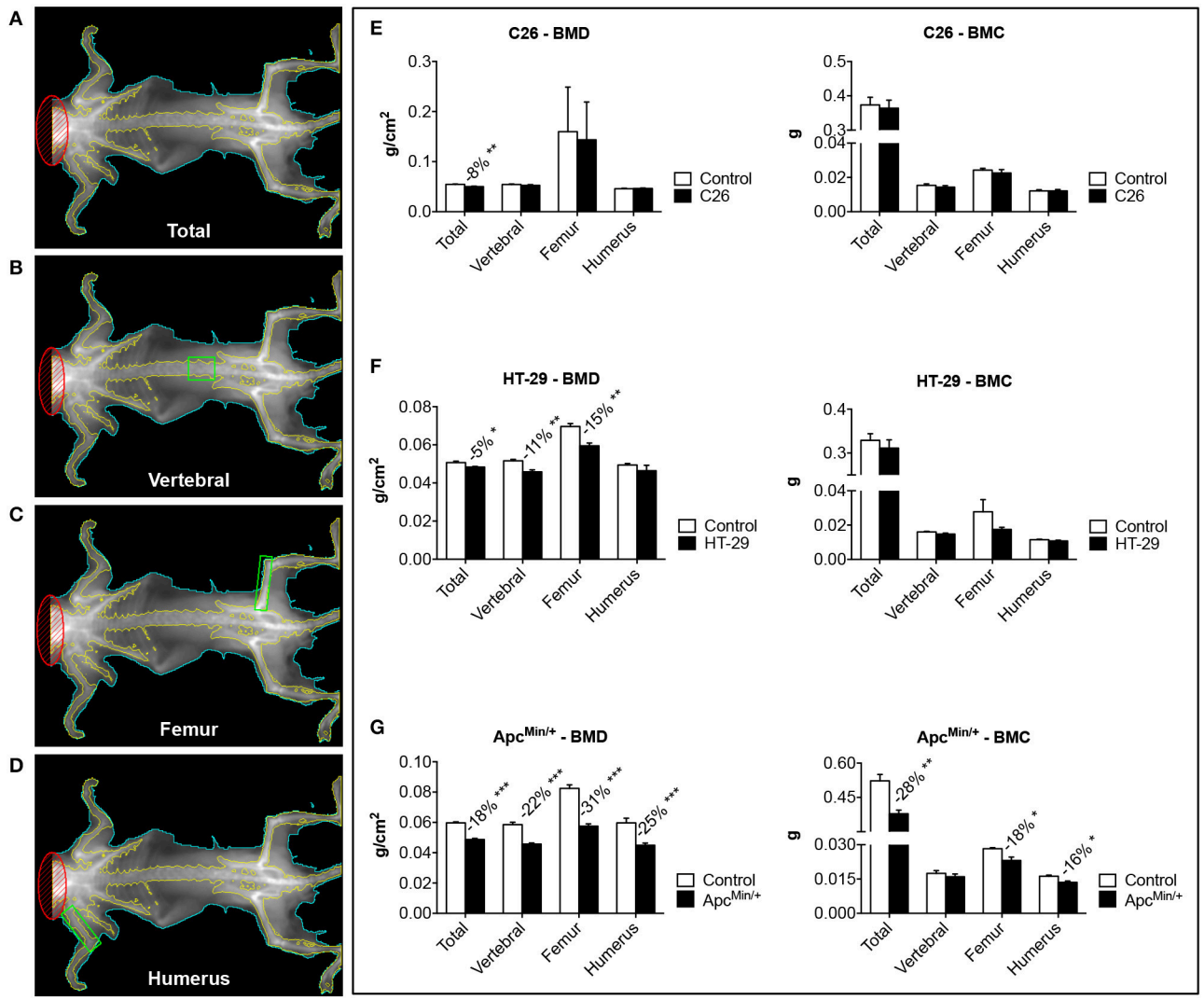

FIGURE 2 | The development of colorectal cancer is associated with decreases in Bone Mineral Density. Representative DXA scans of carcasses from colorectal cancer hosts. Total bone mineral density (BMD) and bone mineral content (BMC) measurements of the whole body are shown in **(A)**. Green boxes indicated ROI for the measurement of regional BMD and BMC (**B**: L5 vertebrae; **C**: femur; **D**: humerus). BMD and BMC quantification in C26-bearing animals **(E)**, HT-29 hosts **(F)** and $Apc^{Min/+}$ mice **(G)**. $n = 4$–8. Data (means ± SEM) are expressed as g/cm^2 (for BMD) or g (for BMC). Significance of the differences: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control.

three models (**Figure 5**). Interestingly, tumor size also seemed to correlate with the degree of wasting and with the length of the experimental period, whereas the C26 hosts show smaller tumors compared to the HT-29 bearers. In the present work, no assessment of tumor size was performed in the $Apc^{Min/+}$ mice, although it was previously shown that the size of the colorectal polyps mainly correlates with the extent of cachexia (Puppa et al., 2011).

Interestingly, our work is consistent with other studies that have shown a concurrent loss of muscle and bone tissue in murine models of cancer cachexia. Indeed, it was recently reported that animals bearing the Lewis Lung carcinoma present muscle wasting associated with decreased BMD, although alterations of bone strength were not taken into consideration (Choi et al., 2013). Similarly, bone tissue and bone strength were significantly affected in murine models of pancreatic cancer (Greco et al., 2015; Zhang et al., 2015). In the present study, we showed that regardless of the type of tumor, muscle and fat loss were generally accompanied by significantly decreased BMD, while BMC was reduced exclusively in the $Apc^{Min/+}$ animals (**Figure 5**). Moreover, HT-29 and $Apc^{Min/+}$ tumor hosts also displayed altered bone structure, consistent with decreased BV/TV, Tb.N and Tb.Th. Only the $Apc^{Min/+}$ mouse, characterized by the longest duration of the disease and the most aggressive cachectic phenotype, showed abnormal mechanical properties, as evidenced by the decreased F_U, S and U_{PY} parameters. Surprisingly, these results are in substantial disagreement with previous reports showing that mutations of

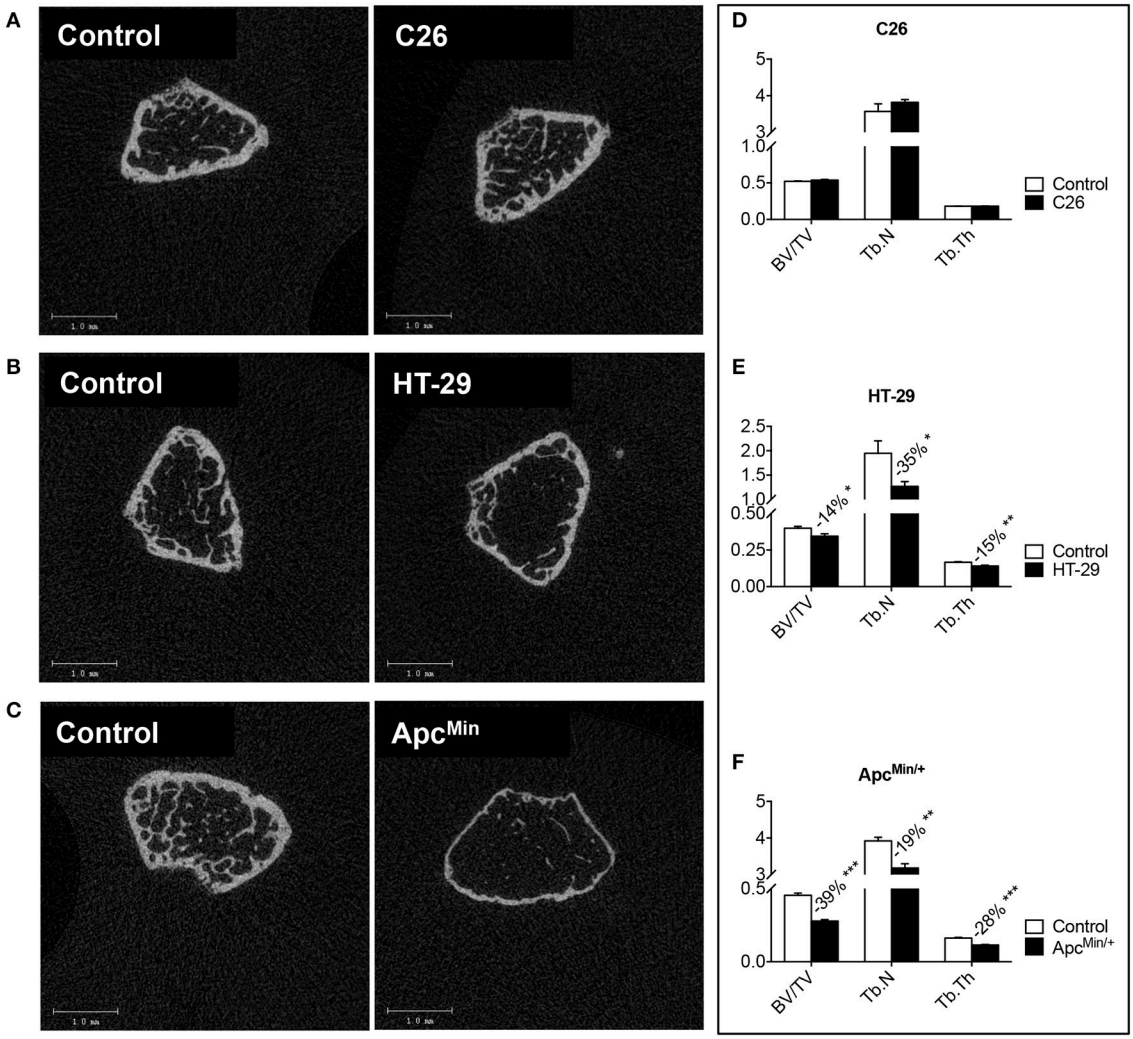

FIGURE 3 | Colorectal cancer promotes bone degeneration in the $Apc^{Min/+}$ and HT-29-bearing mice, but not in the C26 hosts. Representative μCT scan images of femur sections from colorectal cancer hosts **(A–C)**. Quantification of bone volume fraction (BV/TV; %), trabecular number (Tb.N; 1/mm), and trabecular thickness (Tb.Th; mm) in the femur of tumor-bearing mice. C26 **(D)**, HT-29 **(E)** and $Apc^{Min/+}$ **(F)**. $n = 4$–8. Data are expressed as means ± SEM. Significance of the differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control.

the Apc gene, encoding for a protein whose main role is to bind β-catenin, a mediator of the Wnt signaling pathway, are actually associated with increased BMD, both in animal models and in patients with Familial Adenomatous Polyposis (Holmen et al., 2005; Miclea et al., 2010). On the other hand, we cannot exclude that dysfunctions of the gut barrier, as often associated with the development of colorectal cancer in the $Apc^{Min/+}$ mouse model, may not only result into higher inflammation and increased risk of endotoxemia (Puppa et al., 2011), but also in decreased intake of calcium and Vitamin D, required for proper bone formation (Carmeliet et al., 2015; Wesa et al., 2015).

Based on our observations it does appear that the duration of the disease and the extent of muscle loss represent major contributing factors in regulating bone tissue in colorectal cancer. However, our results, generated by analyzing a single endpoint in the three mouse models, cannot establish a definitive relationship between duration of cachexia and severity of bone loss, as they cannot exclude that more severe cancer progression would lead to even more exacerbated bone loss. Moreover, the use of different mouse strains (CD2F1, athymic Nu/Nu, C57Bl6-$Apc^{Min/+}$), either immunocompetent or immunodeficient, may prevent us from generalizing our results and could represent a limitation to our findings. Indeed, strain-specific bone phenotypes have been previously described in a transgenic model with either a BALC/cJ or C57Bl6/J background, as well as in athymic and euthymic mice (Mccauley et al., 1989; Syberg et al., 2012). Similarly, bone regeneration and increased risk of osteoporosis have been shown in NOD/scid-IL2Rγ_c^{null} animals, which exhibit defects in innate and adaptive immunity (Rapp et al., 2016), as well as in patients affected with acquired immunodeficiency

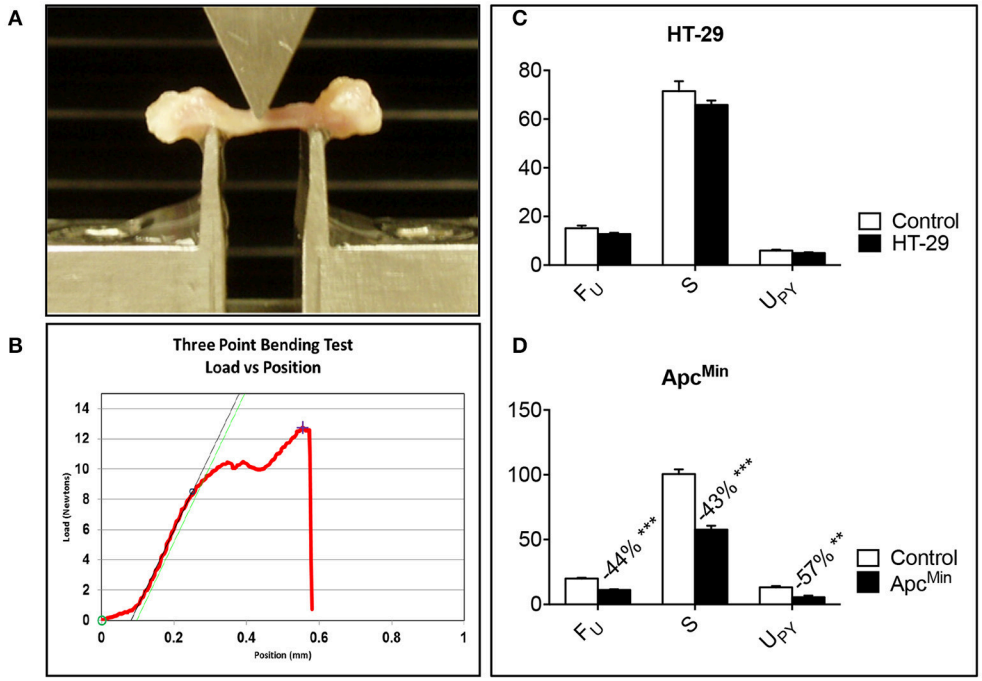

FIGURE 4 | Bone strength is decreased in the Apc$^{Min/+}$ mouse. Representative three-point bending test performed on femur from colorectal tumor-bearing mice **(A)**. Representative Force vs. displacement graph, where the peak of the curve represents the ultimate force (F_U), the slope the stiffness (S), and the area under the curve the energy to failure (U_{PY}) **(B)**. HT-29 **(D)**, Apc$^{Min/+}$ **(C)**. $n = 4$–8. Data (N for F_U; N/mm for S; mJ for U_{PY}) are expressed as means ± SEM. Significance of the differences: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control.

	C26	HT-29	Apc$^{Min/+}$
Duration (weeks)	2	7	27
Body weight	↓↓	↓↓	↓↓↓
Muscle weight	↓↓	↓	↓↓↓
Fat weight	↓↓	↓↓↓	↓↓↓
Bone Mineral Density	↓	↓↓	↓↓↓
Bone Mineral Content	-	-	↓↓
Trabecular volume	-	↓↓	↓↓↓
Bone strength	N.A.	-	↓↓↓

FIGURE 5 | The degree of bone loss in colorectal cancer cachexia depends upon tumor type, burden and duration of the disease. The chart summarizes the phenotypic changes observed upon colorectal cancer growth in the three experimental models (C26, HT-29 and Apc$^{Min/+}$). The number of arrows is indicative of the degree of change (mild, moderate, severe).

syndrome (Annapoorna et al., 2004). Interestingly, the level of physical activity may also contribute to explain the loss of bone tissue across the three tumor models, especially keeping in mind that the interaction between skeletal muscle and bone tissue was initially described as mainly mechanical in nature (Brotto and Bonewald, 2015). Consistently, previous reports showed that the growth of colorectal tumors progressively affected the overall activity and physical performances in C26 hosts and Apc$^{Min/+}$ mice (Baltgalvis et al., 2010; Toledo et al., 2014).

Nonetheless, muscle-derived factors have been shown to significantly affect bone metabolism, although it is not completely clear whether changes in muscle mass *per se* may also affect the integrity of bone tissue (Hamrick, 2012; Brotto and Bonewald, 2015). In the present study, that mainly described the relationship between colorectal cancer and the occurrence of changes in bone mechanical properties, we did not evaluate the levels of any of these factors. However, several mediators, such as BDNF, CXCL-1 (also known as KC), IL-1, IL-5, IL-6, IL-7, irisin, IFN-γ, LIF, TNF, TGF-α/β, and myostatin, have been shown to take part to the biochemical communications between skeletal muscle and bone tissue and to play a role in regulating the complicated balance between bone degradation and bone generation (Saidenberg-Kermanac'h et al., 2004; Mizoguchi et al., 2009; Polzer et al., 2010; Hamrick, 2011, 2012; Schett, 2011; Elkasrawy and Hamrick, 2013; Brotto and Bonewald, 2015; Waning et al., 2015). Importantly, bone-targeting pro-inflammatory cytokines, such as IL-6, IL-7, and IL-15, were originally described in association with muscle contraction and exercise, thus further supporting the idea that mechanical stimulation is fundamental to maintain bone integrity (Nielsen et al., 2007; Pedersen, 2009; Haugen et al., 2010; Hamrick, 2012). Conversely, recent evidence suggests that bone, acting as an endocrine organ, may secrete factors that can target muscle tissue and influence its homeostasis (Dallas et al., 2013).

Indeed, elevated FGF23 was reported to affect cardiac function (Mirza et al., 2009), while osteocalcin levels were shown to have a direct effect on muscle strength (Fernández-Real et al., 2009).

Recent evidence also suggests that anticancer therapies may contribute to both muscle weakness and bone decay. Along this line, we recently showed that therapies routinely used for the treatment of colorectal cancer play an important role in promoting muscle wasting and fatigue, particularly by affecting the muscle oxidative state and causing mitochondrial depletion (Barreto et al., 2016). Of note, significant loss of BMD was described in patients undergoing adjuvant chemotherapy for various gynecologic cancers (Christensen et al., 2016; Lee et al., 2016) or radiotherapy for abdominal tumors (Wei et al., 2016). Regardless of the molecular causes responsible for these side effects, the occurrence of bone fractures in patients with cancer or undergoing chemo-radiotherapy represents a problem of significant concern, causing substantial morbidity and worsening of the quality of life.

In conclusion, the data presented in our study suggest that colorectal cancer associates with muscle wasting and is generally accompanied by bone loss (**Figure 5**). Based on our results, the extent of bone depletion might depend upon tumor type, burden and duration of the disease, although limitations associated with the use of different mouse strains were also identified. Despite all this, the identification of muscle-/bone-derived factors that may result into novel therapeutic targets for the treatment of sarcopenia and osteoporosis is far from being accomplished. Moreover, while it is largely accepted that strategies aimed at preserving muscle mass can improve survival and quality of life in cancer cachexia (Benny Klimek et al., 2010; Zhou et al., 2010), as well as tolerance to the anticancer therapies (Barreto et al., 2016; Hatakeyama et al., 2016), further studies will be required to clarify whether preserving bone mass in cachexia may represent a novel strategy to improve outcomes and survival in colorectal cancer.

AUTHOR CONTRIBUTIONS

AB, LK, and TZ conceived of the studies. JC, TG, KM, AR, MC, LK, and TZ provided essential equipment, expertise and resources. AB, VP, RM, MP, KK, and RB performed experiments. AB, JK, VP, RM, TZ, KM, and AR analyzed and interpreted data. AB, JK, MC, LK, and TZ wrote and edited the manuscript.

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A Rat Immobilization Model Based on Cage Volume Reduction: A Physiological Model for Bed Rest?

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Bed rest has been an established treatment in the past prescribed for critically illness or convalescing patients, in order to preserve their body metabolic resource, to prevent serious complications and to support their rapid path to recovery. However, it has been reported that prolonged bed rest can have detrimental consequences that may delay or prevent the recovery from clinical illness. In order to study disuse-induced changes in muscle and bone, as observed during prolonged bed rest in humans, an innovative new model of muscle disuse for rodents is presented. Basically, the animals are confined to a reduced space designed to restrict their locomotion movements and allow them to drink and eat easily, without generating physical stress. The animals were immobilized for either 7, 14, or 28 days. The immobilization procedure induced a significant decrease of food intake, both at 14 and 28 days of immobilization. The reduced food intake was not a consequence of a stress condition induced by the model since plasma corticosterone levels –an indicator of a stress response– were not altered following the immobilization period. The animals showed a significant decrease in soleus muscle mass, grip force and cross-sectional area (a measure of fiber size), together with a decrease in bone mineral density. The present model may potentially serve to investigate the effects of bed-rest in pathological states characterized by a catabolic condition, such as diabetes or cancer.

Keywords: disuse-induced atrophy, physical inactivity, body composition, muscle performance, bone density, corticosteroids

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INTRODUCTION

Bed rest has been an established treatment in the past prescribed for critically illness or convalescing patients, in order to preserve their body metabolic resource, to prevent serious complications and to support their rapid path to recovery. However, in the past 50 years numerous scientific studies have reported that physical inactivity can exert negative effects to the entire system (Corcoran, 1991). Prolonged bed rest can have detrimental consequences that may delay or prevent the recovery from clinical illness including insulin resistance, thromboembolic disease, degenerative joint disease, disuse osteoporosis, respiratory, and musculoskeletal complications (Dittmer and Teasell, 1993). In addition to different pathological catabolic conditions –such as trauma, cancer and sepsis- healthy

aging (sarcopenia) and disuse (bed rest, microgravity) are also associated with muscle atrophy and wasting. Particularly, disuse-induced muscle atrophy is a catabolic condition commonly manifested in patients enforced to periods of muscle inactivity often associated with situations such as hindlimb immobilization, prolonged bed rest due to aging or the recovery from injuries, sepsis, and illnesses or weightlessness, as occurs in spaceflight (Musacchia et al., 1988). It is important to point out that unlike the muscle wasting caused by some disease states, disuse atrophy is initiated by a reduction in muscle contractile activity and muscle tension, rather than by inflammatory cytokines (Jackman and Kandarian, 2004; Chopard et al., 2009).

Physical inactivity is often achieved in humans using the bed rest or the unilateral limb suspension model, whereas the most frequently used models in rodents are denervation, casting immobilization or tail suspension (Morey-Holton and Globus, 2002; Frimel et al., 2005; Midrio, 2006). Each procedure has specific advantages and strengths which promote its use, as well as disadvantages which limit data interpretation and differ each other in term of degree of reproduced inactivity (Machida and Booth, 2004) and distinct protein degradation profiles induced (Bialek et al., 2011).

In this regard, we have designed a non-invasive, practical and low-cost model for rodents that better mimics human's reduced daily ambulatory motions, reproducing the effects of acute transitions from high to low levels of physical activity, and that better simulates the metabolic state of a patients in bed rest condition. Although immobilization through the restriction of locomotion is not a novel approach in animal experimentation—it has already been applied in biobehavioural research, particularly for the study of the stress response (Hauger et al., 1988; Wood et al., 2003; Buynitsky and Mostofsky, 2009)—its application on the study of muscle atrophy is completely new. Bearing all this in mind, the object of the present study was to design and test an immobilization method for rodents based on reduction of cage volume, to mimic the situation encountered in humans in bed rest. In particular, this initial study concentrates on the metabolic, functional, and morphometric characterization of the temporal progression of changes in muscle and bone mass, together with changes in glucose metabolism.

MATERIALS AND METHODS

Setting and Procedure

Male Wistar rats (11 weeks-old) were housed individually and maintained on a regular light-dark cycle (light on from 08:00 a.m. to 08:00 p.m.) controlled temperature (22°C) and humidity (45%) and they had free access to water and food (AIN93M diet) (Reeves et al., 1993). They were divided into two groups: control (standard cage, $n = 5$) and immobilized (IMMO, reduced volume cage, 7–28 days $n = 9$; 14 days $n = 12$). The immobilized animals were kept for 7, 14, and 28 consecutive days in a reduced volume cage (*Tecniplast 2150*), the space being restricted to 12 × 12 × 8 cm (approximately an 80% reduction in the total standard cage volume) (**Figure 1**). Body weight, food and water intake were recorded daily. Rats were sacrificed at day 7, 14, and 28. Prior to sacrifice rats were weighed and anesthetized (3:1 mixture of ketamine (Imalgene®) and xylazine (Rompun®). Blood was collected from the aorta and post-prandial plasma separated by centrifugation at 3,500 g for 10 min at 4°C and stored at −80°C. Muscles and other tissues were rapidly excised, weighed and frozen in nitrogen liquid. All tissues were stored at − 80°C until analysis. For a better comprehension, a timeline of the experimental plan is represented in **Figure 2**. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. They were cared for in compliance with the *Policy on Humane Care and Use of Laboratory Animals* (ILAR 2011). The Bioethical Committee of the University of Barcelona approved the experimental protocol.

Outcome Parameters

Corticosteroids

At day 1, 4, 8, and 14, blood samples were collected from the distal extreme of the tail at the same time (3:00 p.m.) in order to avoid the "screen" effect of the circadian rhythm on the response to stress (Smith, 2012). The number of animals used to measure the corticosteroid levels were the following: day 1: CONTROL $n = 4$ and immobilized group (IMMO) $n = 5$; day 4: CONTROL $n = 5$ and IMMO $n = 5$; day 8: CONTROL $n = 5$ and IMMO $n = 4$; day 14: CONTROL $n = 4$ and IMMO $n = 4$. Plasma corticosterone levels were quantified by ELISA kit (*Arbor assays, Chicago, USA).*

Oral Glucose Tolerance Test (OGTT)

The OGTT was performed 4 days prior to sacrifice in order to minimize the influence of the fasting on the final body weight, body composition and physical activity tested the day of sacrifice. Animals were fasted overnight (12 h) and blood was collected in heparinised wells from the distal extreme of the tail, prior to the glucose solution administration, to assess the fasting levels of glucose and insulin (baseline time). To reduce an infection, a topical germicide (Betadine® solution) was applied to the tail following blood collection. Blood collection was obtained 15, 30, 60, and 120 min after the glucose solution administration (2 g/kg rat) by gavage. Glucose levels were measured by the Glucometer (*Accutrend, GCT, Mannheim, Germany, Roche*).

Grip Strength

Skeletal muscle force in rats was quantified by the grip-strength test once a week. The grip strength device (*Panlab-Harvard Apparatus, Spain*) comprised a pull bar connected to an isometric force transducer (dynamometer). Basically, the apparatus was positioned horizontally and the rats were held by the tail and lowered toward the device. The animals were allowed to grasp the pull bar by their forelimbs and were then pulled backwards in the horizontal plane. The force applied to the bar just before the animals lost grip was recorded as the peak tension. At least three measurements were taken per rat on both baseline and test days, and the results were averaged for analysis. This force was measured in grams/grams initial body weight (Toledo et al., 2011).

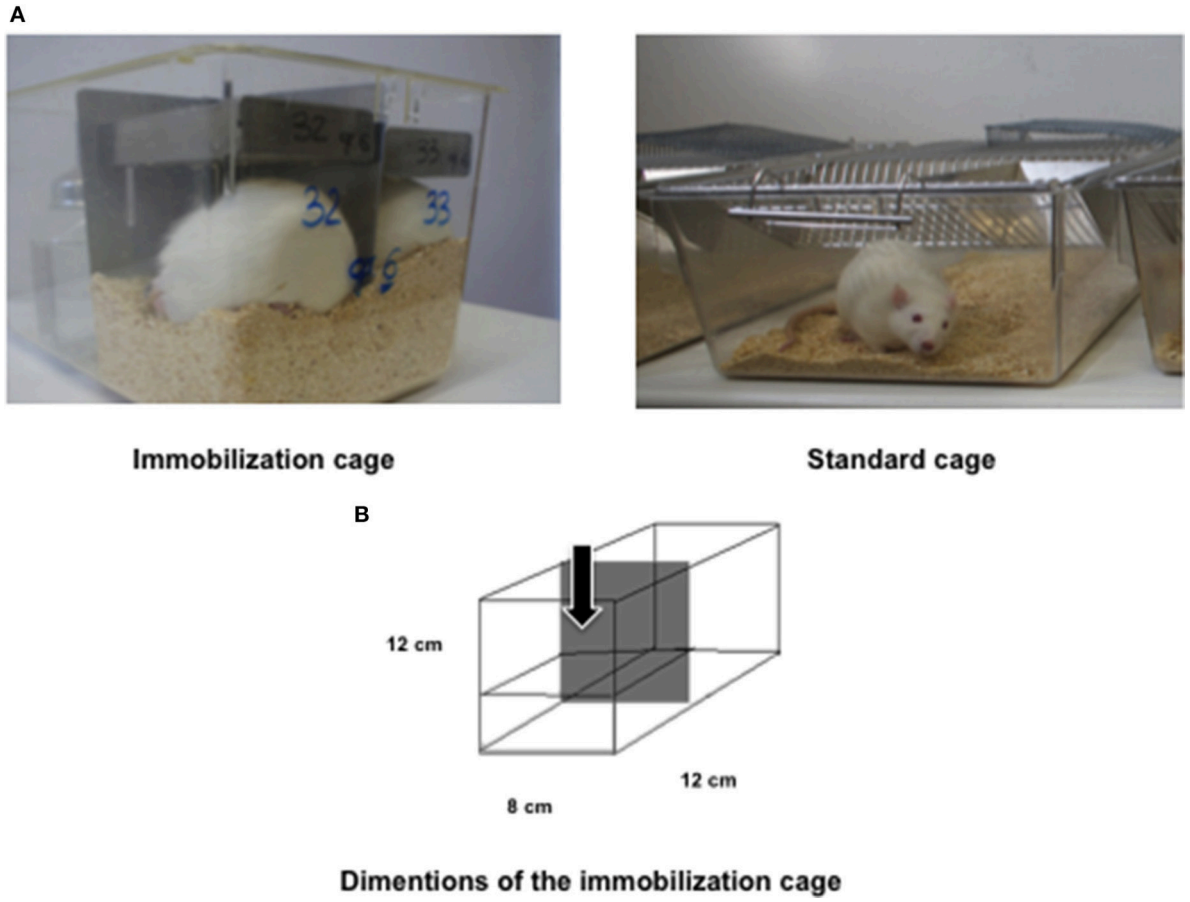

FIGURE 1 | Immobilization model. (A) Representative pictures of immobilized rats and control rat in a standard cage. Each immobilization cage is able to host two animals and it is equipped (in its upper part) with a grating to dispose food and water. **(B)** Schematic representation of the immobilization tested model. The cage had a reduction in volume of 80% in relation with control standard cage. The arrow indicated the housing area of a single rat.

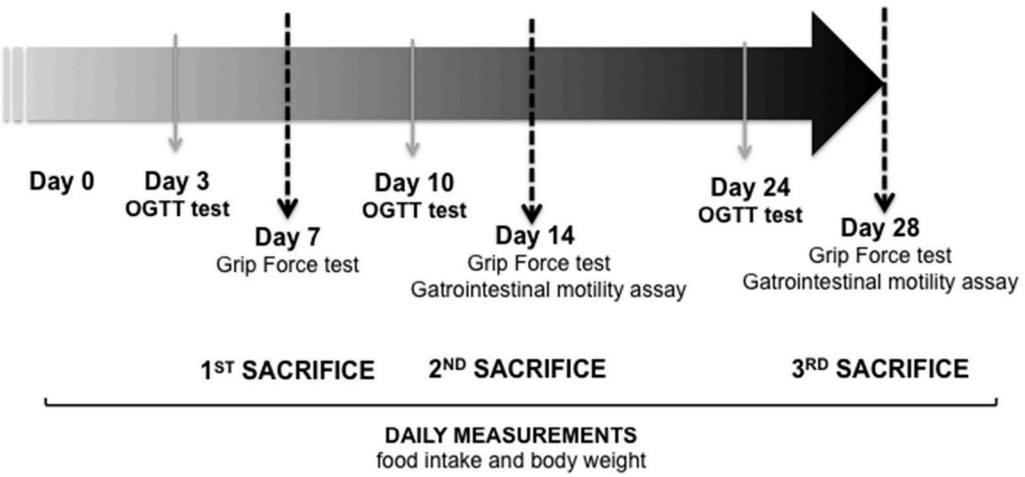

FIGURE 2 | Timeline of the experimental plan. Day 7: CONTROL ($n = 5$) and immobilized group (IMMO) ($n = 6$); day 14: CONTROL ($n = 9$) and immobilized group (IMMO) ($n = 12$); day 28: CONTROL ($n = 5$) and IMMO ($n = 6$). OGTT: Oral glucose tolerance test.

Gastrointestinal Motility

Gastrointestinal motility in rats was tested by a method described (Arbós et al., 1993). An oral glucose load (4 mmol) containing 2 μCi of [^{3}H]inulin was administrated to each rat 2 h before the sacrifice. The gastrointestinal tract was extracted and divided into stomach and intestine with their contents and they were processed for [^{3}H]-scintillation counting. The intestine was divided into six equivalent segments (duodenal to colon: I1–I6) and the amount of labeled retained calculated for each of them. They were mixed with 3% (w/v) perchloric acid and homogenized in a Waring Blender. After centrifugation, 5 mL of the supernatant were neutralized with potassium hydroxide 30% and then centrifuged at 1,000 g for 5 min to accelerate the ClO_4^- precipitation under form of KClO4. Finally, 5 mL of the neutralized supernatant were added to 10 mL of scintillation fluid for the measurement of total radioactivity.

Fiber Cross Sectional Area

During the sacrifice, the soleus muscle was rapidly excised from each limb, and quickly frozen in liquid-nitrogen cooled isopentane, maintaining the correct orientation to allow cross section. Ten micrometers of transverse sections from the mid-belly of the muscles were cut on a cryostat at −20°C. The slides obtained were stained by haematoxylin-eosin staining protocol, mounted with permount mounting media (*Fisher, United States*) and photographed at 10X magnification. Fiber cross-sectional area (CSA) was determined on randomly chosen 100 individual fibers per animal by the *Image J software* and expressed in pixels (Abramoff et al., 2004). Photo magnification and resolution were maintained fixed within each experiment.

Body Composition Analysis

Body composition was determined *post-mortem* in the all body of the animals, excluding tissues used in other measures (muscles, organs, and blood), by quantitative magnetic resonance (QMR) by means of an Echo MRI-100 rodent whole body composition analyser (Echo Medical Systems, Houston, Texas, USA) (Nixon et al., 2010).

Bone Mineral Density Analysis

Bone mineral density was measured *post-mortem* in tibia, femur, lumbar vertebras (LV 2-5), forearm, and humerus by peripheral Dual-energy X-ray Absorptiometry (pDEXA) analysis (Norland Corp., Fort Atkinson, WI, USA) (Griffin et al., 1993).

Statistical Analysis

To summarize and describe the results, average (arithmetic mean), and standard error of the mean (SEM) were calculated for each studied variable. Intergroup differences were evaluated using analysis of variance (ANOVA) and linear mixed models. *Post hoc* pairwise comparisons (Duncan test) were performed when appropriated. In order to assess the validity of the ANOVA results, the normality of data and homogeneity of variances were check for each variable. All the statistical analysis was performed using SPSS (version 21).

RESULTS

In order to study disuse-induced changes in muscle and bone, as observed during prolonged bed rest in humans, we have designed a new model of muscle disuse for rodents. The immobilization device is depicted in **Figure 1**. Basically, the animals are confined to a reduced space not permitting displacement (but allowing them to drink and eat in an easy way similar to a bed rest condition) for 7, 14, and 28 consecutive days (**Figure 2**). However, it has to be pointed out that, in terms of lifespan, the immobilization periods used here are far longer than those previously used in human studies. Although bed rest is a unique model to investigate mechanisms of underlying defects induced by physical inactivity in healthy subjects, it is important to remember that bed rest induces a level of physical inactivity likely different (quantitatively and qualitatively) from that observed under other conditions.

The average daily food intake was decreased due to the immobilization procedure without inducing variations in the body weight of the animals (**Table 1**). The animals consumed a reduced amount of food to maintain the energetic balance. However, quantitative magnetic resonance data, shown in **Table 1**, do not evidence changes of the lean and fat mass composition after the immobilization period. No differences were observed in plasma corticosteroid levels between immobilized and non-immobilized animals (**Table 2**).

We analyzed the effects of the proposed disuse model on glucose metabolism. Unexpectedly, a significantly variation on glucose tolerance was observed in the rats only after 7 days of immobilization (**Figure 3**).

Concerning muscle weights, immobilization resulted in a significant decrease in soleus weight (−7.3, 10.5, and 13.2% for 7-, 14- and 28 days-following immobilization, respectively) (**Table 3**). In addition, heart weight was also decreased after 7 and 14 days of immobilization (**Table 3**). In order to assess if the muscle wasting induced by our immobilization model was translated in an altered muscle performance, we measured grip force of the fore limbs of the animals. The data presented in **Table 3** showed significant decrease of this parameter after 7 and 14 days of physical inactivity. The results presented in **Table 4** clearly showed that physical inactivity significantly decreased bone mineral density in the vertebrae (LV-25) of the immobilized animals.

DISCUSSION

It is known that a period of bed rest leads to physical inactivity status with an associated reduction of energy requirements and appetite. Consequently, food intake generally declines, resulting in an inadequate dietary protein consumption to allow proper muscle mass maintenance (Wall and van Loon, 2013). This is also observed in our study. It could be speculated that the feeding behavior observed in cage-restricted rats could reflect the environmental stress at which apparently the animals were submitted (Zylan and Brown, 1996). Indeed, the inhibition of vegetative functions, such as appetite and feeding, is considered an acute physiological response resulting from the effects of

TABLE 1 | Body weight gain, food intake, energetic efficiency, and body composition in immobilized Wistar rats.

Parameters	7 DAYS		14 DAYS		28 DAYS		ANOVA		
	CONTROL	IMMO	CONTROL	IMMO	CONTROL	IMMO	*I*	*T*	IxT
(FBW-IBW)	6.2 ± 3.8(5)	9.2 ± 2.3(6)	36.7 ± 4.5(9)	22.3 ± 4.8(12)	43.4 ± 9.1(5)	43.4 ± 5.1(6)	ns	0.001	ns
DAILY FOOD INTAKE	7.4 ± 0.4(5)	6.9 ± 0.2(6)	6.1 ± 0.1(9)	5.5 ± 0.2(12)	5.3 ± 0.3(5)	5.0 ± 0.2(6)	0.015	0.001	ns
ENERGETIC EFFICIENCY	4.1 ± 2.7(5)	7.0 ± 1.8(6)	13.6 ± 1.5(9)	9.0 ± 1.9(12)	9.1 ± 1.5(5)	9.9 ± 0.9(6)	ns	0.016	ns
% FAT MASS	10.3 ± 0.7(5)	10.0 ± 0.3(6)	12.2 ± 0.4(9)	11.8 ± 0.4(12)	12.2 ± 0.8(5)	11.7 ± 0.8(6)	ns	0.004	ns
% LEAN MASS	85.0 ± 1.0(5)	85.4 ± 0.6(6)	83.0 ± 0.6(9)	83.0 ± 0.4(12)	82.6 ± 0.7(5)	83.9 ± 0.7(6)	ns	0.030	ns

Data are summarized as mean ± SEM, number of animals (replicates) indicated in parentheses for control (CONTROL) and immobilized (IMMO) groups. IBW, initial body weight, FBW, final body weight (in grams). The formula used to calculate the ENERGETIC EFFICIENCY was [(FBW-IBW)/food ingested] × 100, ingested food in grams. The formula used to calculate the DAILY FOOD INTAKE was (food ingested per day within the period/IBW) × 100, food intake are expressed as grams per 100 grams of initial body weight. Upper body composition (FAT and LEAN MASS percentage) was measured by Magnetic Resonance Imaging analysis. Statistical significant differences were evaluated by a full factorial two-way ANOVA (fixed factors: I, immobilization, T, Time; IxT denotes the interaction term), p-values are shown in the three columns on the right (ns, non-significant differences, which implies a p-value equal to or greater than 0.05).

TABLE 2 | Plasmatic corticosteroid levels and adrenal glands weight.

	CONTROL	IMMO
CORTICOSTEROID LEVELS (ng/mL)		
Day 1	335 ± 57 (4)	242 ± 33 (5)
Day 4	212 ± 67 (5)	271 ± 66 (5)
Day 8	314 ± 48 (5)	213 ± 20 (4)
Day 14	190 ± 61 (4)	384 ± 74 (4)
ADRENAL GLANDS (mg/100 g IBW)		
Day 7	27.9 ± 2.7 (4)	24.0 ± 2.7 (6)
Day 14	30.2 ± 0.9 (9)	27.6 ± 1.0 (9)

Corticosteroid levels (ng/mL) are expressed as mean ± SEM for the number of animals indicated in parentheses for control (CONTROL) and immobilized (IMMO) groups. Adrenal glands weights are expressed as milligrams per 100 grams of initial body weight. Data were analyzed by a Repeated Measures Linear Mixed Model, being time (T) the repeated measures factor and immobilization (I) a fixed-effects factor. Restricted Maximum Likelihood (REML) method was used to fit the mixed model. According to the values of Akaike Information Criterion (AIC) and Schwarz Bayesian Information Criterion (BIC), identity scaling was finally chosen as the covariance matrix structure. For corticosteroid levels: no significant differences were detected over time (T, p = 0.806) or for the immobilization factor (I, p = 0.697), neither for interaction (IxT, p = 0.052). For adrenal glands: Statistical significance was tested by a full factorial two-way ANOVA (fixed factors: immobilization (I) (p = 0.080), Time (T) (p = 0.516); interaction (IxT) (p = 0.990).

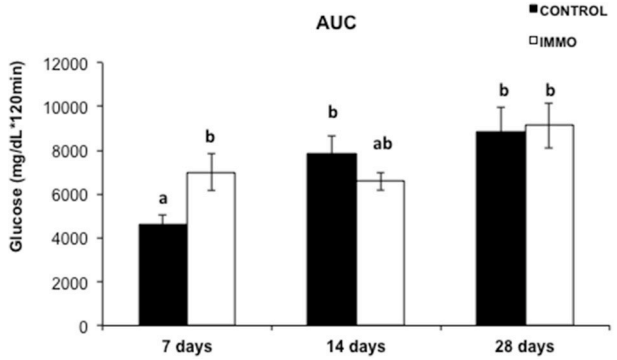

FIGURE 3 | Area under the curve (AUC) of plasma incremental glycaemia values in immobilized Wistar rats. Each bar and segment represents mean values ± SEM for CONTROL ($n = 5$) and immobilized groups (IMMO) ($n = 6$). Statistical significant differences were evaluated by a full factorial two-way ANOVA (fixed factors: I = immobilization, T = Time; IxT denotes the interaction term): I, p = ns; T, $p = 0.033$; IxT, $p = 0.021$. *Post-hoc* pairwise comparisons were performed (Duncan method); different letters (a and b) indicate significant differences between the experimental groups.

stress, induced by the immobilization device, on the appetite-satiety centers in the hypothalamus (Shimizu et al., 1989; Krahn et al., 1990; Charmandari et al., 2005). Certain peptides and neurotransmitters are involved in this response, such as monoamines (Kennett et al., 1987) corticotrophin-releasing hormone (CRH) (Krahn et al., 1988, 1990; Rich, 2005) and others (Charmandari et al., 2005). The body's response to a stressful stimulus is regulated by the hypothalamic-pituitary-adrenal (HPA) axis through hormonal feedback (Cruthirds et al., 2011). The HPA axis involves the release of corticotrophin-releasing hormone (CTH) from the hypothalamus, which modulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary, which then controls the secretion of glucocorticoid from the adrenal glands (Cruthirds et al., 2011). Many immobilization models in rodents are defined as severe chronic stressors (Martí et al., 1994). Ricart-Jané et al. reported that immobilization resulted in decreased body weight gain and food intake, together with an increase in the weight of the adrenal glands. It also resulted in a decrease in liver glycogen, all of them signs of chronic stress (Ricart-Jané et al., 2002). However, the results obtained concerning corticosterone concentrations in our immobilization model confirmed that the changes observed in food intake could not be attributed to stress. In fact, the circulating corticosteroids concentrations together with the unchanged adrenal glands weight (**Table 2**) and the unaltered body weight gain (**Table 1**), confirmed that the immobilization model proposed does not represent a physical stressor for the animal.

No changes were observed on glucose metabolism when the immobilization period was longer than 7 days, possibly as a consequence of the activation of a compensatory response improving the ability of the organism to adapt and increase its chance for the survival. However, further analysis is mandatory

TABLE 3 | Muscle weights and grip force in immobilized Wistar rats.

	7 DAYS		14 DAYS		28 DAYS		ANOVA		
	CONTROL	IMMO	CONTROL	IMMO	CONTROL	IMMO	*I*	*T*	IXT
GSN	597.6 ± 16.1(5)	604.1 ± 8.2(6)	592.2 ± 14.7(9)	607.7 ± 9.7(12)	674.4 ± 8.0(5)	659.0 ± 16.6(6)	ns	0.001	ns
EDL	47.1 ± 2.0(5)	48.4 ± 1.3(6)	45.6 ± 1.3(9)	47.2 ± 0.7(12)	49.2 ± 0.3(5)	48.6 ± 1.9(6)	ns	ns	ns
TIB	184.6 ± 10.2(5)	193.6 ± 2.3(6)	194.0 ± 5.1(9)	195.9 ± 3.1(12)	207.4 ± 5.7(5)	207.5 ± 6.3(6)	ns	0.012	ns
SOL	43.7 ± 2.9(5)	40.5 ± 0.9(6)	42.7 ± 1.5(8)	38.2 ± 0.5(12)	48.2 ± 2.1(5)	41.8 ± 0.6(6)	0.001	0.004	ns
HEART	264.8 ± 7.8(5)b	244.3 ± 3.0(6)a	276.3 ± 3.8(9)b	240.9 ± 7.2(12)a	266.7 ± 5.5(5)b	269.3 ± 7.7(6)b	0.004	ns	0.026
GRIP FORCE	3.8 ± 0.1(5)	3.3 ± 0.1(6)	3.7 ± 0.1(8)	3.5 ± 0.1(11)	4.0 ± 0.2(5)	3.8 ± 0.2(6)	0.005	0.029	ns

Mean ± SEM for the number of animals indicated in parentheses are shown for every period for control (CONTROL) and immobilized (IMMO) groups. Muscle weights are expressed as milligrams per 100 grams of initial body weight. Statistical significance was tested by a full factorial two-way ANOVA (fixed factors: I, immobilization, T, Time; IxT denotes the interaction term), p-values are shown in the three columns on the right (ns, non-significant differences, which implies a p-value equal to or greater than 0.05). It has been detected a significant interaction term in the model for the heart, so that pairwise comparisons were performed (Duncan method); different letters indicate significant differences between the experimental groups. GSN, gastrocnemius, EDL, extensor digitorum longus, TIB, tibialis, SOL: soleus. Muscle force of the animal was tested once a week and was expressed as g/g initial body weight.

TABLE 4 | Bone Mineral density (BMD) of immobilized Wistar rats.

	7 DAYS		14 DAYS		28 DAYS		ANOVA		
BONE	CONTROL	IMMO	CONTROL	IMMO	CONTROL	IMMO	*I*	*T*	IXT
LV-25	175.1 ± 3.9 (5)	173.4 ± 2.6 (6)	183.5 ± 2.4 (9)	172.7 ± 2.2 (12)	189.9 ± 3.5 (5)	180.1 ± 4.2 (6)	0.006	0.011	ns
HUMERUS	159.8 ± 2.1 (5)	162.8 ± 2.2 (6)	169.6 ± 2.9 (9)	165.2 ± 2.0 (12)	175.3 ± 2.0 (5)	175.6 ± 2.6 (6)	ns	0.001	ns
FOREARM	154.9 ± 2.3 (5)	161.8 ± 2.5 (6)	162.2 ± 4.7 (9)	161.0 ± 1.9 (12)	165.9 ± 4.6 (5)	166.9 ± 3.0 (6)	ns	ns	ns
TIBIA	150.7 ± 0.9 (5)	150.6 ± 0.8 (6)	159.2 ± 0.9 (8)	152.9 ± 1.9 (12)	165.6 ± 1.8 (5)	164.2 ± 2.6 (6)	ns	0.001	ns
FEMUR	196.5 ± 3.3 (5)	198.8 ± 1.2 (6)	208.5 ± 2.4 (9)	201.3 ± 4.1 (12)	219.1 ± 2.6 (5)	213.7 ± 4.0 (6)	ns	0.001	ns

Mean ± SEM for the number of animals indicated in parentheses for control (CONTROL) and immobilized (IMMO) groups are shown for every period. Bone Mineral density (BMD) was measured by peripheral Dual-energy X-ray Absorptiometry (pDEXA) analysis and expressed as mg/cm^2. Statistical significance was tested by a full factorial two-way ANOVA (fixed factors: I, immobilization, T, Time; IxT denotes the interaction term). p-values are shown in the three columns on the right (ns, non-significant differences, which implies a p-value equal to or greater than 0.05).

to clarify the molecular pathways underlying the hyperglycaemia observed.

Several studies indicate an inverse relationship between physical activity and risk of gastrointestinal-related disease, such as colon cancer, diverticular disease, cholelithiasis or constipation (Everhart et al., 1989; Aldoori et al., 1995; Colditz et al., 1997; Leitzmann et al., 1998, 1999; Peters et al., 2001). In particular, the last is an uncomfortable gastrointestinal disturb, very common in the Western society, that is strictly associated with diet and physical exercise and characterized by hard stool consistency, straining and incomplete defecation (Sandler and Drossman, 1987; Dukas et al., 2003). For this reasons, we evaluated the effects of reduced physical mobility induced by our model on bowel functionality of the immobilized rats using a methodology based on the gastrointestinal distribution of [^{3}H]inulin, an indigestible carbohydrate (Arbós et al., 1993). From our data, physical inactivity did not influence gastrointestinal motility (**Figure 4**).

In patients, a period of prolonged bed rest, ranging between 10 and 42 days, is accompanied by a variable loss in muscle strength (between 0.3 and 4.2% per day) (Wall et al., 2013). In our study, immobilization resulted in a significant decrease in soleus weight (**Table 3**). This result is in accordance with that observed in many other rodent models of immobilization and hindlimb unloading, which have reported a greater muscle loss in the extensor muscles of the ankle (i.e., soleus and gastrocnemius) rather than the flexor muscles (i.e., tibialis anterior and *extensor digitorum longus*) (Thomason and Booth, 1990; Ohira et al., 2002; Adams et al., 2003; Zhong et al., 2005), demonstrating a preferential sensitivity to unloading of muscles predominately expressing the slow MHC phenotype (Baldwin et al., 2013). In addition of the decreased soleus weight, a significant decrease in the cross sectional area (CSA) of the muscle fibers was observed in all the immobilization groups studied (**Figure 5**). Indeed, a reduced CSA is the main characteristic morphological alteration resulting from muscle atrophy. Other alterations are: sarcomere dissolution, endothelial degradation, build-up of connective tissue between muscle fibers, reduction in the number of mitochondria, and a reduction in capillary density (Tyml et al., 1990; Ohira et al., 2002; Nielsen et al., 2010; Giordano et al., 2014). Our data are in agreement with the reduced CSA value reported in muscle fibers after 16 days of hindlimb suspension (Ishihara et al., 2002), and after 7 days of hindlimb casting immobilization (Talbert et al., 2013). Our model is characterized by a slower rate of muscle wasting in comparison with other immobilization models. For instance, 7 and 14 days of hindlimb suspension induce around 20 and 50%, respectively, of atrophy in the soleus muscle (Isfort et al.,

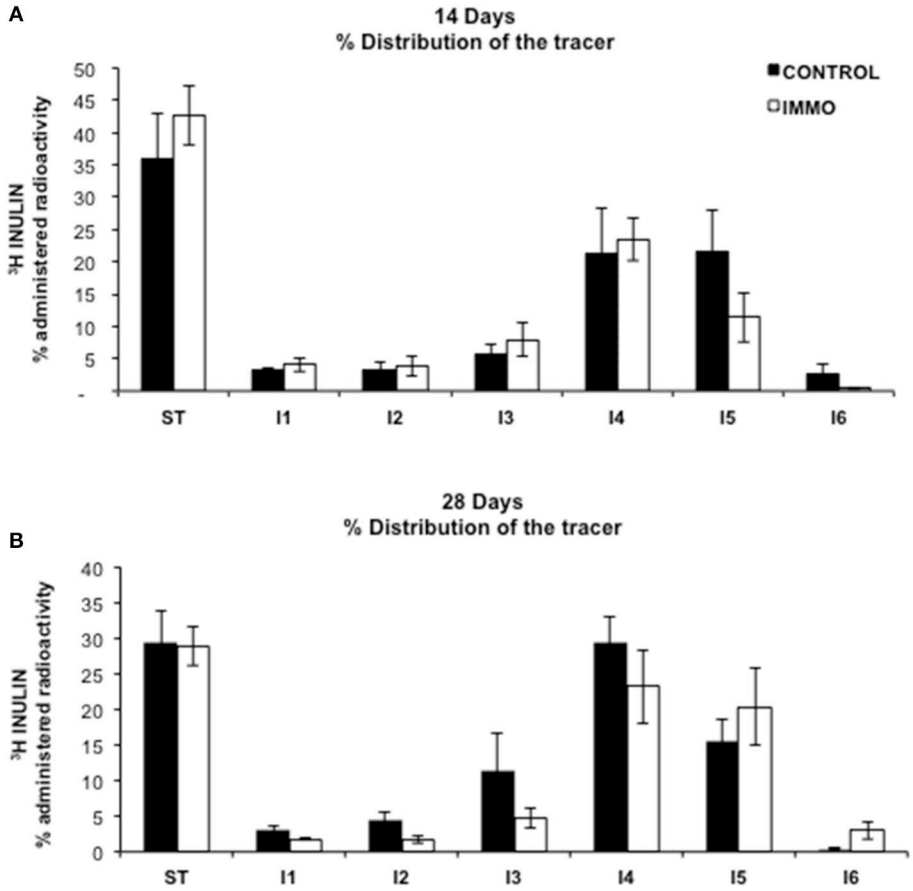

FIGURE 4 | Gastrointestinal motility in immobilized Wistar rats. The represented results are mean values ± SEM for control group (CONTROL) ($n = 5$ animals) and immobilized group (IMMO) ($n = 6$) animals in each gastrointestinal segment. Gastrointestinal distribution of orally-administrated [^{3}H]Inulin. ST: Stomach, I1 to I6: Intestine equivalent segments from duodenal to colon. Data were analyzed by a Linear Mixed Model, being time (T) and immobilization (I) two crossed between-subjects factors, and gastrointestinal segment (G) the within-subjects factor (repeated measures). Restricted Maximum Likelihood (REML) method was used to fit the model. According to Akaike Information Criterion (AIC) and Schwarz Bayesian Information Criterion (BIC), a heterogeneous Toeplitz covariance structure was finally chosen. Only significant differences among the gastrointestinal segments were detected (G, $p = 0.000$).

2002; De Boer et al., 2007). Meanwhile, 8 days of hindlimb immobilization using the plaster-cast model causes an atrophy of 23% in the soleus muscle (Vazeille et al., 2008). Other studies, using animal models, have suggested that the atrophy-induced disuse is driven by a decreased rate of protein synthesis and an increased rate of protein degradation (Booth and Seider, 1979; Tucker et al., 1981; Medina et al., 1995; Taillandier et al., 1996; Krawiec et al., 2005). Thomas on and Booth proposed a model to describe the mechanisms responsible for muscle loss observed in rat soleus muscle following hindlimb unloading. They identify a very fast decrease in protein synthesis rate followed by a gradually increase of the protein degradation rate which reached a peak by 15 days and then declined to below baseline levels (Thomason and Booth, 1990). Conversely, in humans subjects, the muscle atrophy observed during prolonged muscle disuse (>10 days) is a direct consequence of a reduced post-absorptive and post-prandial muscle synthesis rather than due to changes in muscle protein breakdown rates (Wall et al., 2013). Meanwhile during short term disuse (<10 days), the rapid muscle loss is probably due to increased muscle protein degradation that takes place simultaneously to reduced muscle protein synthesis (Wall et al., 2013). Moreover, and in accordance to human studies (Seki et al., 2001), the data presented in **Table 3** showed significant decrease of muscle force after 7 and 14 days of physical inactivity therefore validating further the efficacy of the model to mimic the physiological effects of bed rest in humans.

Heart weight was also decreased after 7 and 14 days of immobilization (**Table 3**). This result is in the agreement with published data obtained in humans showing that 6 weeks of horizontal bed rest cause cardiac atrophy (8%) as a consequence of physiological adaptation to a reduced maximal oxygen uptake and to reserve capacity in perform physical work (Convertino, 1997; Perhonen et al., 2001). On the same lines, Evans and Ivy using experimental animal models, underlined the ability of the hindlimb immobilization technique to induce a generalized catabolic state that was not only restricted to the muscle immobilized but also affected the cardiac tissue, impairing its aerobic capacity and reducing muscle size (Evans and Ivy, 1982).

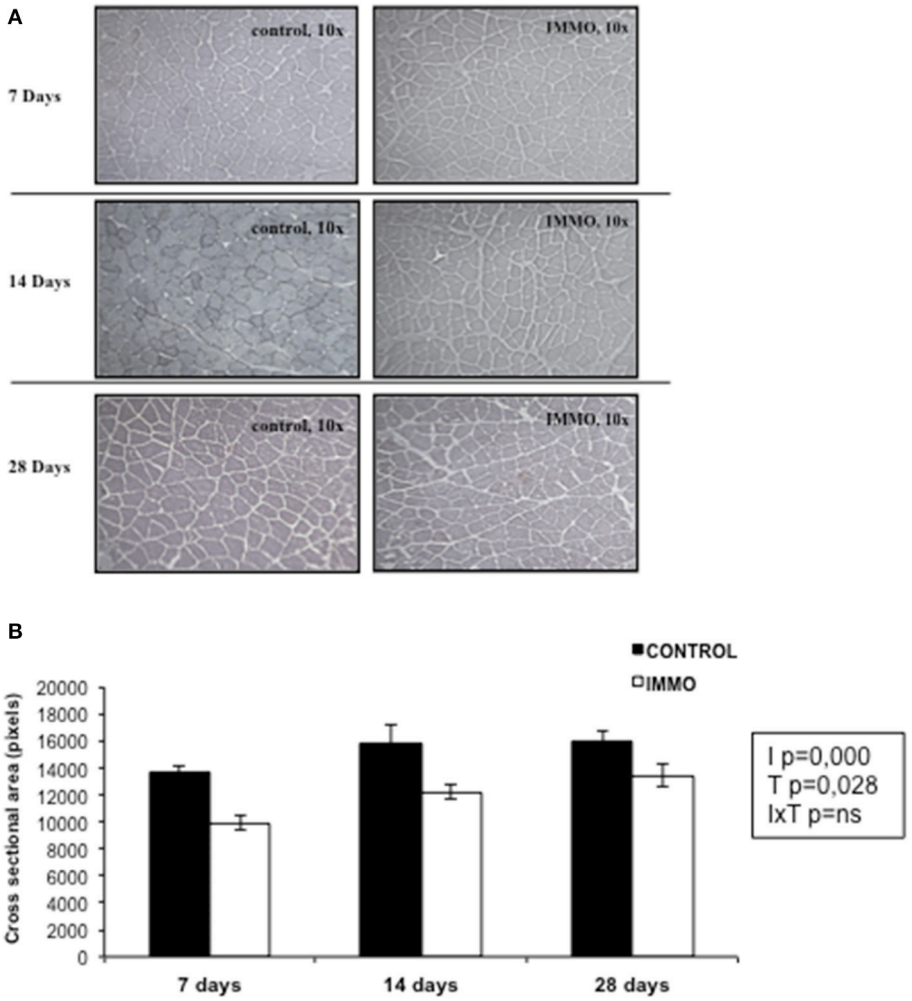

FIGURE 5 | Muscle fiber size in immobilized Wistar rats. (A) Representative images of muscle tissue sections stained with haematoxylin and eosin. **(B)** Muscle fiber cross-sectional area (pixels) of soleus muscle was determined on randomly chosen 100 individual fibers per animal by the Matic Image Plus 2. Bars and segments represents the mean values ± SEM for each group: day 7: CONTROL ($n = 3$) and immobilized group (IMMO) ($n = 5$); day 14: CONTROL ($n = 6$) and immobilized group (IMMO) ($n = 11$); day 28: CONTROL ($n = 4$) and immobilized group (IMMO) ($n = 5$). Statistical significance of the results were assessed by a full factorial two-way ANOVA (fixed factors: I = immobilization, T = Time; IxT denotes the interaction term): I, $p = 0.000$; T, $p = 0.028$; IxT, $p =$ ns.

Several studies have shown that disuse atrophy is associated with bone loss (Bloomfield, 1997; Collet et al., 1997; Kiratli et al., 2000). The bone mineral density (BMD) is the result of a dynamic process, called remodeling, which involves the removal of old or damaged bone by osteoclasts (bone resorption) and the subsequent replacement of new bone formed by osteoblasts (bone formation) (Feng and McDonald, 2011). The absence of intermittent mechanical solicitations, usually produced during loading and muscle contractions, is responsible of progressive deformations of cartilages and alterations of bone remodeling which results in a disorder termed immobilization-induced osteoporosis (Feng and McDonald, 2011). Indeed, studies in healthy subjects have shown that only 24 h of immobilization induced a rise in the osteoclast activity associated with a pronounced increase of bone resorption markers (Suzuki et al., 1994; Heer et al., 2005). In our model this loss of bone density was also observed. In other models, it has been shown that 10 days of hindlimb immobilization by plaster cast caused bone loss as measured by a reduced bone mineral density of the femur (−9%) and a decreased trabecular bone volumen of the tibial metaphysis (−25%) (Hott et al., 2003). The loss of bone weight induced by casting immobilization was mainly due to mineral losses - as indicated by changes in wet weight, ash weight, and calcium content- with a substantial part of the decrease affecting the trabecular bone and not the reduction of external bone volume (Tuukkanen et al., 1991).

CONCLUSIONS

The muscle atrophy together with a significant decline in muscle mass and force, and the loss of bone mass, define the proposed immobilization model as a new tool for the study of disuse-muscle atrophy. Our new procedure overcomes several limitations of the other commonly used immobilization ones,

with the advantages of being a low-cost and non-invasive model, standardized, reproducible and easy to implement. It does not require specific expensive equipment, and it maintains neural innervation to the musculature, while it does not alter the body weight and permits recovery-type studies to be performed with a low level of stress. Furthermore, in terms of lifespan, the immobilization periods used here are far longer than those used previously with human studies, characterizing therefore a "soft," "slow" and long-term model of muscle atrophy that better reproduces the muscle loss of a patient in bed rest condition. The ability of the rat to slightly move in circles inside the restraint cage reflects the real-life circumstances of patients that are not completely immobilized.

Altogether, the results presented here propose a new model for studying the effects of bed rest in experimental animals by reducing cage volume. In this model the number of movements -particularly locomotion ones- are virtually abolished, in a similar situation as is found during bed rest. The muscle atrophy, a significant decline in muscle force together with the loss of bone mass are the main effects of the proposed immobilization model which may potentially serve to investigate the effects of bed-rest in pathological states characterized by a catabolic condition, such as diabetes or cancer.

AUTHOR CONTRIBUTIONS

Each author has participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and conduction of the experiment and for data interpretation (authorship). EM and SB carried out the studies, sample analysis, data analyses, performed the statistical analysis and helped to draft the manuscript. FO helped to realize the statistical analysis and to draft the manuscript. MT, MR, MB, VG helped to carry out the studies, sample analysis and data analysis. JA, JL, MM, RR provide the intellectual input and designs and approves the protocols to be followed in the study. JA, FL, JL, MM, RR conceived the study, participated in the design, coordination of the study, drafted the manuscript and revised it critically.

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REVIEW
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Pathogenesis and Treatment Options of Cancer Related Anemia: Perspective for a Targeted Mechanism-Based Approach

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Cancer-related anemia (CRA) is a common sign occurring in more than 30% of cancer patients at diagnosis before the initiation of antineoplastic therapy. CRA has a relevant influence on survival, disease progression, treatment efficacy, and the patients' quality of life. It is more often detected in patients with advanced stage disease, where it represents a specific symptom of the neoplastic disease, as a consequence of chronic inflammation. In fact, CRA is characterized by biological and hematologic features that resemble those described in anemia associated to chronic inflammatory disease. Proinflammatory cytokine, mainly IL-6, which are released by both tumor and immune cells, play a pivotal action in CRA etiopathogenesis: they promote alterations in erythroid progenitor proliferation, erythropoietin (EPO) production, survival of circulating erythrocytes, iron balance, redox status, and energy metabolism, all of which can lead to anemia. The discovery of hepcidin allowed a greater knowledge of the relationships between immune cells, iron metabolism, and anemia in chronic inflammatory diseases. Additionally, chronic inflammation influences a compromised nutritional status, which in turn might induce or contribute to CRA. In the present review we examine the multifactorial pathogenesis of CRA discussing the main and novel mechanisms by which immune, nutritional, and metabolic components affect its onset and severity. Moreover, we analyze the status of the art and the perspective for the treatment of CRA. Notably, despite the high incidence and clinical relevance of CRA, controlled clinical studies testing the most appropriate treatment for CRA are scarce, and its management in clinical practice remains challenging. The present review may be useful to indicate the development of an effective approach based on a detailed assessment of all factors potentially involved in the pathogenesis of CRA. This mechanism-based approach is essential for clinicians to plan a safe, targeted, and successful therapy, thereby promoting a relevant amelioration of patients' quality of life.

Keywords: cancer-related anemia, erythropoiesis, iron, inflammation, hepcidin, interleukin-6, leptin, erythropoiesis stimulating agents

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INTRODUCTION

Anemia is a clinical status distinguished by a decreased erythrocyte mass with subsequent low hemoglobin (Hb) and hematocrit counts. The World Health Organization (WHO) and National Cancer Institute (NCI) have devised a scale to define anemia grade based on Hb values. As stated by WHO (accessed September/05/2017)[1], normal Hb values are ≥12 g/dL in women, and ≥13 g/dL in men. The NCI grading of anemia is defined as follows: "mild (Grade 1), Hb from 10 g/dL to the lower normal limit; moderate (Grade 2), Hb 8.0–9.9 g/dL; severe (Grade 3), Hb <8 g/dL to 6.5 g/dl; life-threatening (Grade 4), Hb <6.5 g/dL"[2].

Cancer-related anemia (CRA) is a sign that may accompany the evolution of cancer disease and is more commonly diagnosed in patients at advanced disease stages. It can occur independently from concurrent antineoplastic regimen, typically as a consequence of chronic inflammation associated to cancer disease. In fact, CRA biological and hematologic features resemble those described in anemia associated to chronic inflammatory disease. At this regard it should be specified that cancer patients with anemia should be sorted into two main categories: those with normal Hb values before starting medical treatment (often patients with limited, locally advanced resectable disease and candidate to undergo adjuvant cancer therapy) for whom anemia must be interpreted as a specific treatment-related toxicity (chemotherapy-induced anemia); and those with diagnosis of anemia preceding antineoplastic treatment (often receiving chemotherapy for advanced cancers). For the latter group, anemia is a mostly a result of the chronic inflammatory status existing in advanced neoplastic patients; this is the real CRA. This concept is fundamental to better understand the incidence and pathogenesis, and, therefore, the most appropriate treatment strategy for patients with CRA.

Cancer-related anemia is most often normochromic (MCH≥27 pg), normocytic (MCV between 80–100 fl) (Spivak, 2005; Adamson, 2008). Usually it is a hypoproliferative anemia with a reticulocyte count below normal (<25,000/microL) and a low value of reticulocyte index (normal range between 1 and 2), which is a more accurate measure of the reticulocyte count corrected against the severity of anemia on the basis of hematocrit (Rodgers et al., 2017). Additional features include normal/low serum iron concentrations (normal range 55–160 μg/dl for men and 40–155 μg/dl for women) and reduced total iron binding capacity (transferrin saturation <50%) (Rodgers et al., 2017), whilst ferritin values may be normal (30–500 ng/ml) or more often increased (≥500 ng/mL), with increased iron storage (Adamson, 2008). Hence, a defect in iron handling instead of a lack of iron, termed "functional iron deficiency," has been hypothesized to underlie CRA. However, a low ratio of soluble transferrin receptor to ferritin could help distinguish CRA from iron deficiency-related anemia (Wish, 2006). Additionally, bone marrow erythroid hypoplasia is a feature of CRA and circulating erythropoietin (EPO) levels, the main erythrocyte growth factor, are inappropriately low in relation to the degree of anemia and intact renal function (Adamson, 2008).

The anemia prevalence rate in patients with cancer is remarkably high. Although anemia is commonly viewed as a toxicity related to antineoplastic chemotherapy, >30% of patients present with CRA at diagnosis before starting any antineoplastic treatments, rising to ∼67% once treatment is initiated (Ludwig et al., 2004; Birgegård et al., 2005). The prevalence of CRA is influenced by stage of disease (Caro et al., 2001; Knight et al., 2004; Birgegård et al., 2005): indeed, when we consider only patients with advanced cancer, CRA has been observed in a high percentage of men (77%) and women (68%) not undergoing chemotherapy (Dunn et al., 2003). Also advancing age may contribute to a higher incidence of anemia in cancer patients at diagnosis (Schwartz, 2007). Moreover, CRA prevalence differs among cancer types, with the highest percentage of anemic patients reported in lung cancer, gynecologic or genitourinary, and gastrointestinal tumors (Knight et al., 2004; Birgegård et al., 2005; Schwartz, 2007). At this regard a large, prospective, observational study carried out by our group in 888 neoplastic patients, at diagnosis before the implementation of any cancer treatments (Macciò et al., 2015c) showed that 63% of the patients had CRA, whose incidence increased with advanced cancer staging and decreased performance status (PS). We found that lung and ovarian cancer patients had the highest incidence (73.5 and 67.9%, respectively) and severity of CRA. Moreover, in our study Hb inversely correlated with the levels of inflammatory markers, hepcidin, ferritin, EPO, reactive oxygen species (ROS), and the modified Glasgow Prognostic Score (GPS). By contrast, Hb concentration was directly correlated with the levels of leptin, albumin, cholesterol, and antioxidant enzymes. These findings support the conclusion that CRA was a multifactorial inflammation-driven problem, with severity dependent on various components including the nutritional, energy metabolism, iron, and oxidative statuses.

CLINICAL RELEVANCE OF CRA

Cancer-related anemia has a significant clinical impact in cancer patients: it is related with an important decline in PS and quality of life (QL), with progressive worsening of cognitive function and energy-activity levels (Cella, 1998; Crawford et al., 2002). Patients with CRA (Hb range, 8–10 g/dL) exhibit fatigue, lethargy, dyspnea, anorexia, and have difficulty concentrating, which can compromise their overall functional status and significantly reduce adherence to anticancer regimens (Ludwig and Strasser, 2001). In particularly, CRA-related fatigue may negatively influence patient QL and as a consequence the patient tolerance and motivation to sustain the antineoplastic regimen, thus not allowing patients to receive full and timely doses and potentially impairing the therapeutic response (Blackwell et al., 2004).

Moreover, CRA at diagnosis is a negative prognosticator for disease progression, survival (Obermair et al., 1998; Shin et al., 2014; Zhang et al., 2014; An et al., 2015) and overall death risk (Caro et al., 2001). CRA is also an established

[1]http://www.who.int/vmnis/indicators/haemoglobin.pdf
[2]https://evs.nci.nih.gov/ftp1/CTCAE/About.html

negative prognosticator for survival in early stage lung, breast, colorectal, and gynecological cancer patients candidate to surgery (Chamogeorgakis et al., 2008; Zhang et al., 2014; Cybulska et al., 2017). Additionally, CRA is associated with the decreasing efficacy of chemotherapy, radiotherapy, and chemoradiotherapy regimens, with a subsequent detrimental effect on patient prognosis (Thomas, 2001; Knight et al., 2004; Fuso et al., 2005; Gaspar et al., 2015; Zhu and Xu, 2015; Zhang et al., 2017). The relationship between CRA and reduced efficacy of chemo- and radiotherapy could be also a result of the increased aggressiveness of advanced neoplasia and the associated inflammation, which is known to affect prognosis and cause CRA (McMillan, 2009). However, experimental and clinical studies have shown that low oxygen (O_2) levels (hypoxia) had a specific negative effect on the efficacy of antineoplastic treatment (Harrison and Blackwell, 2004; Cosse and Michiels, 2008; Wu et al., 2015; Xia et al., 2018). Cancer-related anemia could worsen tumor hypoxia, which in turn favors disease progression and metastases, and reduces tumor sensitivity to antiblastic treatments via various mechanisms including tissue acidosis, production of ROS, immunodepression, and alterations in tumor cells apoptosis (Höckel and Vaupel, 2001; Shasha, 2001).

Despite the prevalence of CRA and its significant clinical impact, its role at presently has been underestimated to such an extent that the indication for erythropoiesis stimulating agents (ESAs) remains limited to chemotherapy-related anemia (Rizzo et al., 2010; Rodgers et al., 2017). Therefore, specific targeted treatments for CRA have not yet been approved.

Anemia-Related Symptoms

A major negative effect of anemia is the reduced capacity of erythrocytes to transport O_2 around the body; therefore, affecting the metabolic activities and functional specificities of organ systems. Changes in cellular metabolism underlie the signs of anemia leading to compromised patient psychophysical well-being. Anemia symptoms characterize the compensatory mechanisms employed by the cardiovascular system and those subsequent to the decrease in Hb levels and reduced tissue oxygenation (Hare, 2014). O_2 transport to the tissues is dependent on the following factors: erythrocyte Hb concentrations, overall blood Hb levels, O_2 saturation of Hb, Hb-O_2 dissociation curves, and tissue O_2 tension. Changes in these parameters lead to compensatory mechanisms that not only induce the readjustment of the Hb-O_2 dissociation curve but also involve the cardiovascular and renal systems (Woodson and Auerbach, 1982). The most important compensatory mechanism that attempts to balance the decreased capability of blood to transport O_2 is initiated by the cardiovascular system to reduce peripheral vascular resistences and augment stroke volume thereby increasing heart output (Hare, 2014). On the other hand, the kidney reacts to hypoxia by increasing the production of EPO, a major regulator of erythropoiesis (Tsui et al., 2014). However, as mentioned earlier, based on the Hb levels, EPO levels are below the normal value in patients with cancer (Sanz Ortiz, 2008). Tissue hypoxia, causes cessation of glucose metabolism at the lactate level, preventing the conversion of lactate into pyruvate. Furthermore, lactate accumulation exerts a potent vasodilatory action that is strengthened by other vasoactive substances (e.g., bradykinin, adenosine, prostaglandin, nitric oxide, etc.), which are released from hypoxic tissues (Reglin et al., 2009). Consequently, the reduced efficacy of the vascular tone control systems increases blood flow to the periphery. Palpitations and sinus tachycardia are signs associated with an augmented cardiac output, whereas paleness, postural hypotension, and vertigo are correlated with the decrease in erythrocyte mass and lower vascular resistance in the peripheral circulation. Dyspnea, headache, sleep disorders, lethargy, depression, transitory cerebral ischemia, angina pectoris, limited functional capacity, and fatigue are caused by the lack of O_2 availability in various organs and tissues. Anemia-related late signs include rest dyspnea, orthopnea, head vein distension, hepatomegaly, and edemas throughout the body (Pronzato, 2006).

Notably, in CRA, fatigue, weakness, and reduced physical and cognitive capacity are the most common symptoms, subsequent to the metabolic and psychological disturbances induced by tissue hypoxia (Miller et al., 2008; Boushel, 2017). Moreover, CRA has a significant impact on the central nervous system (CNS), particularly those sites more sensitive to hypoxia (Hare et al., 2008). EPO specific receptors are expressed in the CNS and EPO appears to exert a main role in preventing apoptosis and favoring the survival of neurons after a hypoxic, metabolic, or immunologic insult (Buemi et al., 2003).

Cancer-related anemia may strongly affects the immune system causing immunodepression (Bertero and Caligaris-Cappio, 1997), which increases susceptibility to infection and lowers antineoplastic efficacy. In fact, the metabolic damage subsequent to hypoxia is responsible for the lymphocyte functional deficit (Sitkovsky and Lukashev, 2005). Viceversa, recombinant human EPO (rhEPO) is able to augment the antineoplastic efficiency of T cells and humoral immunity (Katz et al., 2007; Nairz et al., 2012). Furthermore, treatment of patients with cancer using rhEPO increased T lymphocyte function regarding blastic response (Ghezzi and Mengozzi, 2007).

ERYTHROPOIESIS

The comprehension of hematopoiesis, particularly erythropoiesis, and its regulatory processes is pivotal for understanding the pathogenesis of CRA, in order to apply the most pertinent and successful treatment choice. Through the erythropoiesis erythrocyte-programmed precursor cells continuously and neatly proliferate and differentiate into mature erythrocytes; thereby stabilizing or expanding the erythrocyte store as required. The rate of new cell production can be regulated by different physiological pathways that can also change under different pathological conditions (Doulatov et al., 2012). Erythrocytes have the main role to deliver O_2 from the lungs to different body sites and CO_2 in the reverse way. Considering that the basal O_2 consumption is 4 mL/kg/min and the body O_2 storage capacity is 20 mL/kg, it is essential to preserve an appropriate and steady erythrocyte mass, which, however, should be able at the same time to spread in response to tissue hypoxia (Benedik and Hamlin, 2014). In fact, under hypoxia, HIF-1

induces the synthesis of erythropoietin at molecular level (Jewell et al., 2001), alongside with vascular endothelial growth factor (VEGF), and several other growth factors to compensate for the negative consequences of low oxygen (Semenza, 2000). As a consequence, there is a log-linear increase in EPO levels that is inversely proportional to Hb or hamatocrit value (Spivak, 2005). Of note, O_2 transported by Hb is essential for glucose metabolism and energy production; therefore, there is a close dependence between energy metabolism and O_2 availability. On the other hand, the cellular O_2 vehicle heme is synthesized from protoporphyrin IX (PPIX) as a product of glucose metabolism via the Krebs cycle. Therefore, although Hb is essential for O_2 transport, glucose is indispensable for heme and, therefore, Hb synthesis. In this sense emerges the fundamental role of nutrients because without glucose and iron, heme synthesis cannot occur (Bennett and Kay, 1981). Erythropoiesis mainly depends from 4 distinct processes as follows: (1) proliferative potential of the erythroid progenitor reserve; (2) potency of the stimuli for erythrocyte production; (3) nutrient disposability (with an emphasis on the importance of glucose and iron); and (4) erythrocyte survival (which is reduced during hemorrhage or by early erythrocyte destruction) (Palis, 2014).

Erythropoiesis and Iron

The differentiation phase of erythropoiesis from proerythroblast to erythrocyte is iron-dependent because of the need for heme and iron-sulfur clusters for the production of Hb. Heme is synthesized at an increased rate during erythroblast differentiation and, in turn, is needed to induce the *globin* gene (Doty et al., 2015).

The first stage of heme synthesis consists in the production of δ-aminolevulinic acid (ALA) through the condensation of succinyl-CoA and glycine in the mitochondrial matrix. This rate-limiting enzymatic step is mediated by 5-aminolevulinate (ALA) synthase 2, which is expressed exclusively in erythroid cells. ALA synthase 2 expression is significantly increased throughout the advanced phases of erythroid maturation, where it is fundamental for the terminal differentiation of erythrocytes. ALA synthase 2 expression is regulated by the presence in its gene of 5′ iron responsive element (IRE), which binds with IRE-binding proteins, thus connecting heme synthesis to iron. ALA is then transported in the cytoplasmic matrix and transformed in coproporphyrinogen III. Thereafter, coproporphyrinogen III inside the mitochondrial intermembrane space is transformed into protoporphyrinogen IX, which is then oxidized to PPIX. Finally, ferrous iron is incorporated, through a reaction catalyzed by ferrochelatase, an iron-sulfur cluster protein, to produce heme in the mitochondrial matrix; this reaction is another rate-limiting step in the heme biosynthesis process. The expression of ferrochelatase is iron-dependent and iron-sulfur cluster synthesis-dependent. Therefore, heme synthesis depends from iron intake by maturing erythroblast because iron is indispensable for the PPIX ring and also regulates the expression of ALA synthase 2 and ferrochelatase (Chiabrando et al., 2014). Considering the essential role of heme for Hb synthesis and erythropoiesis, it should be highlighted that a defect in the synthesis of ALA, which occurs in patients with inherited delta-aminolevulinic acid synthase 2 deficiency, leads sideroblastic anemia (Camaschella, 2008). Therefore, it could be hypothesized that metabolic alterations by affecting the integrity of glucose metabolism via the Krebs cycle and the related synthesis of the ALA precursor succinyl-CoA could negatively influence heme synthesis (McCammon et al., 2003), and then Hb levels (Oburoglu et al., 2016). Additionally, iron contributes to the regulation of EPO synthesis in the kidney through cross-talk with HIF-2-alpha. In detail, iron regulatory proteins bind to the iron responsive element of the kidney *HIF-2-alpha* gene modulating the translation of HIF and consequently, EPO expression. Moreover, an iron-dependent enzyme, prolyl-hydroxylase, catalyzes the degradation of HIF-2-alpha protein to the extent that is negatively related to the degree of hypoxia (Camaschella et al., 2016). Notably, the functional iron deficiency present in chronic inflammation-associated anemia and CRA negatively influences erythropoiesis in patients with advanced cancers. Similarly, it could be hypothesized that anorexia, associated to anemia, characterized by low glucose availability, could heavily interfere with adequate heme synthesis.

PATHOGENESIS OF CANCER RELATED ANEMIA

Cancer related anemia (CRA) refers to a condition occuring without bleeding, hemolysis, neoplastic bone marrow infiltration, kidney and/or hepatic failure. It principally results from the chronic inflammation associated with advanced stage cancer and the synthesis of proinflammatory cytokines by both immune and cancer cells (Weiss and Goodnough, 2005).

The main pathogenetic mechanisms by which inflammation may cause anemia include (Adamson, 2008):

- Shortened erythrocyte survival in conjunction with increased erythrocyte destruction
- Suppressed erythropoiesis in bone marrow
- Effects of inflammation on erythropoietin production
- Alterations in iron metabolism that result in iron-restricted erythropoiesis induced by hepcidin increase

The increased destruction of erythrocytes is mainly due to macrophage activation by different proinflammatory stimuli. The inhibition of erythropoiesis is related to two main mechanisms; iron restriction and direct inhibitory cytokine action on erythropoietic progenitors. Therefore, erythropoiesis is insufficient to compensate for the increased destruction of erythrocytes. Moreover, in patients with chronic inflammatory disease (as cancer) EPO shows a decreased synthesis in reply to hypoxic stimuli and its circulating concentrations are inadequately low for Hb levels, irrespective of intrinsic renal pathologies (Spivak, 2000). A direct effect of proinflammatory cytokines on kidney cells that produce EPO may contribute to the defective synthesis of this hormone (Jelkmann, 1998). A lot of evidence in the literature demonstrate that inflammation mediators exert a major contribution in the etiopathogenesis of CRA. In particular, proinflammatory cytokines (e.g., TNF-α,

IL-1, IL-6), released by the cancer and activated immune cells in response to malignancy, may result in anemia by inducing changes to iron balance, inhibition of erythropoiesis, impairment of EPO synthesis and activity, reduction of erythrocytes lifespan and changes of energy metabolism (Means, 1995). Moreover, IL-1 and TNFα acts by activating the transcription factors GATA2 and nuclear factor-κB, both of which are negative regulators of the hypoxia-inducible factor 1 (HIF1) expression (Spivak, 2005).

Among cytokines, in particular IL-6 is able to induce hepatic synthesis of hepcidin, which regulates iron homeostasis by mediating the degradation of the iron export protein ferroportin 1, thereby inhibiting iron absorption from the small intestine and release of iron from macrophages. As a consequence iron is withdrawn from erythropoiesis (Ganz and Nemeth, 2011).

Moreover, chronic inflammation is correlated with increased concentrations of ROS (Macciò et al., 2005, 2015c), providing a partial explanation for the EPO deficit (Means, 2003). Indeed, ROS ($O^{\circ}\cdot$, H_2O_2, and $OH^{-}\cdot$) can inhibit EPO synthesis, by mimicking a false O_2 signal in the renal peritubular interstitial cells. Oxidative stress can also increase the fragility of erythrocytes, decrease the amount of erythroid maturation, and reduce red cell survival (Sailaja et al., 2003; Olszewska et al., 2012; Lang et al., 2014; Bukowska et al., 2015). ROS also mediate the inhibitory effect of proinflammatory cytokines on erythroid precursor proliferation (Prince et al., 2012). Additionally, an *in vitro* study demonstrated that sustained H_2O_2 levels induce liver hepcidin expression through STAT-3 phosphorylation, by acting synergistically with IL-6, indicating another potential mechanism by which oxidative stress could contribute to CRA (Millonig et al., 2012).

Additionally, in advanced cancer patients, other triggering factors may contribute to anemia through a multifactorial pathogenesis; among them, the following mechanisms can be highlighted:

- Poor nutritional status
- Antineoplastic therapies (chemo- and radiotherapy) that may cause overt and/or aggravate CRA.

Of high relevance are in particular the metabolic and nutritional issues typical of advanced cancer patients and the defect of specific components (such as iron, vitamins, folic acid etc.) essential for erythropoiesis. Notably, the availability of these nutrients (e.g., glucose, iron) influence the synthesis of heme, which also depends on the efficiency of glucose metabolism via the Krebs cycle, and is essential, in combination with iron, for the synthesis of Hb (Chiabrando et al., 2014).

CHRONIC INFLAMMATION IN ADVANCED CANCER PATIENTS

During its development, the neoplastic disease is characterized by immunological alterations, which profoundly influence the patient clinical conditions potentially causing patient's death (cancer cachexia syndrome) (Macciò and Madeddu, 2012; Argilés et al., 2014). In addition to anemia, the immune changes induce different symptoms involving several organs and processes: anorexia, nausea/vomiting, weight loss (with depletion of muscle and fat mass), raised energy metabolism with alterations of glucose, lipid and protein metabolism, fatigue, and immunosuppression (causing greater susceptibility to infections).

Although it is difficult to determine the precise onset of these changes, it has been established that they are consequent to the interplay between cancer and patient immune system (Delano and Moldawer, 2006). Activated macrophages, lymphocytes, and mesenchymal cells produce various soluble factors (cytokines) which are able to activate or inhibit different cells. In particular, IL-1, IL-6, and TNF-α are the principal factors involved in the cell-mediated immune response and also act as second transmitters for the synthesis of IL-2, which play a pivotal role in the control of the anticancer immune response. Some studies from our group have shown that the lymphocyte blastic response to various mitogenic stimuli, including phytohemagglutinin (PHA) and anti-CD3, is impaired in advanced cancer patients (Macciò et al., 1998; Mantovani et al., 2003). The intensity of the immune defect is proportional to cancer stage (Ladányi et al., 2004) and to the amount of inflammatory cytokines, in particularly IL-6, and other acute phase proteins (e.g., CRP) (Macciò et al., 1998). Indeed, the impaired lymphocyte functions should be assumed as a proxy of multiple functional changes, among which extremely important are the immunosuppressive action of macrophage-derived cytokines and the changes in energy metabolism, which are able to induce a status of oxidative stress. In our previous papers we showed that cancer patients are highly subjected to a state of increased oxidative stress (Macciò et al., 2009, 2015c; Madeddu et al., 2014). Notably, inflammatory cytokines not only have specific modulatory immune functions but are involved in the pathogenesis of the main metabolic changes and symptomatic aspects of the neoplastic patient (e.g., body weight loss and cachexia with reduction of muscle mass, anorexia, nausea/vomiting, etc.), thus influencing significantly patient's well-being (Macciò and Madeddu, 2012). Therefore, this behavior of the immune system confers to the cancer the characteristics of a real chronic inflammatory disease with the related complications, such as anemia. In this regard, macrophages seem to be primarily involved in these events.

Specific Actions of Proinflammatory Cytokines

It has been widely demonstrated that proinflammatory cytokines, particularly IL-1, TNF-α and IL-6 may affect erythropoiesis by the induction of: reduced proliferative response of erythroid progenitors; increased erythrocyte destruction by macrophages; and diminished response of erythroid precursors to EPO (Spivak, 2005). Moreover, the chronic activity of these same cytokines is mainly responsible of the multiple metabolic and nutritional changes occurring in advanced cancer patients (Macciò and Madeddu, 2012). Notably, by impairing the energy metabolism and the nutritional status, proinflammatory cytokines contribute to the pathogenesis of CRA.

Considering in detail the action of each cytokine, IL-1 is also able to inhibit erythropoiesis through directly and selectively

suppressing replication and maturation of erythroid (BFU-e and CFU-e) precursors, reducing EPO receptor expression, and impairing EPO synthesis (Faquin et al., 1992; Jelkmann et al., 1992). Moreover, IL-1, together with other proinflammatory cytokines, has been implicated in activating macrophages for erythrophagocytosis, thus inducing a premature destruction and reduced survival of erythrocytes.

Moreover, IL-1 is involved in inducing several changes in energy metabolism and nutritional status. IL-1 induces anorexia associated with reduced food intake by acting in the hypothalamic nuclei of the CNS, where it inhibits the orexigenic factor Neuropeptide Y and indirectly increases the corticotropin releasing factor (CRF) levels (Patra and Arora, 2012); additionally, it stimulates CRF and somatostatin secretion, mediated by prostaglandin E2 (PGE2) (Gautron and Layé, 2010). This results in the inhibition of growth hormone (GH) accompanied by the subsequent reduction in the production of insulin-like growth factor-1 (IGF-1) that, in turn, cause muscle mass reduction typical of advanced cancer patients (Saini et al., 2006). Moreover, IL-1 inhibits the synthesis of insulin by pancreatic beta cells leading to hyperinsulinemia and insulin resistance (Burke et al., 2015). These IL-1 mediated activities may concur to the onset of CRA in advanced cancer patients. In particular, low glucose availability and insulin resistance negatively affect erythropoiesis since commitment into the differentiation stages is strictly dependent on glucose metabolism (Oburoglu et al., 2016). Erythroid differentiation is critically dependent on glucose cell uptake and glucose entry into the tricarboxylic acid (TCA) cycle that allows a high energetic yield for cell proliferation (Vander Heiden et al., 2009) as well as on glutamine-dependent nucleotide synthesis and an increased glucose shunting into the pentose phosphate pathway for the synthesis of carbon sugars (Montel-Hagen et al., 2009). Therefore, glucose uptake and the balance between mitochondrial and non-mitochondrial glucose metabolism affects erythroid differentiation (Oburoglu et al., 2014).

Moreover, there is evidence that links the CNS pathways modulated by IL-1 with anemia. It has been shown that the replacement of GH was related with a significant rise of Hb, Hct, and number of red cells (Christ et al., 1997). Additionally, experimental *in vitro* and *in vivo* data revealed that IGF can induce proliferation and differentiation of both late and early erythroid progenitors. Consistently, some observational clinical studies demonstrated an inverse correlation of blood levels of IGF-1 with Hb and in various populations (elderly, adult, and dialyzed patients) (Iglesias et al., 1998; Nilsson-Ehle et al., 2005; Succurro et al., 2011). A proof of the direct action of IGF in improving anemia is demonstrated by the association of an IGF genetic polymorphism (related to low IGF concentration) with low Hb levels in a large cohort of adult individuals (Marini et al., 2017).

TNF-α has also direct effects on hemopoiesis; it is able to impair erythropoiesis and erythroid differentiation *in vivo* and *in vitro*, to induce an increase in the apoptosis of immature erythroblasts and a decrease in mature erythroblasts, and to reduce the responsiveness of erythroid progenitors to EPO (Buck et al., 2009). Moreover, TNF-α is responsible mainly for the metabolic changes in lipid metabolism typical of advanced cancer patients, particularly those with cachexia (Patel and Patel, 2017). In detail, TNF-α down-regulates the expression and activity of lipoprotein lipase (LPL) that converts circulating triglycerides into free fatty acids (FFA). TNF-α is also able to decrease the adipocyte expression of FFA transporters, in this way blocking the FFA flow inside the adipocytes, to directly reduce the synthesis of enzymes participating to lipogenesis, e.g., acetyl-CoA carboxylase, Acyl-CoA synthase, and fatty acid synthase, and to induce lipolysis (Carbó et al., 1994). TNF-α has been also involved in the occurrence of insulin resistance through the rise of FFA levels, inhibition of the insulin receptor and insulin receptor substrate-1 (IRS-1) production and induction of IRS-1 Ser/Thr phosphorylation. Among the first transcription factors shown to be targeted negatively by TNF-α signaling in adipocytes was the "adipogenic master regulator," peroxisome proliferator-activated receptor gamma (PPARγ) that physiologically exert a crucial regulatory action of lipid metabolism (Cawthorn and Sethi, 2008). Downregulation of PPARγ impairs lipid formation and storage in adipose tissue thus leading to a condition known as lipoatrophy (Bing et al., 2006). Notably, a role of PPARγ has been also demonstrated in the regulation of maturation of erythroid progenitors (Nagasawa et al., 2005).

As regard IL-6, it is greatly involved in the pathogenesis of CRA by influencing erythropoiesis at different levels. IL-6 is able to impair proliferation of erythroid progenitors and their response to EPO, and change iron metabolism by modulating liver gene expression and hepcidin synthesis, which is in turn responsible for the functional iron deficiency, typical of CRA (Adamson, 2008). Of relevance, it has been demonstrated that an additional mechanism by which IL-6 is able to impair erythropoiesis is the inhibition of Hb synthesis, independently from the hepcidin-iron pathway, as a consequence of impaired mitochondrial function (by decreasing membrane potential/oxidative phosphorylation) in maturing erythroid cells (McCranor et al., 2014). The key role of IL-6 in determining CRA has been demonstrated in several publications: in particular one publication by us provided the earliest demonstration that IL-6, in conjunction with the stage of disease, represented an independent predictor of Hb values in a cohort of ovarian cancer patients (Macciò et al., 2005). Additionally, IL-6 is a main contributor of the severe immune and metabolic alterations that characterize advanced cancer and contribute to the pathogenesis of CRA. Several years ago, using an experimental animal model to reproduce cancer-related syndrome, Strassmann et al. (1992) showed a crucial function for IL-6 in inducing the early onset of cachexia symptoms, which were independent from the rate of tumor growth, and were associated with loss of muscle and adipose tissues not only due to appetite decrease. Indeed, IL-6 concentrations were proportional to the severity of cachexia and the removal of primary tumor was followed by a significant reduction in IL-6 levels. Consistently, the onset of cachexia symptoms was prevented with anti-IL-6 monoclonal antibodies (Strassmann et al., 1992). Additionally, in rat experimental models it has been observed that, as shown for IL-1, IL-6 acts directly in the hypothalamus to induce the release of CRF,

mediated by PGE2 (Turnbull et al., 1998) and is able to impair the production of insulin and the metabolism of pancreatic β cells (Sandler et al., 1990). More recently, IL-6 has emerged as the key determinant of muscle mass wasting in advanced cancer patients (Madeddu et al., 2015). In particular, attention has been recently drawn to the IL-6/STAT3-dependent regulation of the PI3K/Akt/mTOR pathway, which is the principal cellular energy sensor and physiologically activates muscle mass growth. The activation of these pathways mediated by IL-6 as well as the associated increased degradation and low availability of amino acids may also contribute to defective erythropoiesis in advanced cancer patients. Indeed, red cell maturation and the synthesis of Hb are dependent on the activation of mTOR signaling consequent to the increased uptake of amino acids. Conversely, when nutrient/amino acid pools are reduced, mTOR activity is reduced and Hb synthesis is inhibited (Chung et al., 2015; Nathan, 2015). Clearly, anorexia, associated with reduced food intake, and insulin-resistance, with impaired glucose metabolism, also contributes to the inhibition of the mTOR pathway. In turn, anemia, defined as a condition of reduced efficient delivery of O_2 to peripheral tissues, may inhibit mTORC1 signaling, mainly as a consequence of impaired oxidative phosphorylation and reduced ATP synthesis that lead to mTOR inhibition. Furthermore, CRA is distinguished by "functional iron deficiency" with reduced iron levels and subsequent reduced production of heme, which is a main molecule of muscle myoglobin (Madeddu et al., 2015). This also suggests that anemia contributes to muscle wasting.

IRON HOMEOSTASIS CHANGES IN CRA

Normally, plasma iron is maintained at a stable level by the control of its intestinal absorption and storage. Iron homeostasis is guaranteed by an endocrine system that involves hepcidin, a hormone that controls circulating iron levels by acting on the pathways mediating iron uptake, storage, and release. A small part (1–5%) of circulating iron derives from the diet while mostly is recycled from senescent erythrocytes. More than 50% of the body iron in humans is linked to Hb in erythrocytes, and approximately a quarter is stored in hepatocytes and macrophages. In the circulation, iron usually is coupled with transferrin that transports iron mainly to the bone marrow for erythropoiesis. Circulating iron is delivered inside cells (macrophages of the reticulo-endothelial system) by endocytosis of transferrin and is stored in complex with ferritin (Ganz and Nemeth, 2015). Since erythrocytes hold a greater amount of iron in comparison to other cells, erythropoiesis is particularly sensitive to reduced circulating levels of iron and is inhibited if the concentration of transferrin-bound iron decreases below physiological values. The hampering of erythropoiesis under iron depletion could be useful to convey iron to more critical processes. In fact, differently from erythropoiesis, energy and intermediary metabolism, neurobehavioral activities and immune defense are unaffected by the sequel of iron limitation, unless this gets extremely serious.

Dietary iron is absorbed mainly from duodenal enterocytes, where it is transferred from the luminal to the vascular basolateral cell membrane, and released into the circulation by ferroportin. Ferroportin is additionally involved in the iron transport from the storage sites (splenic cells, hepatocytes, bone marrow-derived and tissue macrophages) to the blood. Macrophages are the key cell type that recycles iron from Hb through erythrophagocytosis of senescent cells; macrophages modulate the majority of iron in the body. Iron recycling, storage, and export are tightly regulated by hepcidin. Hepcidin, typically produced by the liver and probably by macrophages, is bound to ferroportin and induces its endocytosis, in this way inhibiting the iron export to the blood and sequestrating iron in duodenal enterocytes and in macrophages, thus limiting erythropoiesis (**Figure 1**). Physiologically, hepcidin production is regulated by a classical positive feedback mechanism through high plasma iron levels, and negatively by low plasma iron levels, hypoxia, and increased erythropoiesis (Ganz and Nemeth, 2015). More recently, erythroferrone has been described as an additional regulator of hepcidin: it is synthesized by erythroblasts stimulated by EPO and inhibit hepcidin production, this in turn enables export of intracellular iron and intestinal iron absorption, thus allowing erythropoiesis (Kautz et al., 2014).

Notably, CRA along with anemia associated with chronic inflammatory diseases are characterized specifically by alterations of iron homeostasis; there is increased iron storage in reticulo-endothelial macrophages and limited iron availability for erythroid progenitors with a subsequent decrease of erythropoiesis. Increased intracellular iron influence erythropoiesis also through the promotion of HIF1α degradation and consequent inhibition of EPO synthesis (Kling et al., 1996).

This condition is called "functional-iron deficiency" and it is correlated with normal or even raised iron reserves in the bone marrow, increased ferritin levels and iron-binding capacity, and normal or decreased serum iron levels (Bron et al., 2001). These peculiar changes have been attributed to the increased synthesis of hepcidin from hepatocytes induced by inflammatory cytokines, mainly IL-6 (Alvarez-Hernandez et al., 1989; Torti and Torti, 2002). In mice, transgenic or constitutive hepcidin overexpression leads to severe anemia associated with low iron availability, while inflammation in animals without hepcidin expression does not determine iron deficiency (Nicolas et al., 2002). This finding suggests that, in chronic inflammatory conditions, hepcidin has a notable function in iron traffic diversion by blocking duodenal iron absorbance as well as iron release by macrophages (Laftah et al., 2004; Nemeth et al., 2004).

In advanced cancer as well as in chronic inflammatory diseases, increase in hepatic production of hepcidin is induced by high levels of IL-6. Increased hepcidin degrades cellular ferroportin, blocks iron excretion and thus increases iron storage in hepatocytes, intestinal enterocytes and macrophages; as a result, less iron is delivered to plasma transferrin (Ludwiczeck et al., 2003). As a consequence, there is restricted iron accessibility for heme synthesis thus inhibiting replication and maturation of erythroid precursors.

Macrophages are the main cell type involved in these events; activated by necrosis, and/or specific chemokines produced by tumors, macrophages consume large quantity of iron by phagocytosis of senescent or injured cells (Schmidt, 2015).

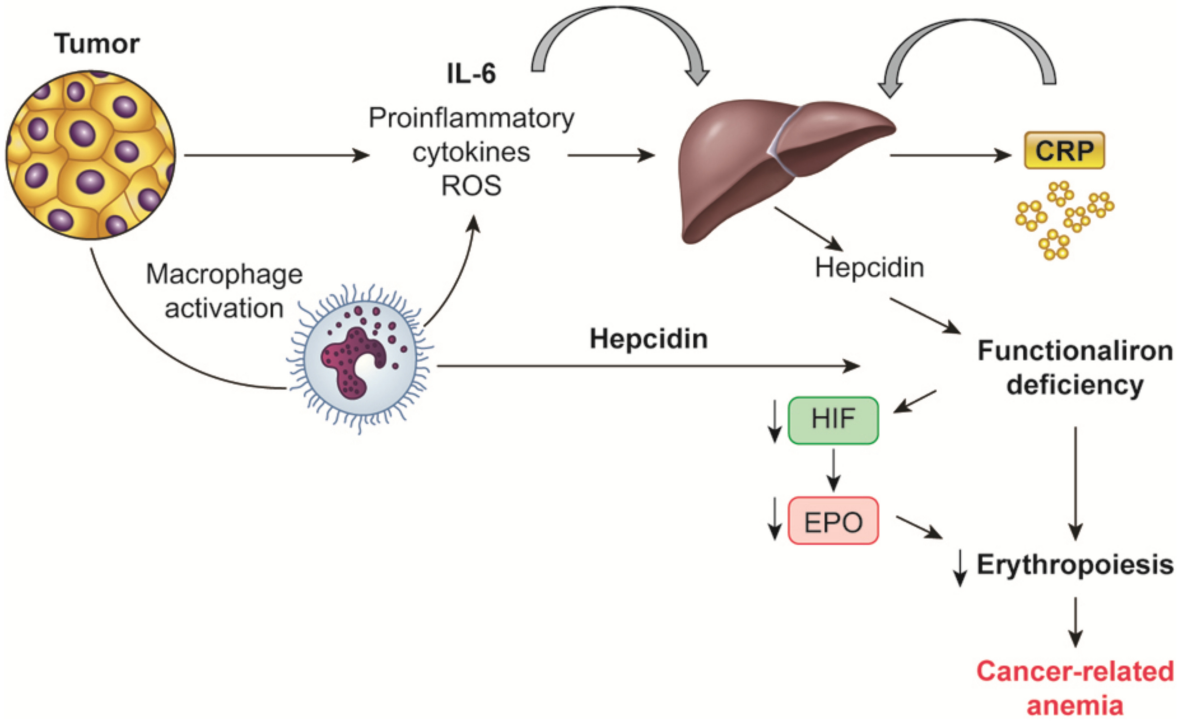

FIGURE 1 | Mechanisms regulating hepcidin synthesis in cancer-related anemia. Tumor and macrophage-derived proinflammatory cytokines, mainly IL-6, induce the liver synthesis of hepcidin, which in turn is responsible of the "functional iron deficiency" typical of cancer-related anemia. Also macrophages by themselves are able to synthesized hepcidin. IL, Interleukin; CRP, C-reactive protein; HIF, hypoxia inducible factor; EPO, erythropoietin.

Macrophages that exert these actions are called M1 macrophages. Indeed, M1 macrophages, which exert proinflammatory activity associated with high expression of proinflammatory cytokines and ROS, are programmed in an iron-retention mode that promotes their antimicrobial and antitumoral actions. It is well known that activation and polarization of macrophages are closely linked to iron homeostasis: in particular, M1 polarized macrophages are characterized by high expression of hepcidin, high ferritin, low levels of transferrin receptor and ferroportin with consequent blockade of iron release and increased iron storage (Recalcati et al., 2012). During bacterial infection, increased iron removal and sequestering by macrophages looks like to have a double role; first to limit iron availability to exogenous micro-organisms and second, to protect the body from the dangerous consequences of elevated iron levels (and iron-derived highly reactive free radicals), which could result from the tissue injury. Considering that the majority of microorganism depend on exogenous iron for survival, it has been assumed that the above changes in iron metabolism have a host defense function (Ganz and Nemeth, 2015). At the same time, by increasing iron retention activated M1 polarized macrophages may contribute to induce anemia associated to chronic inflammation. At this regard, recently we revealed a strong association between M1 polarized tumor associated macrophages and CRA in a population of ovarian cancer patients (Madeddu et al., 2018). We found also that TAM M1 polarization was associated with high levels of hepcidin and IL-6 as well as with a peculiar shift in iron metabolism-related pathways characterized by high ferritin and low free iron levels both in ascites and in serum. Notably, in the same paper (Madeddu et al., 2018) we demonstrated that M1 TAMs were able *in vitro* to release hepcidin and that IL-6 is was the main responsible of macrophage polarization into M1 phenotype. In fact, we found that IL-6 blockade with a specific anti-IL-6 mAb inhibited this polarization and the consequent synthesis of hepcidin. Consistently with the above evidence, in the clinical setting, it has been widely observed that patients with CRA as well as anemia of chronic inflammatory disease have elevated hepcidin values (Basseri et al., 2012; Shu et al., 2014). Recently, an observational prospective study of our group in a wide sample of patients with advanced cancer at different sites strongly confirmed that Hb value was negatively correlated with hepcidin values, which, in turn, were positively correlated notably with IL-6 as well as with and ferritin, and negatively with serum iron and transferrin saturation values.

RELATIONSHIP BETWEEN NUTRITIONAL STATUS AND CANCER-RELATED ANEMIA

During the evolution of the cancer disease, patient nutritional status is severely compromised by symptoms and signs including weight loss associated with significant reduction of muscle

mass, increased resting energy expenditure, anorexia, nausea and vomiting. A recent large observational study supported the central role of weight loss, together with the body mass index (BMI), as a negative prognostic factor in cancer patients at diagnosis independently of other more standardized parameters such as tumor site, stage, and PS. Even cancer patients who exhibit at diagnosis a slight decrease of body weight >2.4% have an increased risk of morbidity and mortality (Martin et al., 2015). Loss of body weight represents the main measure for the diagnosis of cancer cachexia (Fearon et al., 2011), which is a complex inflammatory-driven syndrome often accompanying the advanced stages of the neoplastic disease. The weight loss observed in cachectic cancer patients cannot be justified only by the reduction of the supply of nutrients, caused by anorexia and consequent reduced food intake. Energy metabolism changes, which account for a significant increase in resting metabolic expenditure, significantly contribute to loss of body weight (Friesen et al., 2015). Notably, cancer-associated chronic inflammation concurs to the derangements of energy metabolic pathways inflammatory by modulating glucose metabolism, regulating the functioning lipoprotein lipase, which controls the uptake of circulating triglycerides into adipocytes, and changing protein synthesis and degradation, with subsequent depletion in lean body mass (Madeddu et al., 2015). In fact, in cancer patient muscle protein production is decreased while proteolysis is increased, following the raised activity of proteolytic enzymes. Such metabolic behavior is completely different in the liver, where, despite a stable or reduced albumin synthesis, the synthesis of other proteins, especially those of acute phase inflammation (PCR, fibrinogen and hepcidin), is significantly increased (Porporato, 2016). These peculiar changes can also account for the decrease in albumin levels typically associated with high inflammation that affects the Glasgow prognostic score (GPS), an inflammatory/nutritional status based score value, which is closely linked to the worsening of CRA, as demonstrated by our group (Macciò et al., 2015c). Consistently, it is widely demonstrated that malnutrition, in conjunction with weight loss and reduced food intake, is correlated with anemia in patients with chronic inflammatory disease (Hung et al., 2005). This indicates that, along with inflammation, a main contribution to CRA etiopathogenesis is exerted by the nutritional status. In fact, anemia associated with chronic inflammation in advanced cancer patients is not an isolated symptom, but it is more typically associated with weight loss and remodeling of energy metabolism caused by cancer itself (Macciò et al., 2012). Therefore, we hypothesize that the correction of CRA may be achieved more efficiently with a multifactorial therapy that additionally includes treatment of malnutrition.

Consistently with this evidence we found an association between anemia and leptin, which is one of the main marker of the nutritional and metabolic status (Abella et al., 2017), and inversely related to the level of proinflammatory cytokines and stage in cancer patients (Mantovani et al., 2000, 2001; Macciò et al., 2009). In 2005 for the first time in the literature a study from our group showed that the lowest levels of Hb correlated with the lowest values of leptin in ovarian cancer patients. More recently, in a large prospective observational study including patients with different cancer types (Macciò et al., 2015c), we found that leptin, alongside with albumin, cholesterol, and BMI, was positively correlated with Hb. Of relevance, in the same cohort of patients, leptin, in addition to IL-6 and stage, was a predictive variable of Hb. Consistently, leptin values were found to be a predictor of EPO responsiveness in people with kidney failure with anemia (Axelsson et al., 2005; Hung et al., 2005). Noteworthy, it has been also demonstrated that leptin can influence erythropoiesis and stimulate human erythroid progenitor development *in vitro* (Umemoto et al., 1997). However, because anemia in cancer patients is related to the nutritional state that affects the levels of iron, vitamins and other micronutrients useful for erythropoiesis, it is not surprising that a sensitive nutritional marker such as leptin is correlated with Hb levels (**Figure 2**).

CANCER-RELATED ANEMIA THERAPY

Cancer-related anemia, not associated with concomitant antineoplastic treatment, is underestimated and undertreated. The rationale to treat CRA would be twofold:

(1) CRA is associated with and aggravates multiorgan failure associated with advanced cancer and characterized by a variety of symptoms. Each of these symptoms can, by themselves, compromise patient quality of life;
(2) CRA has a negative prognostic significance.

The approach to treating CRA must begin with an exhaustive assessment of its defining parameters and identifying its multiple potentially treatable causes: because CRA is mostly multifactorial it should be effectively treated with multitargeted therapies.

It should be underlined that CRA reflects the progressive growth of the underlying disease, thus the eradication of cancer should be the only definitive treatment of this particular form of anemia. However, as the neoplastic disease, in many cases, is not curable, the therapeutic strategies against CRA should target the multiple causes that trigger the disease and should include erythropoietic agents, iron supplementation or blood transfusions, nutritional supplementation, and anti-inflammatory therapies (Weiss and Goodnough, 2005). Future strategies could include chelate-iron therapy, use of hepcidin antagonists and cytokines or hormones that can modulate erythropoiesis under severe inflammatory conditions.

Additionally, if we consider that CRA is also associated with significant impairment of patients' functional status and QL with symptoms, such as fatigue, that in turn have themselves a multifactorial basis, the evaluation of the outcomes of CRA treatment is complex and cannot be limited to just the increase of Hb levels. In this regard, it is very important to define both the endpoints of CRA treatment and the target level of hemoglobin to obtain the best therapeutic outcome in terms of improvement of patient's symptoms. The last "Update Committee of the American Society of Hematology/American Society of Clinical Oncology (ASCO/ASH)" published in 2010 (Rizzo et al., 2010) recommended that: "The first objective to achieve is to reduce

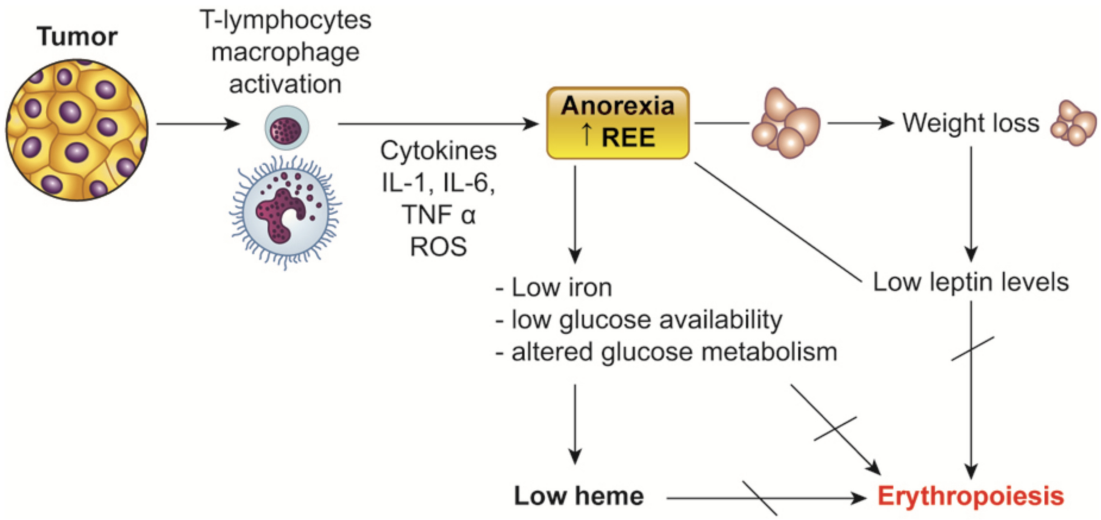

FIGURE 2 | Relationship between nutritional status, leptina and cancer-related anemia. Impaired energy metabolism and low nutrients availability induced by cancer-associated chronic inflammation are involved in the impairment of erythropoiesis. Low iron as well as low heme synthesis as a consequence of altered efficiency of glucose metabolism impair erythropoiesis. Leptin, which decreases as a consequence of such metabolic/nutritional changes, can also influence erythropoiesis by itself. REE, resting energy expenditure.

blood transfusion requirements. The Hb target should be the lowest concentration needed to avoid transfusions, which may vary by patient and condition." Similarly, the NCCN guidelines stated that "elimination of symptoms and avoidance of transfusions are the main goals of ESA therapy" (Rodgers et al., 2017).

It should be underlined that during erythropoiesis, homeostatic mechanisms compensate for increased EPO synthesis when Hb drop to a value lower than 12 g/dl (Finch, 1982). Notably, the target Hb value to prevent the necessity of blood transfusions, should be at least $\geq$ 10 g/dl, but it has been demonstrated that the highest improvement of QL is achieved at Hb values of 12 g/dl, thus indicating the relevance of keeping Hb values within this range (Crawford et al., 2002). More precisely, data from QL assessment related to the amelioration of anemia show that an optimal improvement is achieved between Hb 11 gr/dl and 12 gr/dl.

TRANSFUSION OF CONCENTRATED ERYTHROCYTES

Red blood cells (RBC) transfusions are almost universally successful in raising Hb levels and the oxygen transport capacity of the blood. Therefore, they represent a fast and effective therapeutic intervention useful to ameliorate rapidly the patient's symptoms, e.g., breathlessness, and improve health-related QL (Rodgers et al., 2017). Prior to rHuEPO being available, blood transfusions were the only therapeutic option for the improvement of symptomatic anemia in cancer patients. The major benefit, not provided by any alternative therapy for anemia, is the fast Hb and Hct value rise (Bohlius et al., 2006). RBC transfusions are particularly useful in case of severe symptomatic anemia or anemia compromising patient life (Hb <7–8 g/dl). These thresholds cannot be applicated to people affected by acute coronary syndrome and with complications at risk of bleeding, where blood transfusion may be indicated also for Hb values $\geq$ 8 g/dl (Carson et al., 2016). The need for blood transfusion depends other factors such as age, compromised vital signs and severe tissue hyperoxygenation (Simon et al., 1998). Notably, the last published NCCN guidelines (Rodgers et al., 2017) indicate that RBC transfusions should be considered not on the basis of a specific threshold value of Hb but in patients with symptomatic anemia, in high risk patients (e.g., those undergoing high-dose chemo-or radiotherapy with cumulative decrease of Hb levels) or asymptomatic patients with comorbidities (e.g., heart disease, COPD, cerebral vascular disease). Undoubtedly, patients who are positive for the presence of multiple alloantibodies and those with specific religious beliefs are unable to receive blood transfusions. In advanced cancer patients imminent to surgery, blood transfusions are routinely employed to treat pre-operative anemia and minimize blood loss related to surgical procedures. Perioperative blood management strategies such as pre-surgery autologous blood donation, acute normovolemic blood dilution, intraoperative cell collection, rHuEPO therapy, optimal hemostasis, mini-invasive surgeries may minimize the requirement of allogeneic blood transfusion in case of scheduled surgical procedures (Weber et al., 2008). Some studies evaluating the clinical effect of transfusions showed a survival benefit in neoplastic patients receiving transfusions (Grogan et al., 1999; Kader et al., 2007). In particular, in the study by Grogan et al. (1999), patients that reached a specific Hb value with blood transfusion showed a survival rate similar to patients that had that Hb value spontaneously; then, blood transfusion appeared to reduce the negative prognostic

role of low baseline Hb. Additionally, blood transfusions are beneficial in terms of patient subjective symptoms such as breathlessness (Gleason and Spencer, 1995; Mercadante et al., 2009) and fatigue (Gleason and Spencer, 1995; Tanneberger et al., 2004; Mercadante et al., 2009; Brown et al., 2010). However, blood transfusions have relevant acute and long-term risks, which include fever, transfusion-related and potentially fatal allergic reactions, transmission of infectious diseases, alloimmunization, iron overload and immunosuppression with potential favoring effect on cancer progression (Goodnough, 2005; Toy et al., 2005). The risk of iron overload increases in patients that require frequent transfusions: excessive iron is dumped in critical part of the body such as the heart and the liver causing cumulative toxicity. Moreover, blood transfusions have been correlated with more thromboembolic events and related death in hospitalized neoplastic patients (Khorana et al., 2008). In 2012, a systematic review that evaluated the benefit and risks of blood transfusion in very advanced cancer patients showed a significant increase of Hb levels and a subjective symptomatic response rate, especially in terms of fatigue and dyspnea. Patient survival varied greatly ranging between two and 293 days and a high percentage of patients (23–35%) were dead after only 2 weeks from transfusion. The review concluded that high-quality studies are warranted to establish the risks and effectiveness of blood transfusion in advanced cancer patients and to determine patients who are candidate to obtain a benefit (Preston et al., 2012). Notably, the concern of the premature death after treatment for CRA in very advanced cancer patients raises the question of whether anemia treatment may be causative to the increased risk of death.

TREATMENT WITH rHuEPO

Recombinant HuEPO received its first approval for the therapy of anemia in patients with chronic kidney failure. In 1993 FDA approved r-HuEPO also for the therapy of anemia in cancer patients. Currently different short-and long-acting formulations of rHuEPO are available: r-HuEPOα, r-HuEPOβ, and darbopoetin α. Because of the glucidic component, r-HuEPO has a longer half-life after subcutaneous administration compared to natural EPO that has a half-life of 8.5 h (24 h for r-HuEPOα and 20.5 h for r-HuEPOβ) (Storring et al., 1998). As short-acting drugs, first generation rHuEPO necessitated repeated doses to keep appropriate Hb levels. Conversely, darbopoetin-α is a modified hyperglycosylated epoetin and has a longer half-life after its subcutaneously administration (about 49 h): this allows to increase the interval of the dose schedule necessary for Hb level maintenance (Bohlius et al., 2006). Recombinant HuEPOs are biologically and pharmacologically active intravenously, intraperitoneally but especially subcutaneously (s.c.). All three recombinant erythropoietin proteins have similar efficacy (Halstenson et al., 1991; Storring et al., 1998). The standard therapeutic regimen is 150 IU/kg three times a week for r-HuEPOα, or 40,000 IU once weekly for r-HuEPOα, or 30,000 IU once weekly for r-HuEPOβ. The recommended dose of darbopoietin α is 2.25 μgr/kg weekly or 500 μgr every 3 weeks.

More recently, several biosimilar EPOs have been developed and introduced in the clinical practice: biosimilar epoetin alfa (e.g., Binocrit®, Sandoz) and epoetin zeta (e.g., Retacrit®, Hospira). A biosimilar is a biological drug, which is highly superimposable as for primary structure, mechanism of action, and treatment target, to the approved reference biological drug. The great degree of similarity is revealed by the results of an extensive clinical comparison of pharmacokinetics and pharmacodynamics properties by regulatory authorities. The approvals of biosimilar epoetin alfa and zeta in 2007 were founded on strong assessments including molecular characteristics, preclinical *in vivo* and *in vitro* experiments in animals, clinical trials in the target populations, and pharmacovigilance surveillance studies (Jelkmann, 2010). The safety data indicate that adverse effects among patients treated with epoetin zeta (Retacrit®) are consistent with those known for epoetin α (Michallet and Losem, 2016). Similarly, the majority of safety data for biosimilar epoetin alpha (Binocrit®) are favorable. However, one confirmed and one suspected pure red cell aplasia (PRCA) have been reported during a clinical trial in patients receiving the subcutaneous formulation (Haag-Weber et al., 2012). As a result, biosimilar epoetin alpha is now only accessible for intravenous administration, while epoetin zeta is presented in both intravenous and subcutaneous formulation.

Efficacy of rHuEPO for the Management of CRA

Randomized clinical studies have showed that rHuEPO increased Hb values and reduced the number of hemotransfusion in anemic cancer patients (Ludwig et al., 2009). In particular, regarding CRA, a phase II, double-blind, placebo-controlled trial demonstrated that more patients in the arm receiving darbopoetin-α achieved the hematopoietic response and the target Hb levels in comparison to the placebo arm (Gordon et al., 2008). Another phase III randomized double-blind trial carried out in advanced lung cancer patients demonstrated that treatment with epoetin-α was associated with fewer transfusions and achieved a higher Hb rise than placebo (Wright et al., 2007) The ability of rHuEPO to achieve a significant reduction of RBC transfusions and a higher hematopoietic response was supported by a large Cochrane meta-analysis, which analyzed a total number of 20,102 patients from 91 randomized clinical studies, which evaluated ESAs for the therapy of anemia in cancer patients either undergoing or not undergoing concomitant chemotherapy (Tonia et al., 2012). Moreover, there is a strong evidence that rHuEPO may improve QL, fatigue and other specific anemia-related symptoms (such as dizziness, chest discomfort, headache, trouble walking) (Tonia et al., 2012). A number of open-label non-randomized and community-based trials in advanced cancer patients with CRA demonstrated that a progressive amelioration of QL obtained by ESAs was significantly correlated with

the increased Hb levels (Quirt et al., 2001; Bogdanos et al., 2004; Mystakidou et al., 2005; Wright et al., 2007; Smith et al., 2008). In particular, Crawford et al found that the highest improvement of QL was found if Hb value rises from 11.0 to 12.0 g/dl and ≥1 g/dl (Crawford et al., 2002). In 2014, a systematic meta-analysis confirmed that ESAs determine a clinical significant amelioration of anemia-related symptoms (evaluated by FACT-An), while the improvement in fatigue-related symptoms (FACT-F), did not obtain the value needed for a clinically significant change (Bohlius et al., 2014). These results indicate that fatigue, in advanced cancer patients is influenced by several parameters other than anemia. Therefore, the mere amelioration of Hb levels could not have appropriately counteracted the multifactorial pathophysiology and psychological aspects of fatigue in advanced cancer patients. Otherwise ESAs may have additional beneficial effect other than the correction of anemia, such as neuroprotective, anti-inflammatory, vascular and metabolic actions, directly related to the action of EPO on other target organs such as central/peripheral nervous system and heart (Nekoui and Blaise, 2017).

ADVERSE EFFECTS OF ESAs

Both randomized clinical studies and systematic reviews demonstrated a significantly higher risk of thromboembolic events in patients receiving ESAs for CRA (Wright et al., 2007; Bennett et al., 2008; Smith et al., 2008; Tonia et al., 2012). Excessively high Hb levels at baseline prior to treatment with rHuEPO were related with a significant increased occurrence of thromboembolism in some trials in cancer patients with anemia undergoing chemotherapy (Aapro et al., 2009; Spivak et al., 2009). A survey including five trials by the Agency for Healthcare Research and Quality concluded that there was a trend toward fewer thromboembolic events if ESA administration was started only at Hb < 10 g/dl (Grant et al., 2013). Conversely, in 2012 a Cochrane metaanalysis of 91 controlled studies on ESAs therapy for cancer anemia documented a significantly elevated risk of thrombotic events regardless of the baseline Hb levels (Tonia et al., 2012). As regard the relationship between target Hb values and the frequency of thromboembolism and vascular accidents in end-stage renal disease (ESRD) patients receiving rHuEPO (Singh et al., 2006), it should be remarked that in some of the trials reporting an higher risk of thromboembolism and related mortality, Hb target levels were above standard, ranging from 13 to 15 g/dl (Henke et al., 2003; Leyland–Jones et al., 2005; Juneja et al., 2008). In a large trial evaluating the use of darbepoetin-α for CRA in patients not undergoing concomitant antineoplastic treatment, thromboembolism was not more frequent in people with Hb > 13 g/dl, nor with Hb rise > 1 g/dl in 2 weeks compared to those who did not (Smith et al., 2008). Moreover, it should be highlighted that no randomized clinical trial to date have evaluated any prophilactic approach to counteract the incidence of thromboembolism by administering, for example, anti-coagulant agents especially in the setting of advanced cancer patients, which present by themselves an elevated risk of thromboembolism.

Other adverse events associated with the use of ESAs, such as hypertension, thrombocytopenia/hemorrhage and seizures have been reported. The Cochrane meta-analysis by Tonia et al. (2012) reported and increased risk ratio of hypertension by approximately 30% in cancer patients receiving ESAs. The same Cochrane (Tonia et al., 2012) showed a significant increased risk to develop thrombocytopenia in erythropoietin-treated cancer patients. Some case of seizures have been associated to ESA treatment in cancer patients while the incidence was not significantly different in comparison to control arm (Tonia et al., 2012).

SAFETY ISSUES AND CONCERNS IN CLINICAL PRACTICE

In 2007 FDA published a warning statement limiting the indication of ESAs only for the therapy of chemotherapy-induced anemia and discouraging their administration when the antineoplastic therapy is finished (Goldberg and Goldberg, 2008; Hagerty, 2008); FDA also indicated that ESAs should be used only when the antineoplastic treatment has a palliative intent and clarified there is not a upper range for target hemoglobin but that the objective of ESA therapy should be the lowest Hb value to avoid transfusion (Goldberg and Goldberg, 2008). Such alerts were based on the results of several clinical trials that found that ESAs treatment in cancer patients was associated both with an inferior survival and worse cancer outcomes (Newland and Black, 2008; Bohlius et al., 2009; Pfeffer et al., 2009; Tsuboi et al., 2009): this has been correlated with an excess of thromboembolic events correlated with the levels of Hb reached (Tonia et al., 2012). Additionally, it was also hypothesized that the higher mortality was dependent on EPO ability to stimulate tumor growth (Henke et al., 2006; Brown et al., 2007). However, more recently, in 2009, a pooled analysis, involving more than 13,000 cancer patients, failed to confirm the data about the increased mortality risk associated with ESA (Bohlius et al., 2009).

Following the above concerns, the guidelines from the American Society of Clinical Oncology (ASCO) and American Society of Hematology (ASH) (Rizzo et al., 2010) as well as from the National Comprehensive Cancer Network (NCCN) for the appropriate therapy of anemia of cancer patients undergoing antiblastic therapy recommended considering the utilization of ESAs for selected patients according to FDA indications. In detail, they stated that ESA may be indicated to for patients with chemotherapy-induced anemia with Hb < 10 g/dL to decrease transfusion. Clinicians should start treatment after having discussed with patients the potential harms (e.g., thromboembolism, shorter survival) and benefits (e.g., decreased transfusions) in comparison to the potential harms (e.g., serious infections, immune-mediated adverse reactions) and benefits (e.g., rapid Hb improvement) of transfusions. The Committee cautions against ESA use under other circumstances, in particular in patients who are not receiving concurrent myelosuppressive

chemotherapy. As regard the FDA label that limit the indication for ESA to patients receiving chemotherapy for palliative intent, Rizzo et al. (2010) did not include this point. Indeed, to date no study or meta-analysis has analyzed the outcomes of ESA therapy by dividing patients according to chemotherapy aim. In 2012 a Cochrane meta-analysis assessing 91 trials with 20,102 patients found that if ESAs are used correctly for the therapy of chemotherapy-induced anemia as well CRA only if Hb is below 12 g/dl, no increase in overall mortality and on-study mortality have been observed (Tonia et al., 2012).

In clinical practice, cautions should be used when using ESA concomitant with chemotherapy regimen and cancers associated with increased risk of thromboembolism. Moreover, ESAs are contraindicated in patients with uncontrolled hypertension.

IRON SUPPLEMENTATION

Cancer-related anemia is characterized by both a defective incorporation of iron into developing erythrocytes and by erythropoietin deficiency. Therefore, it has been hypothesized that treatment of CRA could include a combination of ESAs and iron supplementation.

Iron formulation assessed in cancer patients include oral and parenteral forms (low-molecular weight iron dextran, ferric gluconate and iron sucrose.) To date, in a recent systematic meta-analysis (Mhaskar et al., 2016) the addition of iron to ESAs versus ESAs alone for chemotherapy-induced anemia, showed to be associated with increased hematopoietic response, lowered RBC transfusions, and improved Hb changes. The meta-analysis did not show any benefit in time to hematopoietic response nor any improvement in QL in patients supplemented with iron plus ESAs in comparison to ESAs alone. No treatment-related deaths have been reported (Mhaskar et al., 2016). Various routes of iron administration are available: oral intramuscular or intravenously. The bivalent (ferrous) form of oral iron has better bioavailability that the trivalent one (ferric). As for safety, ferrous gluconate is safer than iron dextran (Fishbane and Kowalski, 2000). As for tolerability, parenteral iron may be associated with nausea, vomiting and/or diarrhea, hypotension, pain, hypertension, dyspnea, pruritus, headache, and dizziness. The majority of adverse events in literature were associated with the use of high molecular weight iron dextran, which is anymore recommended and has been today replaced in the clinical practice by the other formulations (Baribeault and Auerbach, 2011; Rodgers et al., 2017).

Nevertheless, the use of iron for CRA is controversial. In fact, in addition to an absolute iron deficiency due to reduced nutritional intake or blood loss, these patients could present an iron functional deficiency with reduced saturation of transferrin and high levels of ferritin, which develops as a consequence of the chronic inflammatory status. This condition is characterized by an increase in iron storage with a limited iron availability or erythropoiesis. Moreover, it is characterized by a compromised iron intestine absorbance system mediated by hepcidin. In particular, this last piece of evidence suggested that cancer patients with anemia should not have any benefit from oral iron formulation, but they should receive intravenous iron. Several studies have compared intravenous versus oral iron administration in combination with ESAs for chemotherapy-induced anemia. The majority of these trials demonstrated that intravenously iron supplementation (in comparison to either orally administered iron or no iron) increases Hb response to ESAs and decreases the number of transfusions (Auerbach et al., 2004; Henry et al., 2007; Bastit et al., 2008; Pedrazzoli et al., 2008). A recent meta-analysis relevant to this issue (Mhaskar et al., 2016) confirmed a superior efficacy of intravenous iron combined with ESAs in comparison to oral iron regarding hematopoietic response, need of RBC transfusions, and improvement in Hb.

Noteworthy, the largest clinical trial performed to date by Steensma et al. (2011) demonstrated the lack of benefit of adding intravenous iron to ESAs in anemic cancer patients with signs of functional iron deficiency. These controversial results may be at least in part attributed to the fact that the clinical trials assessing the effectiveness of the combination of ESAs with orally or intravenously administered iron for chemotherapy-induced anemia, incorporated an heterogeneous population, including both patients in early and advanced stages, where the pathogenesis of anemia and the alterations of iron metabolism are completely different. In this regard, in 2010 we performed a randomized, prospective trial in a selected population of advanced cancer patients with chronic inflammation, which have CRA yet before starting chemotherapy (Macciò et al., 2010). The study had the scope to assess whether the supplementation with orally administered lactoferrin compared to intravenous iron, both added to rHuEPO, was effective and safe, for the therapy of CRA in a cohort of 148 advanced neoplastic patients receiving chemotherapy. Lactoferrin is an iron-binding protein with a molecular weight of 80 kD belonging to the transferrin family, which may play a relevant antimicrobial and antiinflammatory activity in the immune response. Enrolled patients were randomized to receive intravenous ferric gluconate (125 mg/week) or lactoferrin (200 mg/day), each combined with s.c. rHuEPOβ, 30,000 UI/week, for 12 weeks. Hemoglobin increased significantly in the two arms; both arms obtained a similar Hb improvement, hematopoietic response, time to hematopoietic response or mean change in serum iron, CRP, or erythrocyte sedimentation rate. Notably, ferritin levels lowered in the lactoferrin, while they augmented in the intravenous iron arm. Therefore, the results of this trial showed that oral lactoferrin was effective as intravenous iron in terms of Hb increase. The decrease of ferritin in the lactoferrin arm probably suggests that ferritin is better able to modulate iron metabolism and ameliorate iron recycling.

On the basis of currently available data, patients with CRA should receive iron supplementation, especially when they are not responsive to the treatment with erythropoietic agents alone. However, martial therapy could not be recommended for patients with functional iron-deficiency and high levels of ferritin. In this case, iron overload can favor ROS synthesis thus aggravating the

oxidative stress status leading to subsequent endothelial damage and increased risk of cardiovascular complications.

COMBINED MULTITARGETED APPROACH OF CRA

Considering the multifactorial mechanisms leading to CRA, mainly attributable to the condition of chronic inflammation that characterizes advanced cancer patients, some combined targeted approaches have shown a significant benefit in ameliorating anemia and related symptoms. Moreover, not all patients with CRA benefit from the use of ESA alone: up to 15–20% still require RBC transfusions, and only 50–70% have an Hb increment of ≥1 g/dl after administration of ESA for 8–12 weeks (Beguin, 1998). Evidence from several studies in patients with end-stage renal disease and anemia showed that ESAs hyporesponsiveness may be due to several factors: a weakened iron supplying to developing erythroid cells due to functional iron deficiency (Tarng et al., 1999); increased amount of erythropoiesis-inhibiting inflammatory cytokines (Ribeiro et al., 2016); reduced energy and nutrients supply due to the compromised food intake and metabolic changes (Bistrian and Carey, 2000). A multitargeted approach may be useful in circumvent these events and improve the effectiveness of ESAs.

In this regard, a randomized clinical trial (Daneryd et al., 1998) in 108 cancer patients with advanced tumors at different sites with cachexia not undergoing concomitant chemotherapy showed that the administration of rHuEPO in association with the anti-inflammatory agent indomethacin achieved a higher increase in Hb levels as well as increased oxygen uptake, and respiratory activity with a better-preserved exercise capacity in comparison to the arm with indomethacin alone. These results were correlated with a significant reduction in inflammatory markers in the experimental arm. These results have been confirmed in further analysis published by the same authors where the combined approach with indomethacin and rHuEPO showed to improve Hb levels and functional capacity; however, it did not improve significantly subjective measures of QL (Lindholm et al., 2004). Also another randomized study by Lundholm et al. (2004), evaluated whether the addition of a specialized, nutritional support to a combined treatment with indomethacin and rHuEPO, and confirmed that the combined approach prevented CRA and improved significantly the energy metabolism and functional outcome in advanced cancer patients with cachexia. Indeed, indomethacin by reducing cytokine production and inflammation, may improve by itself anemia (Nieken et al., 1995) and increase responsiveness to EPO treatment (Bistrian and Carey, 2000). These properties are common to other NSAIDs that may thus obtain similar results such as aspirin and COX-2 inhibitor.

At this regard, some papers from our group demonstrated that a combined approach for the therapy of metabolic/inflammatory changes and associated symptoms (cachexia) related to advanced cancer was able to significantly counteract CRA. In detail, two case reports published by our group confirmed that a supportive multitargeted anticachectic approach including L-carnitin, curcumin, lactoferrin, rHuEPO and the COX-2 inhibitor celecoxib was able to counteract CRA in a patient with metastatic hormone-refractory prostate cancer (Macciò et al., 2015a) and in a patient with myelofibrosis with associated cachexia (Macciò et al., 2015b), respectively. The combined approach in both patients achieved a rise of Hb and serum iron values, and a concomitant decrease of inflammatory markers, ferritin, and hepcidin. The treatment in both patients was associated with an improvement of weight, lean body mass, grip strength, fatigue, and overall QL.

Therefore, when increased risk factors or comorbidities are lacking, the use of rHuEPO associated with the concomitant administration of low molecular weight heparin, for counteracting the well-known prothrombotic status of advanced cancer, plus the modulation of iron metabolism with appropriate supplements, and an adequate nutritional support, is able to improve CRA, associated symptoms and impairment of QL.

CONCLUSION

Cancer-related anemia has a high incidence and significant clinical impact on patient prognosis and QL. However, so far the clinical studies testing the application of ESAs have been limited to chemotherapy-related anemia. About this point, it should be remembered that ESAs are indicated for Hb values < 10 g/dl, not considering that many patients (particularly in the advanced stages of disease) have already anemia before the start of chemotherapy, with Hb values equal to or lower than this threshold (Ludwig et al., 2004). Considering the evidence regarding iron-restricted erythropoiesis, several studies found that intravenous iron supplementation had higher efficacy than oral iron administration for the therapy of CRA (Henry, 2010). However, more recently the role of intravenous iron administration, in case of functional iron deficiency typical of CRA, has been disputed (Spivak, 2011). Accordingly, in our randomized clinical study we hypothesized the necessity to select the way of iron supplementation on the basis of patient clinical characteristics. Furthermore, we showed that the selective modulation of iron metabolism through oral lactoferrin, in advanced patients with CRA during antineoplastic treatment, decreases excessive iron storage (ferritin) and reduces ferritin levels, and supported the ESAs activity, comparably to intravenous iron administration (Macciò et al., 2010).

It is crucial to underline that cancer growth and related inflammatory response determines alterations of energy metabolism and feeding (cancer-related anorexia and cachexia), that may potentially induce anemia. Consistently, we demonstrated that parameters of the nutritional and energy status of the patient such as leptin, were positively correlated with Hb levels. Also, we showed that the GPS, a score based on the inflammatory/nutritional status, was predictive of Hb levels (Macciò et al., 2015a). This piece of evidence is strongly in accordance with findings observed in chronic kidney failure patients, where the "malnutrition-inflammation score" showed to be widely correlated with anemia (Kalantar-Zadeh et al., 2001; Molnar et al., 2011; Rattanasompattikul et al., 2013).

Until now, the association of the nutritional status with the hemoglobin levels in cancer patients has not been sufficiently assessed. Conversely, in people with chronic kidney failure, where this correlation has been largely found, the role of an adequate nutritional status to improve anemia, particulary in those patients candidate to ESAs, is now strongly recognized (Takeda et al., 2002; Axelsson et al., 2005; Hung et al., 2005).

A proper characterization of cancer patients with anemia on the basis of tumor staging and inflammation/metabolic-related symptoms is thus warranted to identify specific parameters that will enable the design and implementation of the best therapeutic strategy to treat CRA, in which inflammation and metabolic impairments seem to play pivotal roles (Steensma, 2008). In this context, the finding that treatment with anti-IL6 mAb in patients with advanced cancer at different sites is able to significantly increase Hb levels is of high relevance (Bayliss et al., 2011; Coward et al., 2011). In conclusion, on the basis of the evidence discussed here, we can conclude that the comprehension of the multifactorial pathogenetic pathways leading to CRA is pivotal to identify the most adequate and effective treatments. Currently, the leading international scientific societies have developed protocols that have focused primarily on the treatment of chemotherapy-induced anemia. The data discussed herein indicate that an accurate assessment of the neoplastic patient must be achieved prior to start the therapy for CRA; besides ESAs, the approach should provide a specific multitargeted therapy, including, e.g., adequate caloric supplementation, an antioxidant/anti-inflammatory treatment and personalized iron supplementation.

AUTHOR CONTRIBUTIONS

CM and AM contributed to the conception of the work, analysis and interpretation of data, drafting of the manuscript, and critical revision of the manuscript. GG, GA, VA, and ES contributed to the analysis and interpretation of data and drafting of the manuscript. RD contributed to the analysis and interpretation of data, and drafting of the manuscript in particular as regard the paragraph "safety issue and concerns in clinical practice." All the authors approved the final version to be published; all authors agreed to all aspects of the work.

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The MEK-Inhibitor Selumetinib Attenuates Tumor Growth and Reduces IL-6 Expression but Does Not Protect against Muscle Wasting in Lewis Lung Cancer Cachexia

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Cachexia, or wasting of skeletal muscle and fat, afflicts many patients with chronic diseases including cancer, organ failure, and AIDS. Muscle wasting reduces quality of life and decreases response to therapy. Cachexia is caused partly by elevated inflammatory cytokines, including interleukin-6 (IL-6). Others and we have shown that IL-6 alone is sufficient to induce cachexia both *in vitro* and *in vivo*. The mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor Selumetinib has been tested in clinical trials for various cancers. Moreover, Selumetinib has also been shown to inhibit the production of IL-6. In a retrospective analysis of a phase II clinical trial in advanced cholangiocarcinoma, patients treated with Selumetinib experienced significant gains in skeletal muscle vs. patients receiving standard therapy. However, the use of Selumetinib as a treatment for cachexia has yet to be investigated mechanistically. We sought to determine whether MEK inhibition could protect against cancer-induced cachexia in mice. *In vitro*, Selumetinib induced C2C12 myotube hypertrophy and nuclear accretion. Next we tested Selumetinib in the Lewis lung carcinoma (LLC) model of cancer cachexia. Treatment with Selumetinib reduced tumor mass and reduced circulating and tumor IL-6; however MEK inhibition did not preserve muscle mass. Similar wasting was seen in limb muscles of Selumetinib and vehicle-treated LLC mice, while greater fat and carcass weight loss was observed with Selumetinib treatment. As well, Selumetinib did not block wasting in C2C12 myotubes treated with LLC serum. Taken together, out results suggest that this MEK inhibitor is not protective in LLC cancer cachexia despite lowering IL-6 levels, and further that it might exacerbate tumor-induced weight loss. Differences from other studies might be disease, species or model-specific.

Keywords: cachexia, atrophy, cancer, cytokines, chemotherapy, Interleukin-6, MAP Kinase, lung neoplasms

INTRODUCTION

Cachexia is a devastating consequence of cancer and other chronic diseases recognized by dysmetabolism leading to a progressive reduction in skeletal muscle and adipose tissue (Fearon et al., 2011; Argilés et al., 2015; Tsoli et al., 2016). Muscle wasting reduces function, quality of life and decreases response to therapy. Low muscle mass increases chemotherapy toxicity, while chemotherapy in turn can cause muscle wasting and contribute to cachexia (Chen et al., 2015; Barreto et al., 2016; de Lima Junior et al., 2016; Toledo et al., 2016). Currently there are no approved, effective therapies for cachexia. However, blocking muscle loss in cancer cachexia prolongs function and life, indicating that anti-cachexia therapies will be an essential adjunct to anti-tumor therapies for treatment of cancer (Benny Klimek et al., 2010; Zhou et al., 2010; Hatakeyama et al., 2016).

There are several underlying mechanisms that directly contribute to cachexia. It has been referred to as a syndrome of energy imbalance, where intake is decreased and expenditure is increased. However, even with a controlled energy intake, this imbalance persists (Evans et al., 1985). The loss of skeletal muscle mass is largely attributed to abnormalities in protein metabolism, where degradation outweighs synthesis caused in part by increased activity of the ubiquitin-proteasome pathway as well as autophagy (Acharyya and Guttridge, 2007; Mammucari et al., 2007). Loss of myofibrillar proteins leads directly to muscle atrophy, weakness, and fatigue. Common catabolic pathways involved in turnover of skeletal muscle proteins are induced by a multitude of inflammatory cytokines, both tumor- and host-derived. These cachectic mediators include TNFα, Myostatin, Activin, other members of the TGF-β superfamily, and the well-known driver of cachexia, Interleukin-6 (IL-6) (Jackman and Kandarian, 2004; Fearon et al., 2012; Tsoli and Robertson, 2013; Narsale and Carson, 2014; Londhe and Guttridge, 2015). IL-6 binds IL-6 receptor and the common signaling receptor GP130 to activate the ERK, AKT, and STAT3 pathways (Belizário et al., 2016). Others and we have shown that IL-6 alone is sufficient to induce muscle wasting both *in vitro* and *in vivo* (Bonetto et al., 2011, 2012; Zimmers et al., 2016), largely through activation of STAT3 (Zimmers et al., 2016) downstream of GP130 and JAK. Inhibition of IL-6, IL-6 receptor, or STAT3 all reduce cachexia in experimental systems (Strassmann et al., 1992; Oldenburg et al., 1993; White et al., 2011; Silva et al., 2015). Moreover, anti-IL-6 therapies have shown promise in human lung cancer cachexia (Bayliss et al., 2011).

In addition to IL-6, a variety of mitogenic and inflammatory stimuli can activate the Mitogen Activated Protein Kinase (MAPK)/ERK pathway, including other cytokines and growth factors signaling through tyrosine kinase receptors (Guan, 1994). MEK1/2 phosphorylates ERK and influences survival, growth, proliferation, and inflammatory processes (Zheng and Guan, 1993; Hommes et al., 2003). The MEK pathway is also activated by oncogenic Ras, and has been targeted for anti-cancer therapies (Neuzillet et al., 2014). The selective small molecule MEK1/2 inhibitor Selumetinib decreases phosphorylation and activation of ERK1/2 (Yeh et al., 2007) and shows efficacy in cancers of the lung, skin, ovary and liver (Miller et al., 2014; Facciorusso et al., 2015; Heigener et al., 2015; Shoushtari and Carvajal, 2016).

A Phase II study of Selumetinib showed weight gain in patients with bililary cancer, a condition typically associated with severe wasting (Bekaii-Saab et al., 2011). Retrospective re-analysis of those data showed that patients who received Selumetinib experienced significant gains in skeletal muscle while those on standard therapy experienced muscle loss (Bekaii-Saab et al., 2011; Prado et al., 2012). Inhibition of the ERK pathway, via a dominant negative form of Raf or a pharmacological inhibitor, results in robust myotube hypertrophy (Rommel et al., 1999). Additionally, ERK inhibition de-represses myogenic differentiation caused by cardiotrophin-1, a member of the IL-6 family of cytokines (Miyake et al., 2009). Pharmacological inhibition of ERK1/2 significantly increases mRNA levels of the transcription factor myogenin, promoting differentiation and expression of muscle specific genes and the myogenic program (Adi et al., 2002). Finally, ERK inhibition has also been shown to prevent muscle wasting in a C26 colon carcinoma mouse model of cancer cachexia (Penna et al., 2010; Quan-Jun et al., 2016).

Given the promising results of Selumetinib in patients and of ERK inhibition in mice, we sought to investigate Selumetinib in a LLC model of cancer-induced cachexia (Bennani-Baiti and Walsh, 2011). Here we report *in vitro* hypertrophy and *in vivo* tumor killing and inhibition of IL-6 production by Selumetinib in mice, but no evidence of anti-cachexia effects either *in vivo* or *in vitro*.

MATERIALS AND METHODS

Cell Cultures

Lewis lung carcinoma cells were maintained at low confluence at 37°C in a humidified atmosphere of 5% CO_2 in DMEM, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (pen/strep). Cells were trypsinized, counted and resuspended in PBS for injection.

Murine C2C12 myoblasts (ATCC) were grown in DMEM, 10% FBS and pen/strep. Confluent cells were switched to differentiation medium (DM), consisting of DMEM with 2% horse serum and pen/strep for 96 h. After this time, the medium was replaced with DM containing 10 nM Selumetinib or vehicle for an additional 48 h. For the LLC plasma experiment, C2C12 cells were differentiated for 96 h before being switched to media consisting of DMEM with 2% plasma from control or LLC tumor bearing mice and pen/strep, either with or without 10nM Selumetinib, then incubated for an additional 48 h.

Animals

All experimental animal protocols were approved by and used in compliance with the Indiana University School of Medicine Institutional Animal Care and Use Committee. Eight-week old male C57BL/6J mice were obtained from The Jackson Laboratory. All mice were maintained on a regular light-dark cycle and allowed free access to food and water throughout the duration of the experiment. Mice were grouped as follows: Control + vehicle ($n = 6$), LLC + vehicle ($n = 8$), and LLC + Selumetinib ($n = 8$). Tumor bearing mice were subcutaneously

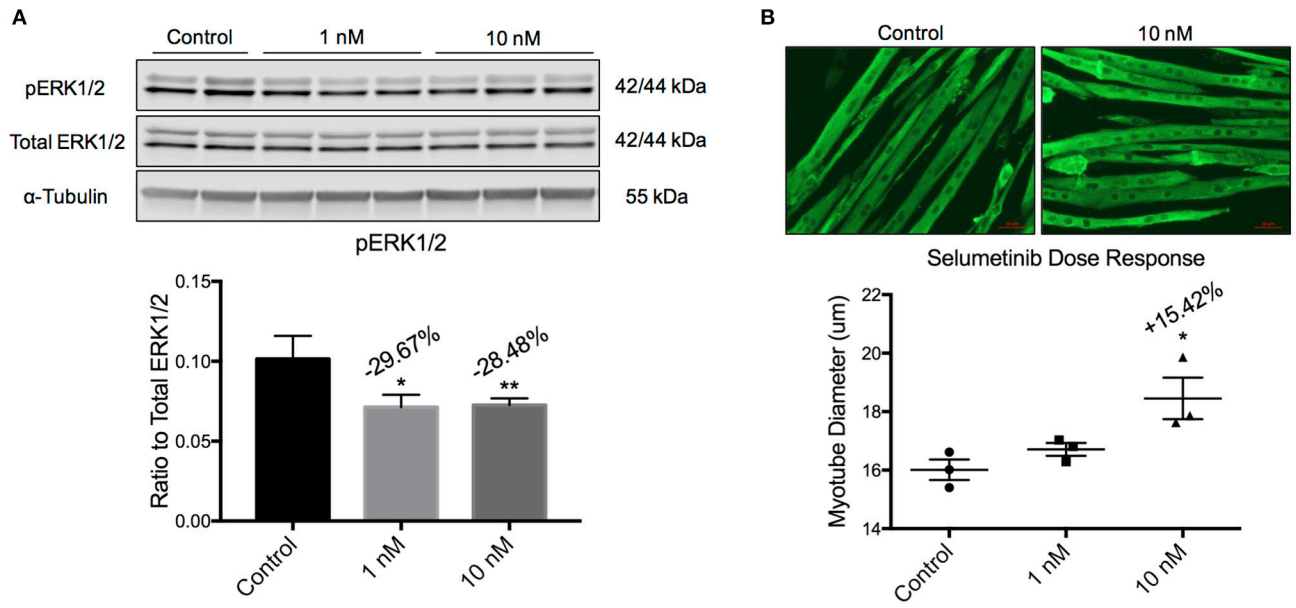

FIGURE 1 | Selumetinib induces myotube hypertrophy. (A) Western blotting analysis of C2C12 myotubes shows that 1 and 10 nM of Selumetinib reduced phosphorylated ERK1/2. pERK1/2 was normalized to α-Tubulin. **(B)** Representative myotubes treated with 10 nM Selumetinib or vehicle and stained for myosin heavy chain. Treatment with 10 nM Selumetinib induces myotube hypertrophy (+15.24%). Conditions were performed in triplicate. Data are means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

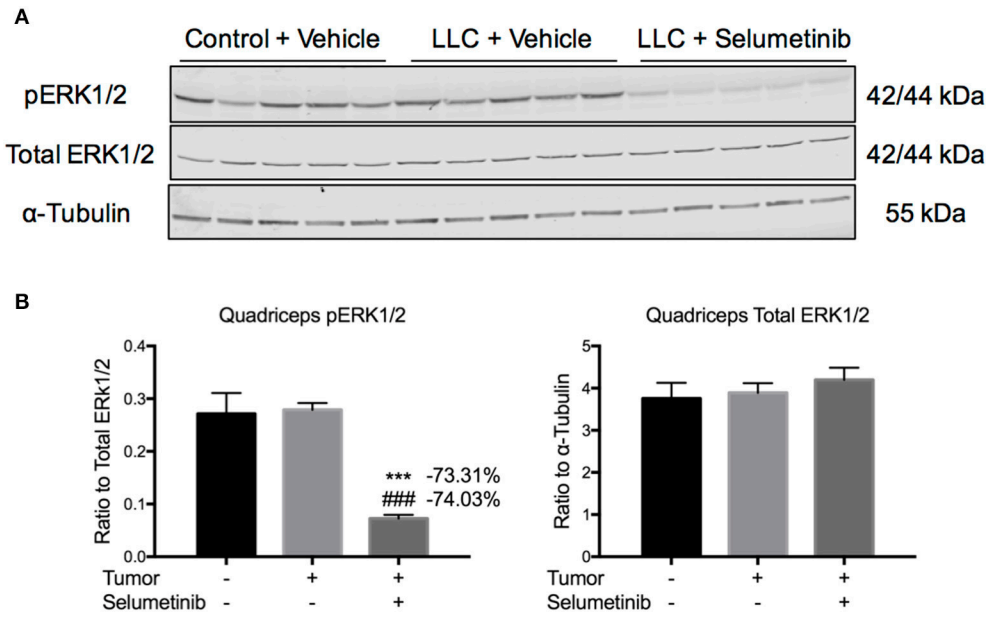

FIGURE 2 | Selumetinib inhibits ERK phosphorylation in skeletal muscle *in vivo*. (A) Western blotting analysis of quadriceps lysates shows reduced pERK1/2 in Selumetinib treated mice, consistent with MEK inhibition. **(B)** Quantification of Western blotting analysis. pERK1/2 was significantly reduced in tumor bearing mice treated with Selumetinib compared to vehicle treated control and tumor bearing mice, while total ERK1/2 was unchanged. Data are expressed as means ± SEM. $^{***}p < 0.001$ vs. Control + Vehicle, $^{\#\#\#}p < 0.001$ vs. LLC + Vehicle.

injected with 10^6 LLC cells in the intrascapular region on day 0, with treatments beginning 24 h later. Selumetinib (Selleckchem) in vehicle (0.5% methylcellulose/0.2% Tween 80) or vehicle alone was administered twice daily at 25 mg/kg by gavage (Shannon et al., 2009; Troiani et al., 2012; Huang et al., 2013). Body weights of the mice were recorded daily. Mice were euthanized

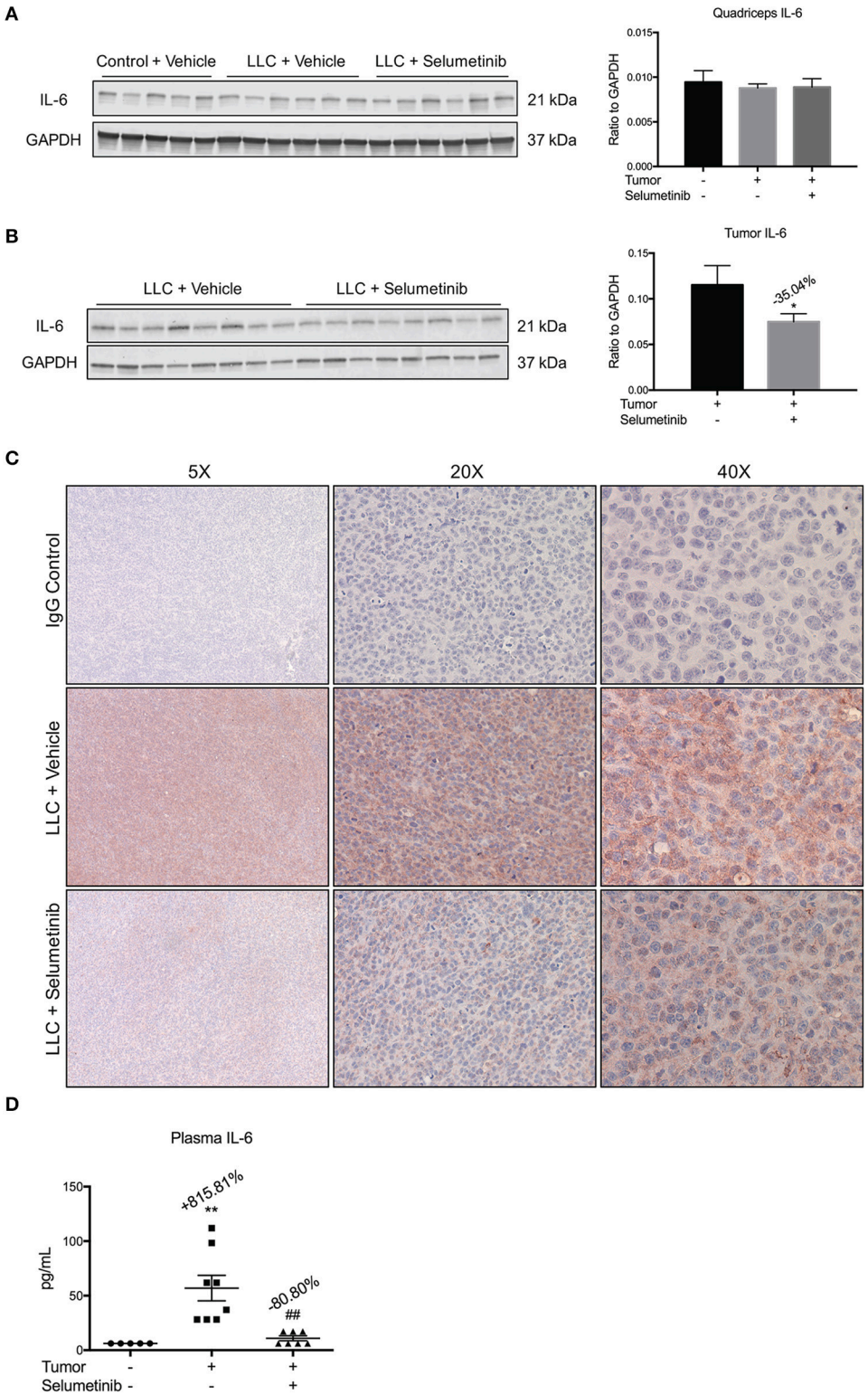

FIGURE 3 | Selumetinib reduces IL-6 levels in blood and tumor, but not muscle. (A) Western blotting analysis shows quadriceps IL-6 was not changed in cachexia or with Selumetinib. **(B)** IL-6 expression was reduced in lysates of tumors from mice treated with Selumetinib. Data are expressed as the means ± SEM. **(C)** Representative images of immunohistochemistry. Selumetinib-treated mice show reduced staining for IL-6 in tumor. **(D)** Plasma IL-6 levels were increased in vehicle-treated LLC mice. Selumetinib treated LLC mice showed a significant decrease in circulating IL-6 vs. vehicle-treated LLC mice. Data are expressed as means ± SEM. $^{*}p < 0.05$ vs. LLC + Vehicle; $^{**}p < 0.01$ vs. control + Vehicle; $^{\#\#}p < 0.01$ vs. LLC + Vehicle.

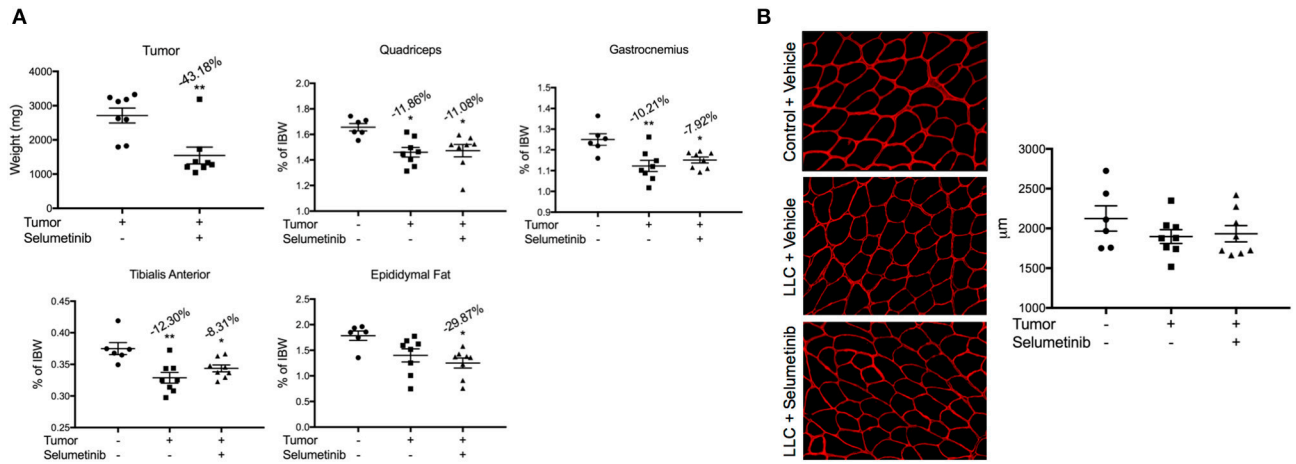

FIGURE 4 | Selumetinib reduced tumor mass, but did not protect against cancer-induced cachexia. (A) Tumor mass was significantly reduced in mice treated with Selumetinib. When compared to controls, LLC-bearing mice treated with either vehicle or Selumetinib showed similar wasting in the quadriceps (−11.86 and −11.08%,), gastrocnemius (−10.21 and −7.92%), and tibialis anterior muscles (−12.30 and −8.31%). Epididymal fat loss was significant in the LLC-Selumetinib group. Greater carcass loss was observed in the LLC-Selumetinib mice, while heart loss was greater in vehicle-LLC mice. Data are expressed as means ± SEM. $^{*}p = <0.05$, $^{**}p = <0.01$. **(B)** Representative images of muscle fiber cross-sectional area and quantification. Data are expressed as means ± SEM.

under general anesthesia on day 17 when some mice reached the criteria for a humane endpoint. Muscles, tumors and organs were dissected, weighed, snap frozen in liquid nitrogen, and stored at −80°C. Tissue weights are expressed as a percentage of initial body weight to normalize for small differences in starting size.

Immunofluorescence and Immunohistochemistry

C2C12 cultures were fixed and permeabilized in ice cold acetone/methanol (1:1) at −20°C for 20 min. After 10 min of rehydration in PBS at room temperature (RT), cells were blocked in an 8% BSA solution for 1 h at RT. Primary antibody against myosin heavy chain (Developmental Studies Hybridoma Bank) was incubated overnight at 4°C with gentle agitation. Washed cultures were incubated with AlexaFluor 488-labeled anti-mouse IgG (Life Technologies) for 1 h at RT. Nuclei were stained with DAPI and images were captured on an Axio Observer.Z1 (Zeiss). Myotube diameter was measured using ImageJ analysis software (Wayne Rasband, U.S. National Institutes of Health).

For analysis of muscle fiber cross-sectional area, tibialis anterior muscles were mounted on cork discs with Optimal Cutting Temperature compound, and frozen in 2-methylbutane cooled in liquid nitrogen before being stored at −80°C. Fresh frozen sections were cut using a Leica CM1860 Cryostat (Leica Microsystems Inc.). Muscle sections were fixed in 100% acetone at −20°C before being rehydrated with PBS and blocked in an 8% BSA solution for 1 h at RT. Following overnight incubation with a primary antibody against Dystrophin (Vector Laboratories), sections were incubated for 1 h at RT with an AlexaFluor 594-labeled anti-mouse IgG. Muscle fiber cross-sectional area was measured using an ImageJ macro developed by Dr. Richard Lieber (Minamoto et al., 2007).

Formalin-fixed, paraffin-embedded tumor tissue sections were deparaffinized in xylene and ethanol. Slides were boiled in 10 mM sodium citrate buffer pH 6.0 for 10 min, and cooled at RT for 30 min, then blocked with 8% BSA in PBS for 1 h, followed by overnight incubation at 4°C with antibody against IL-6 (Abcam) or normal rabbit IgG (Santa Cruz Biotechnology). Antibody detection used the ImmPRESS HFP Anti-Rabbit IgG (Peroxidase) Polymer Detection kit and ImmPACT DAB Peroxidase (HRP) Substrate per manufacturer's instructions (Vector Laboratories).

Western Blotting

Muscles and tumor were homogenized on ice in lysis buffer containing 25 nM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and fresh protease and phosphatase inhibitor cocktail tablets (Roche). Homogenates were centrifuged at 4°C at 14,000 rpm for 15 min, and supernatant was collected and stored at −80°C. Protein concentration was measured by BCA protein assay kit (Thermo Scientific). Protein extracts (30 µg) were denatured at 95°C for 5 min in loading buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 1% bromphenol blue, and 10% 2-mercaptoethanol). Samples were resolved on Tris-Glycine gels and transferred to nitrocellulose (Bio-Rad Laboratories). Membranes were blocked in SEA BLOCK Blocking Buffer (Thermo Scientific) and incubated overnight at 4°C with antibodies against: IL-6 (EMD Millipore), α-Tubulin (Sigma-Aldrich), Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), p44/42 MAPK (ERK1/2), and GAPDH (Cell Signaling Technology). Anti-mouse and anti-rabbit IgG conjugated to DyLight 680 and 800 fluorescent dye (Cell Signaling Technology) respectively, were detection antibodies incubated for 1 h at RT. Membranes were imaged and quantified using an ODYSSEY CLx Infrared Imaging System and software (LI-COR).

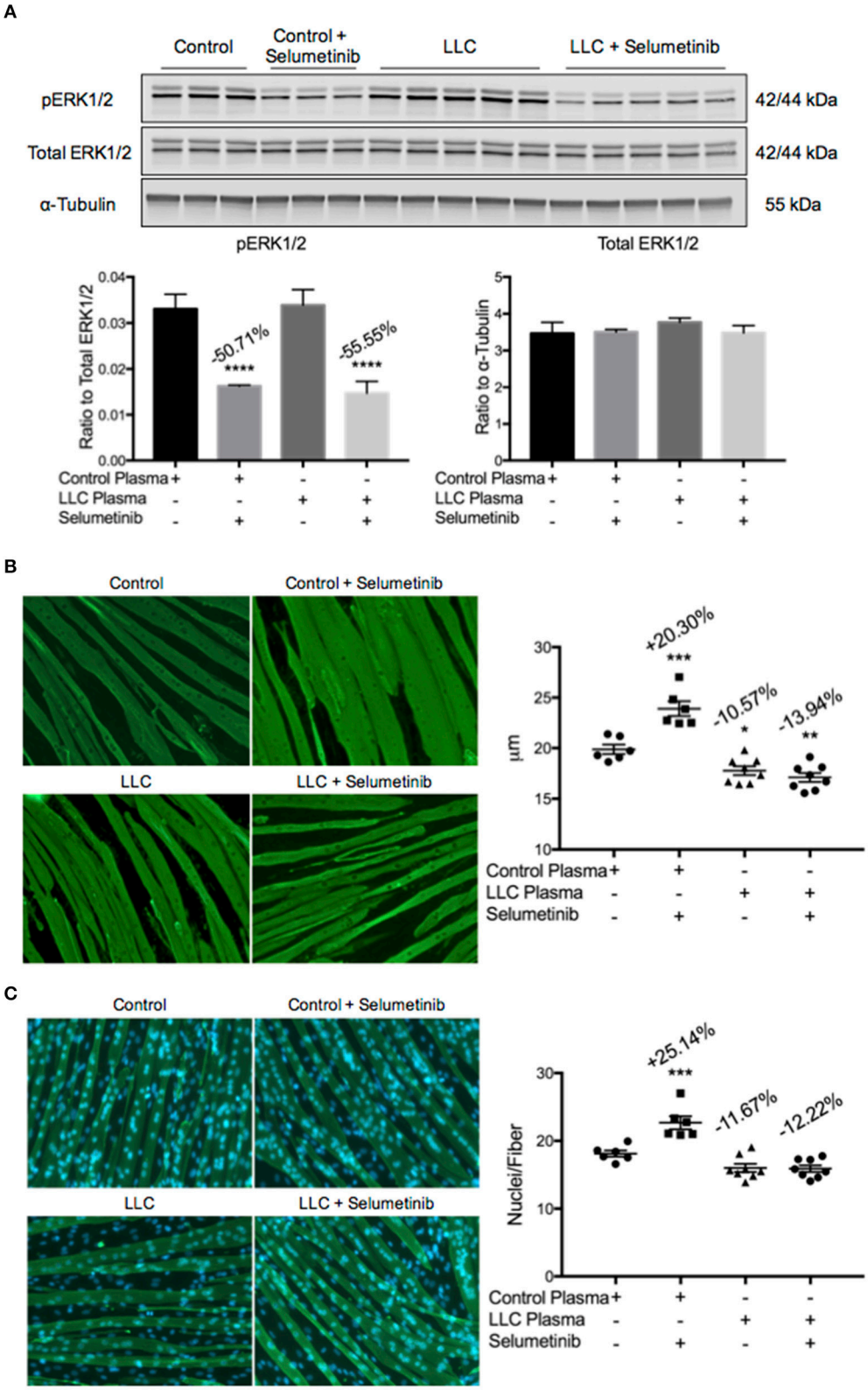

FIGURE 5 | ERK inhibition does not block LLC plasma-induced atrophy. C2C12 myotubes were treated with control plasma, control plasma + 10 nM Selumetinib, LLC plasma, or LLC plasma + 10 nM Selumetinib. Each data point represents myotubes treated with plasma from one individual mouse. **(A)** Western

(Continued)

FIGURE 5 | Continued
blotting analysis of C2C12 myotubes incubated with control (−50.71%) or LLC plasma (−55.55%) show inhibition of ERK1/2 phosphorylation upon treatment with Selumetinib. Data are expressed as means ± SEM. ****$p = <0.0001$. **(B)** Representative images and quantification of myotube diameter of C2C12 cells. Myotubes incubated with control plasma and treated with Selumetinib showed significant hypertrophy (+20.30%) vs. the control plasma only group. Both the LLC plasma only (−10.57%) and the LLC plasma with Selumetinib (−13.94%) groups showed atrophy vs. control plasma only. Data are expressed as means ± SEM. *$p = <0.05$, **$p = <0.01$, ***$p = <0.001$. **(C)** Representative images and quantification of nuclei per fiber. The total number of fibers counted were the same amongst all groups. Selumetinib treatment increased nuclei per fiber (+25.14%) when compared to control plasma only. LLC plasma showed a decrease (−11.67%) vs. control plasma only, which Selumetinib treatment was unable to block (−12.22%), although these were not statistically significant. Data are expressed as means ± SEM. ***$p = <0.001$.

IL6 Immunoassay

Whole blood was collected at euthanasia, via cardiac puncture, into EDTA tubes (BD Biosciences) and placed on ice. Plasma was separated by centrifugation at 3500 × rpm for 15 min at 4°C and stored at −80°C. IL-6 was detected in duplicate samples by a mouse magnetic 1-plex custom kit as per the manufacturer's instructions (Life Technologies) on a MAGPIX (Luminex).

Data Analysis

For experiments containing only two groups, statistical testing was by unpaired *t*-test. Experiments containing three or more groups, statistical significance was determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. A p-value > 0.05 was considered statistically significant.

RESULTS

Selumetinib Induced C2C12 Hypertrophy

C2C12 myoblasts proliferate as mononuclear cells in growth medium, and are induced to differentiate into syncytial myotubes upon switching to low serum conditions. This system has been used extensively to assess the atrophic or hypertrophic effects of proteins, conditioned medium, serum, or small molecules (Bonetto et al., 2011, 2012). Differentiated C2C12 myotubes were incubated with Selumetinib or vehicle for 48 h, with a media change after the first 24 h. Western blotting showed that 1 and 10 nM Selumetinib reduced ERK1/2 phosphorylation in C2C12 myotubes by ∼30% (**Figure 1A**). Myotube hypertrophy (diameter +15.42%, $P < 0.05$) was observed at a concentration of 10 nM but not 1 nM Selumetinib (**Figure 1B**), and higher concentrations were toxic (data not shown).

Selumetinib Inhibited ERK1/2 Phosphorylation in Skeletal Muscle

To test effects of Selumetinib on tumor growth and body composition in the setting of cancer, we injected mice with LLC cells, a well-validated and traditional model of cancer cachexia. Mice were treated twice daily with 25 mg/kg Selumetinib by gavage. Control mice received PBS injection and vehicle gavage, while tumor-bearing mice received tumor cell injection and vehicle gavage. No differences in overall body weight change or body composition were observed over the course of the experiment (data not shown). Mice were euthanized and necropsied on day 17. To query an on-target effect of Selumetinib in muscle, we performed Western blotting for phospho-ERK1/2. pERK1/2 was decreased 73.31 and 74.03% in the quadriceps of Selumetinib-treated mice vs. vehicle-treated control and tumor-bearing mice respectively (**Figures 2A,B**, $P < 0.001$). Total ERK1/2 was similar among all groups (**Figures 2A,B**).

IL-6 Expression Decreased in Blood and Tumor, but Not Muscle

Given that Selumetinib reportedly blocks production of the pro-cachectic inflammatory cytokine IL-6 (Tai et al., 2007), we measured IL-6 in tissue, tumor and blood. By Western blotting, IL-6 was not decreased in skeletal muscle (**Figure 3A**). However, Selumetinib decreased IL-6 protein by 35.04% ($P < 0.05$) in tumor lysates (**Figure 3B**), a finding confirmed by immunohistochemistry of tumor sections (**Figure 3C**). Furthermore, circulating levels of IL-6 were decreased 80.80% ($P < 0.01$) in Selumetinib-treated LLC mice vs. vehicle-treated tumor bearers (**Figure 3D**). IL-6 levels in the Selumetinib group were not significantly different from non-tumor bearing mice.

Selumetinib Reduced Tumor Size, but Did Not Prevent Muscle Wasting or Fat Loss

Consistent with its anti-tumor effects in other models of non-small cell lung cancer, Selumetinib treatment reduced LLC tumor size by 43.18% ($P < 0.01$) (**Figure 4**). Given that tumor size was greatly reduced, we expected muscle wasting to be attenuated, because in this model severity of cachexia generally correlates with tumor burden. However, in all muscles analyzed, both the Selumetinib and vehicle-treated LLC mice showed similar wasting (**Figure 4A**). Analysis of muscle fiber cross-sectional area displayed the same pattern as those observed in the muscle weights (**Figure 4B**). Greater fat loss and carcass loss were observed in Selumetinib-treated mice (**Figure 4A**).

Selumetinib Did Not Prevent LLC Plasma-Induced C2C12 Myotube Atrophy

While a reduced tumor burden and decreased circulating levels of IL-6 were seen with Selumetinib treatment, we did not observe any protection in skeletal muscle or fat mass. This led us to question whether there were other cachexia drivers in the LLC model that Selumetinib treatment could not modulate. To explore this, we treated C2C12 myotubes with plasma from either control mice or vehicle treated tumor bearing mice. This was done both with and without 10 nM of Selumetinib, the concentration previously used to induce myotube hypertrophy. Western blotting analysis showed that Selumetinib was able to reduce expression of pERK1/2 50.71 and 55.55% in myotubes

incubated with control or LLC plasma, respectively (**Figure 5A**). Consistent with our prior *in vitro* data (**Figure 1**), Selumetinib treatment was able to induce significant hypertrophy in myotubes incubated with control plasma. However, similar to our *in vivo* results, Selumetinib was unable to block myotube wasting induced by LLC plasma (**Figure 5B**). Further analysis showed that in addition to increasing myotube diameter, ERK inhibition also increased the number of nuclei per fiber (**Figure 5C**). Myotubes treated with LLC plasma showed a reduction in the number of nuclei per fiber, although this was not statistically significant, which Selumetinib treatment was again unable to attenuate.

DISCUSSION

Here we show that unlike results reported in patients with biliary cancers, mice with lung cancer do not exhibit reduced lean muscle loss despite tumor response with Selumetinib. This result was surprising for three reasons. Firstly, Selumetinib increased C2C12 fiber size *in vitro*, suggesting a potential pro-anabolic effect in skeletal muscle. In addition, we observed an increase in the number of nuclei per fiber with Selumetinib, suggesting that ERK inhibition increased the differentiation or fusion potential of myoblasts. However, despite reducing pERK1/2 in skeletal muscle, Selumetinib did not result in muscle protection much less hypertrophy in LLC conditions. Secondly, Selumetinib significantly inhibited tumor growth. Tumor mass normally correlates with the severity of muscle wasting, thus reduction of tumor burden should have led secondarily to reduced cachexia. This disconnect between tumor size and cachexia suggests that Selumetinib actually enhanced pro-cachectic pathways in LLC mice. Thirdly, those pathways must also be independent of IL-6, given that circulating and tumor-derived IL-6 were reduced in our study. This conclusion is supported by the observation that Selumetinib was unable to block LLC plasma-induced myotube atrophy. These data suggest that another, or several other, inflammatory cytokines or circulating factors are the essential driver/s of muscle wasting in the LLC model, not IL-6.

It is possible that the effects of Selumetinib on tumor growth and muscle wasting are disease specific, because Selumetinib was associated with increased lean body mass in patients with biliary cancers (Prado et al., 2012) and in the murine C26 colon adenoma cachexia model. Biliary, colon and lung cancers might exert muscle wasting through different effectors. In the C26 studies, ERK inhibition had no effect on tumor mass in one study (Penna et al., 2010), but resulted in an ∼15% decrease in tumor mass in another study (Quan-Jun et al., 2016). However, the studies each used different inhibitors and the mice from both were of a different genetic background than those used here.

The MEK pathway might also play different roles in humans vs. murine cancer cachexia. In the phase II clinical trial, 52% of patients treated with Selumetinib experienced a decrease in target lesion size, similar to what we observed in the present study. This could potentially explain the gain in total body mass of patients treated with Selumetinib, as opposed to the loss in patients receiving standard therapy. The increased muscle mass could be a result of a reduced tumor burden, and not any direct effect on the skeletal muscle itself. The authors hypothesize that the anabolic effect of Selumetinib is likely attributed to the inhibition of cytokine secretion. However, here we observed a significant decrease in both tumor tissue and circulating levels of IL-6, but with no beneficial effects on skeletal muscle.

Finally, it is possible that the lack of muscle preservation is due to the differential regulation and requirements of the MEK pathway during myogenesis. While we did not investigate muscle satellite cells in this study, it is possible that constant inhibition of the pathway led to a defect in proliferation or depletion of the satellite cell pool. Literature shows that ERK signaling can be both stimulatory and inhibitory for muscle differentiation. ERK1/2 activation is necessary for satellite cell proliferation and self-renewal (Ogura et al., 2015; Hindi and Kumar, 2016), but not required for fusion or expression of muscle specific genes (Jones et al., 2001). In addition, ERK2 is necessary for myotube formation, as siRNA-mediated knockdown of ERK2 in C2C12 myoblasts inhibited their fusion into multinucleated myotubes (Li and Johnson, 2006). Akt activation, a positive regulator of muscle mass, leads to inhibition of the MEK pathway in differentiated myotubes, while having no effect on their muscle precursor cells (Rommel et al., 1999). Conversely, leukemia inhibitory factor, an IL-6 family cytokine, inhibits myogenic differentiation through phosphorylation and activation of ERK1/2 (Jo et al., 2005). *In vitro* data show that early ERK1/2 activation, within 24 h post differentiation induction, can repress myogenic differentiation. Inhibition of MEK1 in the latter stages of differentiation displayed similar effects, blocking myotube formation (Jo et al., 2009). These data suggest that myogenic differentiation is coordinated by low MEK1 activity during the initial phases, and high activity thereafter. As such, while constant administration of Selumetinib inhibits tumor growth, achieving an anabolic effect appears to be more complicated.

Due to the requirements for ERK1/2 modulation in myogenesis, constant inhibition of ERK1/2 may be detrimental to skeletal muscle mass. The studies mentioned were able to control the myogenic stages at which the pathway was perturbed. While this would be challenging to accomplish *in vivo*, a potential approach would be to treat intermittently. This approach would allow for pathway activation, instead of remaining under a constant state of inhibition. Based upon the literature, allowing for cycles of activation and inhibition could potentially produce the stimulatory effects necessary for muscle hypertrophy. Future investigation will be necessary to determine a proper dosing regimen in order to determine the therapeutic potential of ERK inhibition as a treatment for cachexia and the potential effects of such cyclic dosing on tumor growth.

Taken together, these data suggest the need to consider the differential regulation of not only the MEK and IL-6 pathways, but also other pathways in muscle wasting of cancer cachexia. Moreover, they point to profoundly different drug-responsive phenotypes in commonly used cachexia models, suggesting diversity in the underlying cellular and molecular mechanisms

and the need for care in extrapolating results across disease states, clinical trials and model systems.

AUTHOR CONTRIBUTIONS

EA and AD carried out experiments, collected and interpreted data. LK and TZ designed and directed experiments. EA and TZ wrote the manuscript. TZ obtained funding for the studies.

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Effect of Oral Docosahexaenoic Acid (DHA) Supplementation on DHA Levels and Omega-3 Index in Red Blood Cell Membranes of Breast Cancer Patients

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Rationale: Docosahexaenoic acid (DHA) in cell membrane may influence breast cancer (BC) patients' prognosis, affecting tumor cells sensitivity to chemo- and radio-therapy and likely modulating inflammation. The possibility of identifying BC patients presenting with low DHA levels and/or low ability of DHA incorporation into cell membrane might help to treat this condition.

Methods: We enrolled BC patients and healthy controls, recording their seafood dietary intake. DHA in form of algal oil was administered for 10 consecutive days (2 g/day). Blood samples were collected at baseline (T0) and after 10 days of supplementation (T1) to assess DHA, omega-3 index, as the sum of DHA + eicosapentaenoic acid (EPA), in red blood cells (RBC) membranes and plasma tumor necrosis factor-alpha and interleukin-6 levels. Pre- and post-treatment fatty acid profiles were obtained by gas-chromatography. Parametric and non-parametric tests were performed, as appropriate, and P-value < 0.05 was considered statistically significant.

Results: Forty-three women were studied, divided into 4 groups: 11 patients with BRCA1/2 gene mutation (M group), 12 patients with familiar positive history for BC (F group), 10 patients with sporadic BC (S group), and 10 healthy controls (C group). DHA and omega-3 index increased from T0 to T1 in the 3 groups of BC patients and in controls ($P < 0.001$). No difference was found in DHA incorporation between each group of BC patients and between patients and controls, except for M group, which incorporated higher DHA levels with respect to controls ($\beta = 0.42$; $P = 0.03$). No association was documented between cytokines levels and DHA and omega-3 index at baseline and after DHA supplementation. Independent of the presence of BC, women considered as "good seafood consumers" showed at baseline DHA and omega-3 index higher with respect to "low seafood consumers" ($P = 0.04$; $P = 0.007$, respectively). After supplementation, the increase in DHA levels was greater in "low seafood consumers" with respect to "good seafood consumers" ($P < 0.0001$).

Conclusion: DHA supplementation was associated with increased DHA levels and omega-3 index in RBC membranes of BC cancer patients, independent of the type of BC presentation, and in controls. BRCA1/2 mutation, as well as low seafood consuming habits in both BC patients and healthy controls, seem to be associated with greater ability of DHA incorporation. Larger samples of BC patients are necessary to confirm our observation.

Keywords: breast cancer, DHA, omega-3 index, omega-3 fatty acids, BRCA

INTRODUCTION

Breast cancer (BC) is the most common cancer in women, with an incidence greater than 1 million of new cases per year worldwide (Bougnoux et al., 2009, 2010). A strong relationship exists between diet, overweight and risk of primary BC and its recurrence (Amadou et al., 2013; Molfino et al., 2016). Diets with a high content in omega-6 polyunsaturated fatty acids (PUFAs), and relatively low omega-3 PUFAs (Molfino et al., 2014), are associated with increased risk to develop BC and its relapse (Chlebowski et al., 2006; de Lorgeril and Salen, 2012; Laviano et al., 2013). Moreover, omega-3 PUFAs, in particular the docosahexaenoic acid (DHA), are able to influence the efficacy of chemo- and radio- therapy in BC patients (Bougnoux et al., 2009, 2010), sensitizing the malignant tumor cells to chemo- and radio-therapy, not increasing the toxicity on non-tumor tissues (Bougnoux et al., 2010; Hajjaji et al., 2011; Laviano et al., 2012). The lipid environment of cancer cells (i.e., the lipid component of cell membranes) may influence tumor sensitivity to chemotherapeutic agents. The membrane lipids of cancer cells are similar to those of storage lipids in terms of fatty acids composition, suggesting that DHA levels may potentially influence the activity of anti-tumor agents (Bougnoux et al., 1999, 2010). The dietary supplementation with DHA might be able to increase the effectiveness of systemic chemotherapy and local mammary irradiation. In particular, DHA from food or from exogenous supplementation, after intestinal absorption, is rapidly incorporated into circulating phospholipids and in those of cell membranes, including the red blood cells (RBC). A study showed that in BC patients the incorporation of DHA in circulating phospholipids is variable, and two different phenotypes of BC patients were identified: "high-incorporator" (with high incorporation of DHA in phospholipids) and "low-incorporator" (with poor or reduced incorporation of DHA in phospholipids) (Bougnoux et al., 2009). The possibility of recognizing "low-incorporator" patients would identify patients who might significantly increase the intake of DHA to increase the sensitivity of the tumor to cancer therapies. In addition, it could be also hypothesized that "low-incorporators" may have an increased susceptibility to develop BC or a tumor recurrence.

Studies have been conducted on a possible relationship between PUFAs metabolism and the pathways involving the Breast Related Cancer Antigens (BRCA) 1 and BRCA2 genes (Bernard-Gallon et al., 2002), which are implicated in inherited predisposition to BC, showing the presence of a possible transcriptional or post-transcriptional regulation of BRCA1 and BRCA2 after omega-3 PUFAs treatment in breast tumor cells (Bernard-Gallon et al., 2002; Shiovitz and Korde, 2015).

Omega-3 fatty acids are incorporated into phospholipids of cell membranes during both reticulocyte maturation and through plasma exchange, making erythrocytes an accurate indicator of dietary fatty acid intake. The omega-3 index measures the percentage of the long-chained omega-3 fatty acids, eicosapentaenoic acid (EPA) and DHA, to total erythrocyte membranes fatty acids (Harris, 2008).

In this light, we aimed at assessing the ability of DHA incorporation in RBC membranes, expressed as omega-3 index, in BC patients and in healthy controls and the potential differences in the DHA incorporation ability, and at determining whether the incorporation of DHA could differ in BC patients with a family history of breast malignancy, either positive or not for BRCA1/BRCA2 gene mutation. We secondarily verified a possible association between omega-3 PUFAs levels in RBC membranes and the inflammatory status.

MATERIALS AND METHODS

This was a spontaneous, single-center, controlled study performed on patients from the Department of Surgical Sciences, Sapienza—University of Rome, Italy. After approval of the local Ethics Committee and after obtaining written informed consent from each participant, women with diagnosis of BC and healthy women with no personal and no family history of BC were recruited. All procedures were in accordance with the ethical standards of the Helsinki Declaration issued in 1975 and later amendments. Exclusion criteria were: self-reported consumption of omega-3 PUFAs supplements and omega-3 PUFAs supplemented foods in the previous 6 months.

The sample size was determined based on a previous study conducted in BC patients observing changes of DHA levels before and after DHA oral supplementation (Bougnoux et al., 2009).

Participants

We recorded participant's demographic and anthropometric characteristics (age, weight, height, body mass index—BMI) and serum nutritional and metabolic biomarkers, including cholesterol, low density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides. Histological diagnosis, tumor staging, and a detailed medical history were collected. Based on the familiar and past medical history, the participants were

divided in: patients with no family history of breast malignancy—sporadic (S) group, patients with BC familial history, but negative for BRCA1 or BRCA2 gene mutation—familiar (F) group, patients with documented BRCA1 or BRCA2 gene mutation—mutated (M) group, healthy subjects—control (C) group matched for age and BMI. Participants were interviewed regarding the presence of body weight change over the prior 6 months, and for the presence of comorbidities such as diabetes, hypercholesterolemia, and hypertriglyceridemia. Questionnaire on participant's self-reported dietary habits was administered. It included questions on the habitual consumption of seafood in the diet, focusing on portion size (i.e., at least a seafood portion of 80 g) and frequency (once a month/once a week/more than once a week), as previously validated (Dahl et al., 2011). Based on the answers given, participants were divided into 2 groups: those who self-reported eating seafood in the diet once per month or less than once per month ("low seafood consumer"), and those who self-reported consuming seafood once per week or more than once per week ("good seafood consumer").

Intervention

DHA in the form of algal oil syrup (from Schizochytrium sp.) containing DHA at 10% (strawberry-flavored Richoil® syrup, DMF, Italy) was administered in patients and controls. The product was provided free of charge by the manufacturer. Each participant took 10 ml of the syrup twice per day for 10 consecutive days, corresponding to 2 g of DHA per day. A standard normo-balanced diet was prescribed during the same days, as well as to maintain the usual physical activity level. The participants were supplied with reference telephone number to contact for ensuring compliance and to discuss any difficulties during intervention period.

Blood Sample Collection

Blood samples were collected at baseline (T0), and after DHA supplementation (after 10 days, T1). Whole blood samples were collected on overnight fasting by vein puncture in serum tubes and in ethylenediaminetetraacetic acid (EDTA) tubes, which were kept into ice and centrifuged at 3,000 rpm for 10 min at +4°C. After removing the plasma and, carefully, the buffy coat, the RBC aliquots were stored at −80°C and then analyzed.

RBC Fatty Acid Assay

RBC fatty acids composition was analyzed by gas chromatography—flame ionization detection (GC-FID; GC 6850 Agilent Technologies, Santa Clara, CA, USA), as previously described (Mazzucco et al., 2010), in the Laboratory of the University of Trieste, Italy. Laboratory personnel were unaware of the clinical status of the participants (i.e., BC patients or controls, type of intervention, dietary habits). Specific fatty acids standards were used to identify fatty acid methyl esters (FAME) by retention times in erythrocyte samples. Area-under-the-curve of each selected peak was determined by highly standardized hand integration performed using commercial software (HP Chem station; Agilent Technologies, Santa Clara, CA, USA).

RBC membrane level of each fatty acid was expressed as percent ratio between area-under-the-curve of each selected FAME peak and the sum of all measured FAME peaks.

Omega 3 index was calculated as sum of the DHA + EPA in erythrocyte membranes, indicating a percentage of total erythrocyte fatty acids.

Serum Cytokines Analysis

Cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha levels were measured in duplicate by commercially available ELISA kits (Abcam, Cambridge, U.K.) on the blood samples collected at T0 and T1.

Statistical Analyses

Patient's characteristics were described using mean ± standard deviation (SD) for continuous normally distributed variables, including DHA levels and omega-3 index overall and separately by group, and percent for dichotomous variables. Not-normally distributed variables were described using median (25th, 75th percentiles). Interactions between treatment and participant characteristics (age, BMI, body weight change) were tested to identify inter-individual differences in omega-3 index response to treatment.

One-sample *t*-tests were used to test for overall change from T0 to T1. Pearson's correlation was used to analyze the association between inflammatory markers and DHA and omega-3 index, at baseline and after supplementation. Categorical variables were utilized using proportions by χ^2 test. Multivariable regression analysis, adjusting for patient characteristics (i.e., age, BMI, comorbidity, inflammation, BC presentation, seafood consumption), was performed to predict DHA and omega-3 index changes before and after supplementation. Adjusted $P < 0.05$ was considered significant. All statistical analyses were performed in R v. 3.0.2.

RESULTS

Participant's Characteristics

A total of 45 women were enrolled, including patients and controls. Two subjects withdrew from the study between T0 and T1, because no longer interested in the study (1 patient in M group and 1 healthy control). Forty-three participants, 33 BC patients and 10 healthy women (C group) completed the study. Breast cancer patients were distributed as follows: 10 patients in S group, 12 patients in F group, 11 patients in M group. Baseline characteristics of the participants are shown in **Table 1**. Mean age was 47.3 ± 8.9 years for BC patients and 48.3 ± 5.66 years for group C.

DHA Levels and Omega-3 Index in RBC Membranes at Baseline and after Supplementation and the Role of Inflammation

At baseline, no significant differences were observed in DHA levels and omega-3 index between BC patients and controls, neither between each group of BC patients (S, F, M group).

TABLE 1 | Participants' characteristics.

All participants $N = 43$	BC patients $N = 33$, Mean ± SD*	Controls $N = 10$, Mean ± SD*
Age, years	47.33 ± 8.88	48.3 ± 5.66
Body weight, kg	63.91 ± 11.84	60.6 ± 7.4
BMI, weight (kg)/height^2 (m)	23.93 ± 3.98	23.68 ± 2.93
Glycemia, mg/dl	94.1 ± 9.57	88.72 ± 9.93
Cholesterol, mg/dl	206.8 ± 28.63	217.7 ± 31.77
Comorbidities:		
Diabetes mellitus (yes/no)	2/31	0/10
Hyperlipidemia (yes/no)	7/26	1/10
IL-6, pg/ml	2.12 (0.9, 9.08)	4.04 (0.78, 7.85)
TNF-alpha, pg/ml	26.04 (12.32, 116.37)	15.3 (9.61, 140.15)
Seafood consuming (yes/no)	25/8	8/2

**Median (interquartile range) is shown for non-normally distributed variable (IL-6, TNF-alpha).*
BC, breast cancer; BMI, body mass index; IL, interleukin; TNF, tumor necrosis factor.

All the participants took the DHA oral supplementation. During and after supplementation, the daily doses of oral DHA were well tolerated with good compliance in all the participants.

After supplementation, DHA levels and omega-3 index significantly increased in all groups of BC patients and in the controls ($P < 0.001$) (**Table 2**). No differences in DHA incorporation and omega-3 index were observed between the three groups of BC patients and between patients and controls, except for M group showing higher ability of DHA incorporation when compared to healthy women (C group) ($\beta = 0.30$; $P = 0.02$) (**Table 3**).

At baseline, no differences in TNF-alpha and in IL-6 were documented in the four groups (**Table 1**), and no correlation was found between DHA levels or omega-3 index and cytokines levels at baseline and after supplementation.

Self-Reported Dietary Seafood Consumption, DHA, and Omega-3 Index

According to the self-reported seafood eating habits, 33 participants resulted as "good seafood consumers," 25 BC patients (7 in S, 11 in F, and 7 in M group) and 8 controls. Ten participants, 8 BC patients (3 in S, 1 in F, and 4 in M group) and 2 controls resulted as "low seafood consumers" (**Tables 1**, **4**). At baseline "good seafood consumers" showed higher DHA levels and omega-3 index with respect to "low seafood consumers" (**Table 4**) ($P = 0.01$). After supplementation, we observed a significant increase in DHA levels and omega-3 index in "good seafood consumers" ($P < 0.001$) (**Table 4**), as well as in "low seafood consumers" ($P = 0.002$ and $P = 0.006$, respectively) (**Table 4**). The increase in DHA levels was higher in "low seafood consumers" with respect to "good seafood consumer" ($P < 0.001$) (**Table 3**). No association was found between the type of BC presentation and seafood consumption.

TABLE 2 | DHA levels, EPA levels, and omega-3 index in RBC membranes in the four groups of participants at baseline (T0) and after DHA supplementation (T1).

All participants ($N = 43$)	T0, Mean ± SD	T1, Mean ± SD	P
S GROUP ($N = 10$)			
DHA	6.14 ± 1.21	7.54 ± 0.97	0.002
EPA	0.53 ± 0.23	0.68 ± 0.22	0.014
Omega-3 Index	6.67 ± 1.33	8.21 ± 1.08	0.002
F GROUP ($N = 12$)			
DHA	6.09 ± 1.11	7.35 ± 1.12	0.003
EPA	0.62 ± 0.27	0.77 ± 0.33	0.004
Omega-3 Index	6.71 ± 1.32	8.12 ± 1.35	<0.001
M GROUP ($N = 11$)			
DHA	5.86 ± 1.51	7.4 ± 1.32	<0.001
EPA	0.60 ± 0.34	0.7 ± 0.27	0.018
Omega-3 Index	6.43 ± 1.75	8.1 ± 1.53	<0.001
C GROUP ($N = 10$)			
DHA	6.1 ± 0.88	7.23 ± 0.7	0.006
EPA	0.52 ± 0.11	0.6 ± 0.1	0.004
Omega-3 Index	6.62 ± 0.9	7.0 ± 0.74	0.002

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; RBC, red blood cell; S group, sporadic group; F group, familiar group; M group, mutated group; C group, controls.

TABLE 3 | Multivariate regression models to predict variation of DHA levels in RBC membranes between baseline (T0) and after DHA supplementation (T1).

Clinical characteristics	Beta coefficient (95% CI)	P
TYPE OF BC PRESENTATION		
S group vs. C group	0.12 (−0.21, 0.44)	0.47
F group vs. C group	0.21 (−0.13, 0.55)	0.21
M group vs. C group	0.30 (0.05, 0.55)	0.02
DIETARY SEAFOOD HABITS		
Good vs. Low seafood consumer	−0.49 (−0.68, −0.30)	<0.001

DHA, docosahexaenoic acid; RBC, red blood cell; BC, breast cancer; S group, sporadic group; F group, familiar group; M group, mutated group; C group, controls.

TABLE 4 | DHA levels, EPA levels and omega-3 index in RBC membranes in "good" and "low seafood consumers" at baseline (T0) and after DHA supplementation (T1).

All participants ($N = 43$)	T0, Mean ± SD	T1, Mean ± SD	P
GOOD SEAFOOD CONSUMERS ($N = 33$)			
DHA	6.29 ± 1.10	7.5 ± 1.03	<0.001
EPA	0.61 ± 0.29	0.74 ± 0.26	<0.001
Omega-3 Index	6.9 ± 1.26	8.24 ± 1.19	<0.001
LOW SEAFOOD CONSUMERS ($N = 10$)			
DHA	5.24 ± 1.04	6.97 ± 0.97	0.002
EPA	0.41 ± 0.09	0.52 ± 0.12	0.014
Omega-3 Index	5.64 ± 1.09	7.49 ± 1.05	0.006

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; RBC, red blood cell.

DISCUSSION

Several studies have addressed the therapeutic effects of omega-3 PUFAs in cancer showing that omega-3 PUFAs can improve efficacy and tolerability of chemotherapy (Bougnoux et al., 2009; Nabavi et al., 2015). There are clinical trials where DHA alone or combinations of omega-3 PUFAs are being tested for cancer prevention, support, or therapy (Berquin et al., 2008; Nabavi et al., 2015). DHA as a treatment strategy is often combined with chemotherapeutic drugs since DHA most likely enhances the cytotoxic effects of these drugs (Nabavi et al., 2015).

Our study aimed at verifying the ability of DHA incorporation in RBC membranes of BC patients after oral DHA supplementation compared to heathy women. We found that DHA levels, and omega-3 index had a significant increase after a short period of supplementation (10 days) in BC patients, with no difference related to the type of BC presentation, as well as between patients and controls. Interestingly, we observed that only M group had significantly higher DHA increase with respect to control group. Arterburn et al. (2006) found that DHA supplementation in healthy humans led to a dose-dependent increase in RBC DHA and plasma phospholipid and contents. The data available in the literature are mostly centered on the effect of DHA supplementation during chemo- and radio-therapy in reducing adverse side effects and in improving the outcome of chemotherapy, when highly incorporated (Bougnoux et al., 2009). Also, studies in experimental models showed that, under DHA-supplemented diet, peroxisome proliferator-activated receptors β (PPARβ) is a crucial player capable of regulating different PPAR mRNA expressions, which downregulate BC cell growth and mammary tumor growth (Wannous et al., 2013). The possibility of recognizing in BC patients potential differences in DHA absorption was previously evaluated (Bougnoux et al., 2009). In fact, in a phase II trial, metastatic BC patients were categorized in "low or high incorporator" according to patient's ability to incorporate oral DHA supplementation (Bougnoux et al., 2009). Interestingly, authors documented that DHA "low incorporators" showed a worse outcome in term of reduced response to therapy and increased side effects, such as anemia and thrombopaenia (Bougnoux et al., 2009). In this respect, we were not able to demonstrate in our study a similar behavior in BC patients. In particular, we did not identify one or more groups of patients with this characteristic, possibly because of several factors, including age (Walker et al., 2014), that might have influenced the difference in DHA incorporation described in our study and by Bougnoux et al. (2009).

Studies in BC patients, where DHA was combined with the chemotherapeutic drugs epirubicine, cyclophosphamide, and 5-fluorouracil, emphasize that an interindividual uptake, and incorporation of DHA can alter the treatment response (Bougnoux et al., 2009) and reduce BC cell proliferation (Corsetto et al., 2012). Patients were supplemented with DHA daily during the chemotherapy cycles and were then divided into high and low incorporating groups based on the DHA levels in plasma and RBCs. The high incorporating group was characterized by delayed time to tumor progression and longer overall survival compared to the low incorporating group (Bougnoux et al., 2009). This observation is in line with other studies showing that DHA incorporation differs between individuals due to dissimilar rates of metabolism, enzymatic activity, background diet, age, and gender (Arterburn et al., 2006; Rusca et al., 2009).

The clinical setting in which the patients were previously treated for a longer duration of time is different from ours. In fact, BC patients were supplemented during the entire duration of chemotherapy (Bougnoux et al., 2009). Our patients were not on chemotherapy and were orally supplemented with DHA to assess the capacity of incorporation and not to observe effect associated with anticancer treatments.

Moreover, when we considered in all the participants the dietary seafood habits, which were considered a reliable instrument to assess omega-3 fatty acids intake (Dahl et al., 2011), we found that "good seafood consumers" had higher DHA levels at baseline with respect to "low seafood consumers," confirming the reliability of our questionnaire.

After supplementation "low seafood consumers" showed higher DHA increase with respect to "good seafood consumers." One possible explanation is represented by the fact that "good seafood consumers" at baseline presented with higher DHA levels, and possibly, reaching the maximum rate of absorption of DHA. In this light, our data are in accordance with those obtained in the study by Bougnoux et al. (2009), where the highest DHA levels described in "high incorporators" after supplementation reached values comparable to the ones obtained in our study.

Since DHA is a highly unsaturated PUFA, it is susceptible to peroxidation and can cause accumulation of a surplus of reactive oxygen species that cannot be scavenged by the cancer cells. Addition of anti-oxidants to cells incubated with DHA diminishes the toxic effects, strengthening this theory (Lindskog et al., 2006; Gleissman et al., 2010). In our study we did not assess this specific effect.

Several metabolic derangements are described in BC patients, mostly represented by insulin-resistance and alterations in lipid metabolism (Amadou et al., 2013; Molfino et al., 2016). However, in our cohort of BC patients we did not observe a high prevalence of diabetes (only 2 patients), and this might have probably reduced the possibility to observe effect of DHA supplementation in this clinical setting.

In the recent years, the composition of the cell membrane fatty acids has been investigated, not only as a factor influencing the response to treatments, but also as an element influencing BC prognosis independent of the treatment received (Bougnoux et al., 2009, 2010; Straka et al., 2015). Therefore, the identification of different cell membrane composition at baseline, and a possible variation in DHA incorporation among the different type of BC presentation, could be useful in clinical setting. In this light, our data reveal differences only between BRCA mutation carriers (M group) and controls. In fact, although patients of M group did not show differences at baseline in terms of DHA levels and omega-3 index with respect to the other groups, they showed greater and significant increase of DHA levels and omega-3 index after supplementation when compared to healthy women. We are not able to describe a mechanism underlying this behavior and

we cannot exclude the possibility that reduced basal DHA level in BRCA mutation carriers might be at least in part determined by low dietary DHA intake and/or by impaired absorption of food-derived DHA amount.

BRCA is the major tumor suppressor gene associated with hereditary predisposition to BC, and the risk of BC is known to be increased by a lack of BRCA1/2 protein function (Shiovitz and Korde, 2015). Interestingly enough, an experimental study found that DHA supplementation significantly reduced the incidence of BC and led to 60% increase in BRCA1 protein level with respect to the control group (not supplemented with DHA), indicating that BRCA1 up-regulation mediated by DHA might be protective against the risk to develop BC (Jourdan et al., 2007). Moreover, Bernard-Gallon et al. observed an increase of BRCA1 and BRCA2 mRNA expressions in DHA-treated BC cell lines, suggesting the presence of a possible transcriptional or post-transcriptional regulation of BRCA1 and BRCA2 genes after omega-3 PUFAs treatment in BC cells (Bernard-Gallon et al., 2002).

Linking BRCA to key nutritional factors, such as omega-3 PUFAs, involved in the incidence rate of BC (Brasky et al., 2010; Molfino et al., 2016), opens wide perspectives for nutritional prevention in BC and in possibly modulating inflammatory status. Recently, Roy et al. documented positive associations between erythrocyte and breast tissue omega-3 fatty acids, and suggestive inverse associations between erythrocyte long chain omega-3 PUFAs and tissue C-reactive protein (CRP) (Roy et al., 2015).

Although inflammation is a common condition in BC, our data fail to reveal increased circulating levels of pro-inflammatory cytokines in BC patients and no modification of IL-6 and TNF-alpha were obtained after DHA supplementation. This is in line with recent data available in the literature, demonstrating that inflammation is more common in obese BC patients (Gershuni et al., 2017), although we did not asses high-sensitivity CRP, which might give additional important information. In fact, our cohort showed a normal average BMI and it did not differ between BC patients and controls. Moreover, a recent randomized trial, conducted in healthy young adults daily supplemented with oral EPA + DHA for 5 months, showed a marginal decrease in serum TNF-alpha and no change in IL-6 levels (Flock et al., 2014).

Although our study involved a homogeneous population of BC patients, enrolled in a single cancer unit, all receiving the same DHA supplementation and the intervention being conducted only by one medical team, it presents several limitations. Our cohort of BC patients is small and may not be representative of larger BC patients' population. The small sample size of the groups enrolled might have limited the possibility of identifying association between patient's characteristics, in particular inflammatory status, and basal DHA and omega-3 index and DHA absorption overtime (during and after supplementation). Additionally, the dietary assessment tool utilized to assess seafood intakes, as an estimate of omega-3 PUFAs intake, might not be accurate, because it does not include questions on omega-3 PUFAs intakes from other dietary sources.

In conclusion, our study serves as the basis for development of larger trial to clarify the clinical impact of different DHA levels and omega-3 index in BC and, in particular, if DHA supplementation, performed for a short, as we did, or longer duration of time, has an impact on robust outcomes and if different subgroups of patients (i.e., BRCA mutation carriers) present a different and clinically relevant behavior and to potentially develop novel therapeutic strategies.

ETHICS STATEMENT

The local Ethics Committee (Policlinico Umberto I, Sapienza University of Rome, Italy) approved the study. Consent procedure for human research was obtained. All procedures were in accordance with the ethical standards of the Helsinki Declaration issued in 1975 and later amendments.

AUTHOR CONTRIBUTIONS

AM designed research, conducted research, analyzed data, wrote the paper. MA conducted research, analyzed data, wrote the paper. SM and CR performed laboratory dosages. GB provided essential reagents necessary for research and analyzed and interpreted laboratory data. AF performed statistical analysis. SA interpreted laboratory data. MMo reviewed the paper. MMu designed research, reviewed the paper, and had primary responsibility for final content. All authors read and approved the final manuscript.

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Validation of the CAchexia SCOre (CASCO). Staging Cancer Patients: The Use of miniCASCO as a Simplified Tool

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The CAchexia SCOre (CASCO) was described as a tool for the staging of cachectic cancer patients. The aim of this study is to show the metric properties of CASCO in order to classify cachectic cancer patients into three different groups, which are associated with a numerical scoring. The final aim was to clinically validate CASCO for its use in the classification of cachectic cancer patients in clinical practice. We carried out a case -control study that enrolled prospectively 186 cancer patients and 95 age-matched controls. The score includes five components: (1) body weight loss and composition, (2) inflammation/metabolic disturbances/immunosuppression, (3) physical performance, (4) anorexia, and (5) quality of life. The present study provides clinical validation for the use of the score. In order to show the metric properties of CASCO, three different groups of cachectic cancer patients were established according to the results obtained with the statistical approach used: mild cachexia ($15 \leq \times \leq 28$), moderate cachexia ($29 \leq \times \leq 46$), and severe cachexia ($47 \leq \times \leq 100$). In addition, a simplified version of CASCO, MiniCASCO (MCASCO), was also presented and it contributes as a valid and easy-to-use tool for cachexia staging. Significant statistically correlations were found between CASCO and other validated indexes such as Eastern Cooperative Oncology Group (ECOG) and the subjective diagnosis of cachexia by specialized oncologists. A very significant estimated correlation between CASCO and MCASCO was found that suggests that MCASCO might constitute an easy and valid tool for the staging of the cachectic cancer patients. CASCO and MCASCO provide a new tool for the quantitative staging of cachectic cancer patients with a clear advantage over previous classifications.

Keywords: cachexia, wasting, anorexia, weight loss, physical performance, quality of life, classification, score

INTRODUCTION

Cancer cachexia is a syndrome present in a large number of cancer patients that results in body weight loss, inflammation, reduced physical performance, and decreased quality of life (Evans et al., 2008; Muscaritoli et al., 2010; Fearon et al., 2011; Cederholm et al., 2016). For instance, according to Evans et al: "cachexia, is a complex metabolic syndrome associated with underlying illness and

characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance, and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption, and hyperthyroidism and is associated with increased morbidity" and it can be classified using the following criteria: (a) if the patient has a 5% loss of edema-free body weight during the previous 12 months or less and (b) the presence of at least three of following five characteristics: decreased muscle strength, fatigue, anorexia, low fat-free mass index, or abnormal biochemistry (Evans et al., 2008). Although several definitions exist, they share common features (Argilés et al., 2010). In spite of the fact that, in addition to definition, diagnostic criteria have been established (Evans et al., 2008), only few studies deal with cachexia staging and classification of patients (Bozzetti and Mariani, 2009; Gabison et al., 2010). From this point of view, Fearon et al. (2011) have established a classification of the syndrome based on inflammation and body weight loss. Indeed, according to this study: "Severity can be classified according to the degree of depletion of energy stores and body protein (lean body mass) in combination with the degree of on going weight loss. Assessment for classification and clinical management should include the following domains: anorexia or reduced food intake, catabolic drive, muscle mass, and strength, functional, and psychosocial impairment." However, this study only allows a qualitative classification of the different cachectic patients, such as precachexia, cachexia, and refractory cachexia. A couple of recent papers also proposed a grading system: (1) incorporating the independent prognostic significance of both BMI and percentage of weight loss (Martin et al., 2015) or (2) according to changes on biochemistry (high C-reactive protein or leukocytes, or hypoalbuminemia, or anemia), food intake, weight loss, and performance status (Vigano et al., 2016). CASCO was designed to fulfill the gap of a numerical classification system and therefore enable the proper quantitative staging of cachectic cancer patients.

CASCO is mainly based on the following constituents: (1) body weight loss and composition, (2) inflammation/metabolic disturbances/immunosuppression, (3) physical performance, (4) anorexia, and (5) quality of life (Argilés et al., 2011). **Table 1** pictures components and measured parameters in more detail.

Body weight loss and composition (BWC) is essential to all definitions of cachexia. But, the fact that both loss of muscle and fat tissue coexist in the cachectic patient, stresses the importance of assessing any changes in relation to lean body mass. The second component of CASCO is inflammation/metabolic disturbances/immunosuppression (IMD). Inflammation is a key feature of the cachectic response (Fearon et al., 1999; Delano and Moldawer, 2006). It cannot be overlooked that there are also a number of metabolic disturbances present in many cachectic patients such as: glucose intolerance, anemia, and low levels of plasma albumin, most of them included in CASCO (see **Table 1**). Immunosuppression may also be an early marker of cachexia (Faber et al., 2009); therefore, assessment of the immune response could also be a valid indication for a cachexia staging system. The third component relates to physical performance (PHP). Even with a relative small decrease in muscle mass in cachexia, there may be a significant decrease in physical activity which are related to muscle performance (Fouladiun et al., 2007; Maddocks et al., 2010). Anorexia (ANO) constitutes the fourth parameter included in CASCO. Indeed, anorexia is present in cachexia in many diseases (Laviano et al., 2005). A decrease in food intake, by itself, promotes changes in quality of life and also conditions many metabolic alterations. Finally, the last component of CASCO is quality of life (QoL). Quality of life reflects not just changes in weight and physical performance but also in metabolic alterations (Fouladiun et al., 2007; Granda-Cameron et al., 2010). Therefore, it is an important point to consider.

These five different factors mentioned above, clearly interact between each other and represent the most important set of variables to assess the severity of the cachectic syndrome.

Bearing all this in mind, the aim of the present investigation was to clinically validate CASCO for its use in the classification of cachectic cancer patients. In addition, a simplified form of CASCO (miniCASCO) was designed to cope with the limitation of assessment methods and tools in some clinical centers.

TABLE 1 | Components of CASCO.

Component	%	Measurement	Parameter
Body weight loss and composition (BWC)	40	Body weight loss	
		Lean body mass	
Inflammation/metabolic disturbances/ immunosupression (IMD)	20	Inflammation	Plasma CRP
			Plasma IL-6
		Metabolic disturbances	Plasma albumin
			Plasma pre-albumin
			Plasma lactate
			Plasma triglycerides
			Plasma urea
			Anaemia
			ROS plasma levels
			Glucose tolerance test/HOMA index altered
		Immunosupression	Absolute lymphocyte number
Physical performance (PHP)	15		Questionnaire of 5 questions related to physical activity.
Anorexia (ANO)	15		Questionnaire of 4 questions extracted from SNAQ of St. Louis VA Medical Centre.
Quality of life (QoL)	10		Questionnaire of 25 questions from QLQ-C30.

PATIENTS AND METHODS

Patients

An observational prospective case-control study has been performed and a total of 186 carcinoma patients and 95 age-matched control subjects were included (see Participant flow chart in **Figure 1**). All the participants in the study were recruited at the Department of Medical Oncology (University of Cagliari, Cagliari, Italy) from June 2011 to September 2014. Inclusion criteria for the cancer patients were histologically confirmed cancer at any site, age≥18 years old, and the absence of diagnosed mental disease or severe cognitive deterioration. Inclusion criteria for the control subjects were absence of neoplasia, to be over 18 years old and absence of diagnosed mental disease or cognitive deterioration. Those patients affected by either non cancer-related nutritional alterations or inflammatory states leading to body weight loss were excluded from the study. All the enrolled patients and controls were evaluated for all parameters with the same methodology descripted below, reaching the CASCO score for all subjects. The clinical protocol was fully approved by the Ethics Committee of the University of Cagliari (Cagliari, Italy; control and patient subjects) and by Ethics Committee of the University of Barcelona (control subjects), and all patients and controls signed the approved written informed consent. Subject characteristics are presented in **Tables 2, 3**. Data were extracted, and the quality of the included studies was evaluated using the STROBE checklist.

CASCO

The CAchexia SCOre was applied and calculated for each of the subjects as previously described (Argilés et al., 2011). The different elements of the score are shown in **Table 1**. More information related to the questionnaires and related calculations can be found in: http://hdl.handle.net/2445/65137 and http://www.ub.edu/cancerresearchgroup/.

MCASCO

The simplified version of CASCO, miniCASCO (MCASCO), was applied and calculated for each of the subjects. The components of the MCASCO are shown in **Table 5**.

Body Weight Loss and Composition (BWC)

Body weight was measured at questionnaire time using an electronic balance (Health-o-Meter, Bridgeview, IL, USA), pre-illness weights being obtained by interview or by patient's data collection in clinical practices. Lean body mass (LBM) was assessed through different methods based on the planned instrumental oncological assessments. These included: (i) conventional bioelectrical impedance analysis (BIA) (Simons et al., 1995; Ellis, 2000) using a Bioelectric Impedance Analyser STA/BIA101 (Akern, Firenze, Italy) in 70% of the patients;

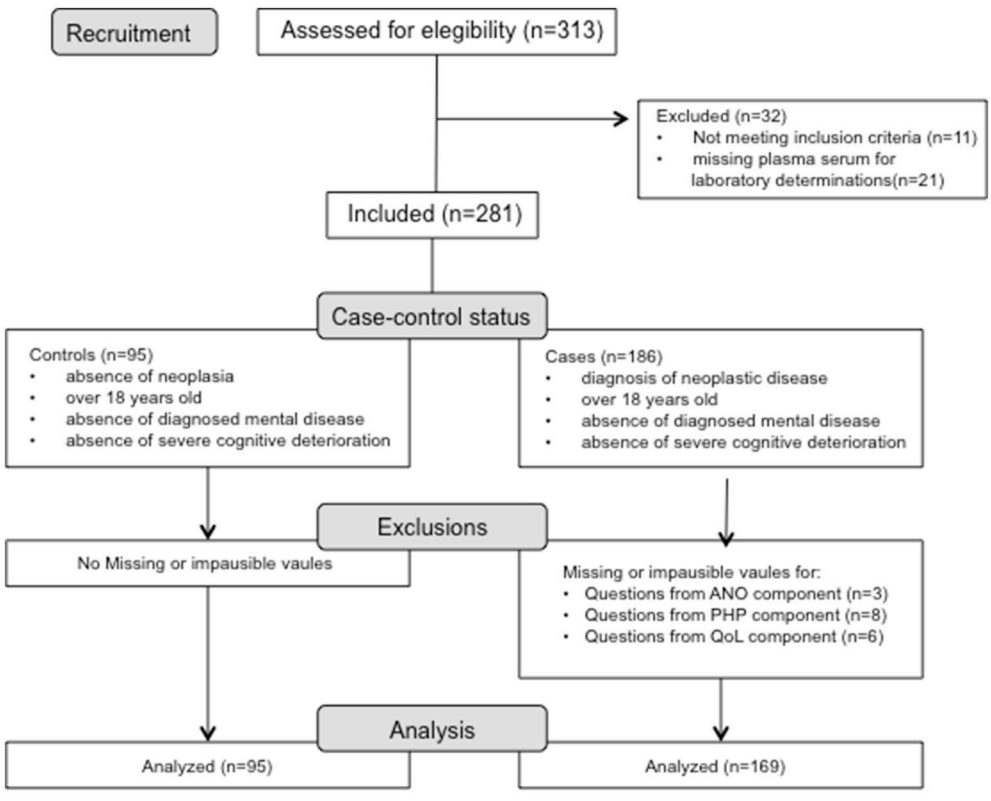

FIGURE 1 | Flow diagram of excluded participants in the CASCO Study with missing or implausible data.

TABLE 2 | Descriptive statistics for each group.

Group	Gender (%)	Age	Weight	Diagnosis	Ecog Ps	Stage
Controls ($n = 95$)	males = 55%	56.34 ± 0.677 (95*)	76.73 ± 1.58 (93*)	Healthy subjects ($n = 75$)	0 (100%)	NV
	females = 45%			Patients suffering from non-neoplastic diseases[#] ($n = 20$)	0 (100%)	NV
Oncologic patients ($n = 186$)	males = 59%	65.27 ± 0.877 (186*)	74.00 ± 1.92 (186*)	Carcinoma ($n = 178$)	0–1 (38%) 2–3 (62%)	I –IIIA 20% IIIB– IV 80%
	females = 41%			Mesothelioma, and sarcoma ($n = 8$)		

*Results are mean ± S.D and n (the number of patients and controls in parentheses, * indicates the number depending of missing data).[#] asthma, hypertension, allergic rhinitis, muscle pain, high cholesterol levels.*

TABLE 3 | Carcinoma site diagnosed in cancer group.

Tumor site	n	Age
Lung	32	70.81 (2.75)
Breast	27	59.78 (2.67)
Head and neck	21	63.19 (2.36)
Colon	17	69.16 (2.16)
Ovary	13	55 (3.79)
Pancreas	11	64.72 (3.87)
Prostate	10	76.3 (1.38)
Upper gastrointestinal	10	63 (3.29)
Rectum	8	68.71 (1.01)
Bile glands	7	69 (5.68)
Endometrium	4	64.5 (4.03)
Liver	3	64.66 (14.30)
Kidney	3	65.33 (5.66)
Other*	17	69.14 (2.49)

*Results are mean (S.D) and n the number of cancer patients. *Other carcinoma sites: peritoneum, cervix, appendix, bladder; and other tumor types: lung sarcoma, pleura sarcoma, myelofibrosis, pleural mesothelioma and lung heteroplasia.*

(ii) dual-energy X-ray absorptiometry (DXA) (Plank, 2005; Ellegård et al., 2009) using a Hologic Delphi W scanner (Hologic Inc., Bedford, MA) in 10% of the patients; (iii) regional computed tomography (CT) scan analysis at L2-L3, currently considered the highest precision method, trough measurement by Tomovision Slice-o-Matic Software (Montreal, Canada) in 20% of the patients. It has to be pointed out that, although different approaches for the determination of body composition were used, good correlation have described between the different methodologies used (Fürstenberg and Davenport, 2011). LBM depletion was defined at baseline accordingly to standard international range for each method.

Inflammation/Metabolic Disturbances/Immunosuppression (IMD)

Inflammation

Peripheral venous blood samples were obtained from all patients and controls by venipuncture (BD Vacutainer, California, USA). The serum levels of CRP were measured by automatic centralized nephelometric analyser (AU640, Olympus, Germany), the results were expressed in mg/L. IL-6 were assessed by ELISA "sandwich" test, using monoclonal antibodies against specific molecular epitopes (DRG International, Springfield, NJ, USA by IAM Consulting, Parma, Italy). The assays were performed in semiautomatic ELISA analyser (DiaSorin Etilab, Guidonia, Italy) and the results expressed in pg/mL. The coefficients of variation for all these methods, in accordance with the quality control procedures, were always <5%.

Metabolic Disturbances

Metabolic disturbances included the determination of albumin, pre-albumin, lactate, triglycerides, hemoglobin, urea, reactive oxygen species (ROS), and HOMA index. Albumin, pre-albumin, lactate, triglycerides, and urea levels were obtained during oncological clinical routine by hospital central laboratory (METROLAB 2300 (Wiener Lab) and the results expressed in mg/dL for pre-albumin, triglycerides, and urea and g/dL for albumin. HOMA index was calculated for each patient (Matthews et al., 1985). Determination of plasma levels of ROS were assessed by reactive oxygen species colorimetric assay (FORT test, Callegari SpA, Italy; Mantovani et al., 2003; Pavlatou et al., 2009). Haemoglobin levels were obtained carried out the routine blood count (Coulter LH750, Beckman-Coulter) and expressed in g/dL.

Immunosuppression

The immunosuppression was evaluated for each patient by absolute lymphocyte count, obtained from de routine blood count. Lymphocyte count has been well recognized as a valid marker of immune function as well as a prognostic marker (Bouwhuis et al., 2011). It is included in validated nutritional tools (such as the Mini Nutritional Assessment; Kabata et al., 2015; Bourdel-Marchasson et al., 2016). It has to be pointed out that the total lymphocyte count is not a fully convincing measure of immunosupression, although it is an "affordable" easy reliable measurement in a standard hospital. This, therefore, represent a minor limitation of the score particularly since it only represents 4% of CASCO.

Physical Performance (PHP)

In order to be able to assess the functional state of a cachectic patient, a physical performance questionnaire was used at the evaluation time. One question from EORTC QLQ-C30 (question

number 1) was included. Its use is under permission of 1995 EORTC Quality of Life Group (Aaronson et al., 1993). The text of the questionnaire is: *During the past week:* ***1.*** *Have you noticed any particular decrease in the physical activities (i.e., at work, at home, at leisure etc...) that you normally carry out during the day?;* ***2****. Have you had any problems doing strenuous activities, like carrying a heavy shopping bag or a suitcase?;* ***3.*** *Have you noticed any loss of handgrip force?;* ***4****. Did you have to put more effort on climbing stairs?;* ***5****. Have you felt tired after walking approximately half a kilometer?*

Anorexia (ANO)

Anorexia was estimated using a standard questionnaire [Simplified Nutrition Assessment Questionnaire (SNAQ)] (Wilson et al., 2005). Its use in CASCO is under permission of St. Louis GRECC Program of St. Louis VA Medical Centre. The text of the questionnaire is: *1. My appetite is (very poor, poor, average, good, very good); 2. When I eat (I feel full after eating only a few mouthfuls, I feel full after eating about a third of a meal, I feel full after eating over half a meal, I feel full after eating most of the meal, I hardly ever feel full); 3. Food tastes (very bad, bad, average, good, very good); 4. Normally I eat (less than one meal a day, one meal a day, two meals a day, three meals a day, more than three meals a day).*

Quality of Life (QoL)

Concerning quality of life, CASCO includes 25 questions from EORTC QLQ-C30 (question numbers: 4–12, 14–17, 19–30). Questions related to physical performance or food intake were withdrawn. Its use in CASCO is under permission of 1995 EORTC Quality of Life Group (Aaronson et al., 1993). The text of the questionnaire is: *During the past week: 1. Do you need to stay in bed or a chair during the day?; 2. Do you need help with eating, dressing, washing yourself or using the toilet?; 3. Were you limited in doing either your work or other daily activities?; 4. Were you limited in pursuing your hobbies or other leisure time activities?; 5. Were you short of breath?; 6. Have you had pain?; 7. Did you need to rest?; 8. Have you had trouble sleeping?; 9. Have you felt weak?; 10. Have you felt nauseated?; 11. Have you vomited?; 12. Have you been constipated?; 13. Have you had diarrhea?; 14. Did pain interfere with your daily activities?; 15. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?; 16. Did you feel tense?; 17. Did you worry?; 18. Did you feel irritable?; 19. Did you feel depressed?; 20. Have you had difficulty remembering things?; 21. Has your physical condition or medical treatment interfered with your family life?; 22. Has your physical condition or medical treatment interfered with your social activities?; 23. Has your physical condition or medical treatment caused you financial difficulties?; 24. How would you rate your overall health during the past week?; 25. How would you rate your overall quality of life during the past week?*

Statistical Analysis

The *Statistical Package for Social Sciences* (IBM v.21) was used to analyse the effects between groups. From a psychometric perspective, reliability, as an internal consistency parameter, was estimated using the Cronbach's α. In addition, Confirmatory Factorial Analysis (CFA) was conducted to estimate construct validity through EQS software (v6.0) and normative data were obtained from a classical point of view with position indexes. All the statistical techniques were carried out with a significance level of $\alpha = 0.05$, correcting for reduction of type I error using the Bonferroni correction. Finally, cluster analysis between groups was performed to determine the breakpoints within the scale and estimate the maximum inertia centroids values. In addition, each statistical contrast includes the specification of sample size due to missing data presence.

RESULTS

Analysis of Reliability

The reliability coefficients were estimated using Cronbach's α for each of the general factors derived from the questionnaire (**Table 4**). The values obtained indicate high reliability for each of the factors studied

Construct Validity

To estimate the validity of the construct, a model involving Confirmatory Factor Analysis (CFA) from the Correlation Matrix of Pearson was used. The value of the Maximum Likelihood Estimation (ML), applied to the matrix R, provided initial results in an adjusted model ($\chi^2 = 675.11$; $df = 253$, $p < 0.001$; Comparative Fit Index (CFI) = 0.912; Tucker Lewis Index (TLI) = 0.941; Root Mean Standard Error (RMSEA) = 0.04; Adjusted and Standardized Root Mean Residuals (SRMR) = 0.039; 95% Confidence Interval of SRMR 0.02–0.05; Ratio $\chi^2/df = 3.277$). All indicators, except for the statistical χ^2, are compatible with a valid model, particularly the criterion derived from χ^2/df. Additionally, it should be remarked that each of the latent factors assumes a significant proportion of the initial variance. Thus, PHP took 15% while ANO 18% and QoL 45%, the three representing 78% of the initial value, which is regarded as a high level of variance accounted for by the reduction of dimension.

Discriminant Validity

Using the CASCO score the two groups (patients and controls) were compared in order to estimate the discriminant validity of the score. The results depicted in **Figure 2** show a very significant difference between groups ($t = 145.77$, $df = 273$, $p < 0.001$; $r = 0.74$; Confidence interval of mean difference at 95% between 14.01 and 29.86), indicating a high capacity in the discriminative ability of the total score. Thus, in the cancer group, the CASCO

TABLE 4 | Reliability estimation through Conbrach's α.

Variable	n*	Cronbach's α
Physical performance	276	0.928
Anorexia	279	0.793
Quality of life	275	0.945

*n, the number of patients and controls, * indicates the number depending of missing data.*

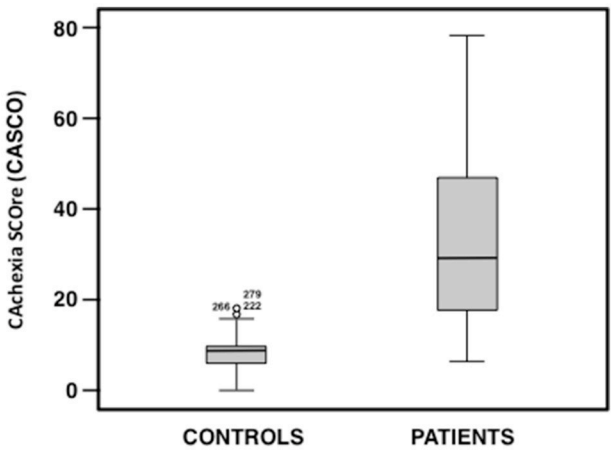

FIGURE 2 | Box plot of CASCO total score for each group (patient and control groups).

value was 32.54 with a standard deviation of 17.58, while in the control group it was 8.72 with a standard deviation of 3.56.

Concurrent Validity

The correlation between the total CASCO values and those obtained using a subjective diagnosis of specialized oncologists (the Oncologist Team from the Department of Medical Oncology, University of Cagliari, Cagliari, Italy) was established. The subjective evaluation was based on the following question: "*Before applying CASCO, what is your perception of severity of patient's cachexia according to the following scale 0 (normal, absence of cachexia), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (terminal, evident cachexia).*" **Figure 3A** shows the scatter plot between the two variables, characterized by estimating the Spearman correlation coefficient ($r_s = 0.412$, $p < 0.001$). The results indicate a clear positive relationship between the two variables and therefore, a high level of concurrent validity. Moreover, other external criteria were used: the Eastern Cooperative Oncology Group (ECOG) scale, which is a widely used score involved in assessing cancer patients. **Figure 3B** shows the scatter plot between the ECOG and CASCO scores. The Spearman correlation coefficient was: $r_s = 0.290$, $p < 0.001$, indicating a clear positive relationship between the two variables a moderate level of concurrent validity.

Estimated Classification

Using the CASCO values, three cut-off values were estimated by means of the application of a hierarchical cluster. Four groups were originally described, one exactly below the observed mean, and the other exactly over the mean; and the two last zones adjusted to every cue (inferior and superior). The three cut-off values were estimated through the maximization of the classification function using 95% confidence levels. This was accomplished by using a similarity matrix according to the metric properties of the variables and assuming multinormal distribution. The results show that the four groups were: no cachexia (≤14), mild cachexia (15–28), moderate cachexia (29–46) and finally, severe cachexia (>46). **Figure 4** shows the distribution observed for each of the groups derived from the above criterion, indicating that the differences between groups

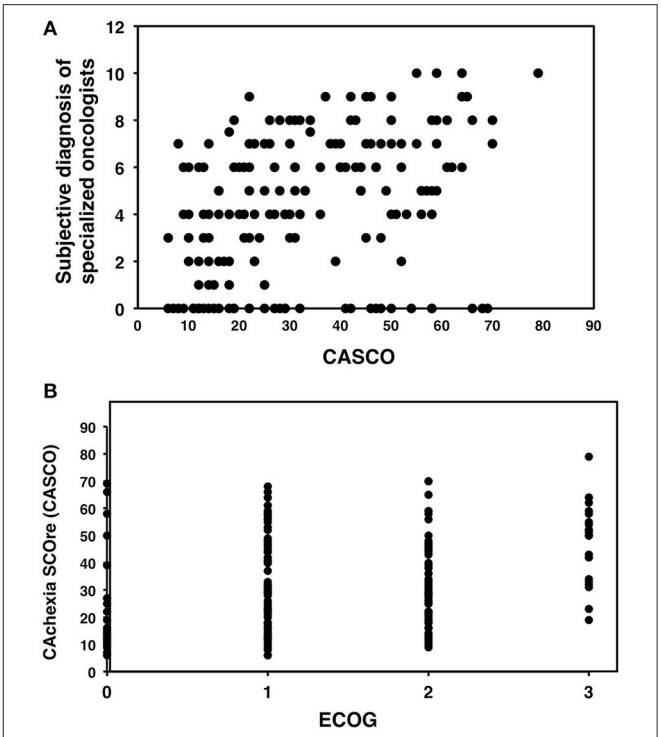

FIGURE 3 | Scatter plots of CASCO against other values of clinical assessment. (A) Correlation between CASCO and the subjective diagnosis of specialized oncologists. **(B)** Correlation between CASCO and the Eastern Cooperative Oncology Group (ECOG) scale.

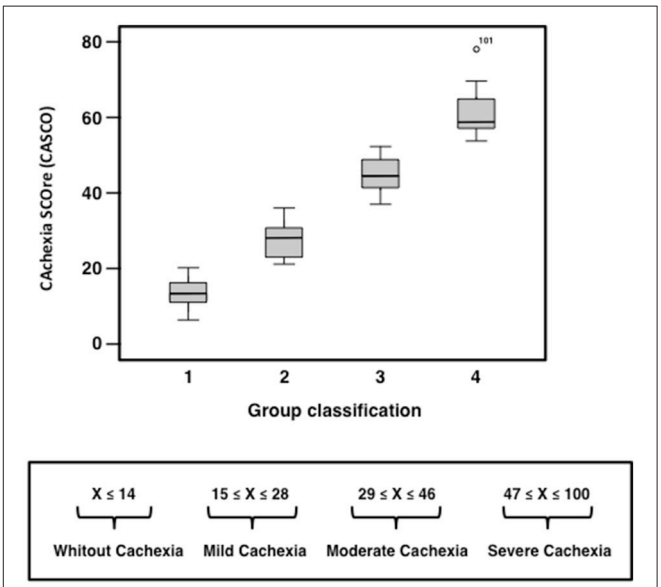

FIGURE 4 | CASCO distribution for each of the groups set for the diagnosis of the severity of cachexia.

were highly significant ($F = 743.12$, $df = 3, 244$; $p < 0.001$; $\varepsilon = 0.61$).

MiniCASCO

A simplified version of CASCO, miniCASCO (MCASCO), was designed to avoid an excessive amount of clinical measurements which in some medical centers may be limiting. The components of the MCASCO are shown in **Table 5**. The correlation between the two values, i.e., the original CASCO and the reduced version MCASCO showed a highly significant coefficient ($r = 0.964$; $df = 19.50$; $p < 0.001$; **Figure 5**). This result ensures that the psychometric properties of CASCO are also present in the MCASCO test, therefore suggesting a feasible and quick assessment of the cachexia stage.

DISCUSSION

With the aim of validating the previously published CAchexia SCOre (CASCO) (Argilés et al., 2011), 186 cancer patients (males and females in a similar percentage) were recruited in this study (**Table 2**). As a reminder, one has to take into consideration that CASCO includes a combination of the following components: (1) body weight loss and composition, (2) inflammation/metabolic disturbances/immunosuppression, (3) physical performance, (4) anorexia, and (5) quality of life. CASCO was only slightly modified from the original published version (Argilés et al., 2011); thus absolute lymphocyte number was taken as a measure to evaluate immunosuppression (**Table 1**).

The study includes a heterogeneous cancer patient population. The most abundant type of cancer was lung carcinoma while kidney and liver cancer and other carcinoma sites included the smaller number of patients (see **Table 3** for more information). Control subjects were either healthy ($n = 75$) or suffering from non-neoplastic diseases (asthma, hypertension, allergic rhinitis, muscle pain, high cholesterol levels; **Table 2**).

Interestingly, other cachexia classification studies also agree with the results obtained here (Fearon et al., 2011; Vigano et al., 2016). Indeed, Fearon et al. proposed a classification of cachectic patients based on identifying the following stages: precachexia, cachexia, and refractory cachexia (Fearon et al., 2011). The additional advantage of the classification proposed in the present study is that it involves a numerical scale and therefore can discriminate between patients in any of the three cachectic groups. Then, the study basically classifies patients into the following three groups that are associated with a numerical scoring: mild cachexia, moderate cachexia and severe cachexia. Conversely, other cachexia classifications do not provide any discrimination between patients in the same subgroup.

Although the obtained data seem to follow a logical pattern as compared with previous studies, we undertook a more rigorous validation based on correlating CASCO with other scores. From this point of view, we chose the ECOG (Oken et al., 1982) scale, which is a widely used score involved in assessing cancer patients. Although ECOG is not an specific scale fo cachexia, it has to be pointed out that it is widely used in cancer patients and also that there is no other quantitative cachexia scale at present to establish an alternative validation. Additionally, a subjective diagnosis of specialized oncologists was used (the oncologist team from the Department of Medical Oncology, University of Cagliari,

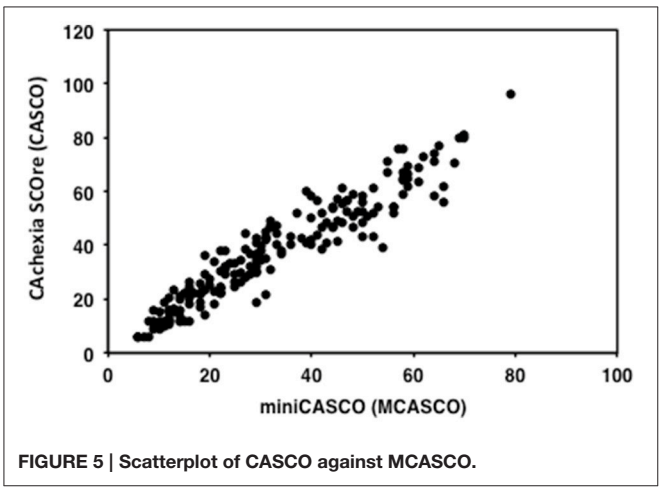

FIGURE 5 | Scatterplot of CASCO against MCASCO.

TABLE 5 | MiniCASCO.

Weight	Body weight loss	
	Lean body mass	
Blood parameters	Plasma albumin	Metabolic disturbances
	Anaemia	
	CRP	Inflammation
	Absolute lymphocyte number	Immunosuppression
Questionnaires	Did you have to put more effort on climbing stairs?	Physical performance
	Have you felt tired after walking approximately half a kilometer?	
	My appetite is...	Anorexia
	When I eat...	
	Do you need to stay in bed or a chair during the day?	Quality of life
	Where you limited in doing either your work or other daily activities?	
	Were you limited in pursuing your hobbies or other leisure time activities?	
	Have you had pain?	
	Did you need to rest?	
	Did you feel weak?	
	Did pain interfere with your daily activities?	
	Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	
	Has your physical condition or medical treatment interfered with your family life?	
	How would you rate your overall health during the past week?	

Cagliari, Italy) and adequate statistically significant correlations between CASCO and the two scales were observed for both (only considering the patient values; **Figures 3A,B**).

In spite of the fact that this study clearly demonstrates that CASCO is a valid score in the clinical context, it can be argued that the large number of items (both measurements and questionnaires) could be a serious obstacle for its routinely use. Bearing this in mind, we developed a simplified version, the so-called MiniCASCO (MCASCO), containing only a reduced number of items (see **Table 5**). To reduce the number of items a component analysis was performed. The reduction of items was done based on factorial loadings of the items in the component and the discrimination index. This process was done for each component (PHP, ANO, and QoL). In addition to body weight loss and composition, blood measurements include only albumin, anemia, CRP, and absolute lymphocyte number (**Table 5**); together with a questionnaire containing two questions related with PHP, two related with ANO and 11 related with QoL (**Table 5**). It can be seen that there is an excellent correlation between CASCO and MCASCO ($r = 0.964$; **Figure 5**).

Altogether, the information presented here, first, serves to clinically validate CASCO for the staging of cachectic cancer patients and second, it provides a significantly plausible tool (MCASCO) to perform the staging in almost any clinical setting, since the majority of hospitals and clinics have access to the determination of the parameters included in MCASCO. It has to be pointed out that CASCO and MCASCO provide a new tool for the quantitative staging of cachectic cancer patients with a clear advantage over previous classifications (Fearon et al., 2011; Martin et al., 2015; Vigano et al., 2016).

AUTHOR CONTRIBUTIONS

Each author has participated suficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and conduction of the experiment and for data interpretation (authorship). SB, AB, and RS carried out the studies, sample analysis, data analyses, performed the statistical analysis and helped to draft the manuscript. SB, RS, JA, CM participated in the design and coordination of the study, carried out the studies, and helped to draft the manuscript. JG, MP, FL helped to carry out the studies. JA, SB, and RS conceived the study, participated in the design, coordination of the study, drafted the manuscript, and revised it critically.

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