Consequences of late-stage non-small cell lung cancer cachexia on muscle metabolic processes.

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Conflict of Interest

Funding was received from The University of Nottingham to help support the analytical costs associated with the study. Dr Maddocks is funded by a National Institute of Health Research (NIHR) Post-Doctoral Fellowship. The associated funding bodies did not have any involvement in the design or execution of the study, nor in the decision to publish. The authors confirm that they have no conflict of interest.

Micro Abstract

The loss of muscle is a common consequence of advanced lung cancer and impairs an individual's quality of life while often shortening their time until eventual death. Animal models of cancer have highlighted potential mechanisms that could explain this debilitating loss of muscle mass, but the impact of cancer on muscle metabolism in lung cancer patients has been less well explored, particularly in advanced stages of the disease. To address this, we surveyed in muscle biopsies obtained from late-stage non-small cell lung cancer patients the impact of the disease on a number of key metabolic processes; observing changes consistent with an impairment of muscle to manufacture new muscle proteins and a reduction in the amount of lipid stored in muscle cells. Collectively, it highlights the need to understand the potential contribution of impaired fat metabolism and muscle protein synthesis in the development of lung cancer-induced muscle loss.

Abstract

Introduction: Loss of muscle is common in patients with advanced non-small cell lung cancer (NSCLC), and contributes to the high morbidity and mortality of this group. The exact mechanisms behind the loss of muscle are unclear. **Methods:** To investigate this, 4 patients with stage IV NSCLC meeting the clinical definitions for sarcopenia and cachexia were recruited, along with 4 age-matched healthy volunteers. Following an overnight fast, biopsies were obtained from the vastus lateralis and key components associated with inflammation and the control of muscle protein, carbohydrate and fat metabolism assessed. **Results:** Compared to healthy volunteers, significant increases in mRNA levels for interleukin-6 and NFκB signalling were observed in NSCLC patients along with lower intramyocellular lipid content in slow-twitch fibres. While a significant decrease in phosphorylation of mTOR signalling protein 4E-BP1 (Ser^{65}) was observed along with a trend towards reduced p70 S6K (Thr^{389}) phosphorylation (P=0.06), there was no difference between groups for mRNA levels of MAFbx and MuRF1, chymotrypsin-like activity of the proteasome, or protein levels of multiple proteasome subunits. Moreover, despite decreases in intramyocellular lipid content, no robust changes in mRNA levels for key proteins involved in insulin signalling, glycolysis, oxidative metabolism or fat metabolism were observed. **Conclusions:** These findings suggest that an examination of the contribution of suppressed mTOR signalling in the loss of muscle mass in late-stage NSCLC patients is warranted and reinforces our need to understand the potential contribution of impaired fat metabolism and muscle protein synthesis in the aetiology of cancer cachexia.

Key words: Muscle protein synthesis; proteolysis; cachexia; mTOR signalling; ubiquitin proteasome system

Introduction

Cachexia, characterised by the unintended loss of skeletal muscle mass, is common in patients with non-small cell lung cancer (NSCLC), with about half presenting with severe muscle depletion at the time of diagnosis [1]. Of concern, cachexia is associated with reductions in tolerance to anticancer therapy, quality of life and survival [2]. Given that no standard treatment exists for cancer cachexia and it cannot be reversed by conventional nutritional support, it represents a major unmet clinical need. For logical developments in therapy to occur, the physiological and cellular mechanisms which lead to the loss of muscle mass in these patients need to be better understood.

While reports of the impact of cancer on muscle metabolism in patient populations are limited, evidence accumulated to date suggests cancer-induced alterations in muscle metabolism are reversed on cancer resection [3, 4]. This implicates cancer burden as a key driver in cancer cachexia. NSCLC, in common with a number of cancers, is associated with the increased synthesis and elevated circulating concentrations of pro-inflammatory cytokines including tumor necrosis factor-α (TNFα) and interleukin-6 (IL6) [5]. Animal and cell culture work has routinely demonstrated inflammation to negatively impact upon multiple facets of muscle metabolism (see [6]) and thus inflammation may act as a central causative feature for the cachexia that develops with cancer progression. In support, the administration of an IL6 receptor antibody to impair the action of IL6, has been shown to negate 84% of the muscle atrophy typically observed in the gastrocnemius of a murine model of cancer cachexia [7].

The loss of muscle mass is thought to develop as a consequence of enhanced muscle proteolysis, predominantly through activation of the ubiquitin proteasome system (UPS), with a concomitant suppression of muscle protein synthesis [8]. Specifically, a reduction in mTOR signalling has been reported in the Apc(Min/+) mouse model of cancer, with hypo-phosphorylation of the downstream targets p70-S6k and 4E-BP1 [9] which would be projected to lead to reduced initiation of translation [10]. Importantly, the suppression of mTOR signalling parallels the fall in muscle protein synthesis and muscle mass in experimental cancer cachexia [9], and a dose-dependent suppression of IL6 on mTOR activity has been reported [11], suggesting it plays a key role. Likewise, a number of studies in animals and in patients with various cancers have reported an increase in components of the UPS [12-14], including increased transcription of the two ubiquitin ligases, MuRF1 and MAFbx (atrogin-1), which appear instrumental in the specific targeting of muscle proteins for degradation by the 26S proteasome [15, 16]. Reports of IL6 receptor antibody administration preventing proteasome activity and muscle atrophy in an experimental model of cancer cachexia [7] viewed in conjunction with the ability of pharmacological inhibition of the proteasome pathway to blunt cancer cachexia [17], highlights the central role ubiquitin-proteasome mediated proteolysis is thought to play in the progression of cancer cachexia. However, of studies that have attempted to examine components of the proteasome pathway in patients with NSCLC and none have demonstrated an increase in UPS activity [5, 18, 19]. Thus, the contribution of ubiquitin-proteasome mediated proteolysis in NSCLC cachexia remains unclear.

In addition to the described changes in protein metabolism, cancer induced alterations in muscle carbohydrate and lipid metabolism could be underpinning impaired muscle function experienced by cancer patient populations. The

development of peripheral insulin resistance is commonly observed in experimental cancer models and in cancer patients [20]. Inflammation, including via action of the cytokines IL6 and TNFα, has been associated with an impairment of the AKT insulin signalling pathway. Likewise, administration of IL-6 to healthy individuals to increase plasma IL6 concentrations leads to profound increases in muscle lipolysis and fatty acid oxidation [21]. However, the impact of late-stage NSCLC on muscle metabolism remains poorly defined.

The purpose of this study was to examine the impact of late-stage NSCLC on inflammatory signalling and key parameters that regulate muscle protein, fat and carbohydrate metabolism, thereby providing unique insight into the potential mechanisms responsible for cachexia and functional impairment in this patient population.

Methods

Ethical Approval

Research was conducted in accordance with the Declaration of Helsinki (2008) of the World Medical Association regarding the use of human subjects in medical research and approval gained from the East Midlands Research Ethics Committee (ref. 09/H0403/65). Participants were given a full explanation of the study and provided written informed consent.

Participants

Patients were recruited from an existing study examining the effectiveness of an exercise regimen in adults scheduled to receive first-line palliative chemotherapy following a diagnosis of advanced NSCLC [22]. Recruited patients underwent the protocol described below before beginning chemotherapy or undertaking the exercise intervention required of the parent study [22]. Patients had an Eastern Cooperative Oncology Group performance status [23] between 0 and 2. Lumbar CT muscle mass was determined using diagnostic computed tomography images and SliceOMatic analysis software following previously described methods [24]. Appendicular muscle mass was determined using dual-energy X-ray absorptiometry (LUNAR Prodigy Advanced, GE Lunar, USA) with the accompanying encore software (Version 13.6) using standard imaging and positioning protocols [25]. Mean daily protein ($g.day^{-1}$) and energy ($kJ.day^{-1}$) intake were calculated using Compeat™ (version 5.8.0, Nutrition Systems UK) based on a prospective 3-day diary which included one weekend and two week days. Relevant anthropometric and blood pathology results were obtained from the patients' medical records. Healthy

volunteers of a similar age, gender, smoking history and physical activity level to the patient group, and without a history of metabolic or cardiovascular disease or vegetarian diet, were recruited (Table 1). Healthy volunteers underwent a battery of blood tests including full blood count, liver function tests, and urea and electrolytes, with all values found to be within normal ranges. Physical activity level was matched using the 12-item Modified Baecke Questionnaire [26], which summarizes habitual physical household (e.g. housework, meal preparation and stair climbing) and leisure activities in the previous year. The questionnaire was selected as it has been shown to be a valid and repeatable assessment of physical activity, suited to the older population, and has previously been used to match volunteers to NSCLC patient groups [27].

Study protocol

Participants attended the laboratory facility at 09:00h following an overnight fast and having abstained from alcohol or vigorous exercise the previous day. While resting in a semi-supine position, a venous blood sample was taken from a vein in the antecubital fossa region into pre-chilled EDTA tubes. Samples were kept on ice and within 30 mins of collection centrifuged at 3,000g for 10 mins, with the resultant plasma stored at -80°C prior to analysis. Afterwards, a single percutaneous muscle biopsy specimen was obtained from the vastus lateralis muscle under local anaesthetic according to the method described by Bergstrom [28]. The muscle sample was snap frozen and stored in liquid nitrogen prior to qPCR and western blot analysis.

Plasma IL6 and TNFα concentration by ELISA

The cytokines TNFα and IL6 are both known to be systemically elevated in a multitude of cancers, including in NSCLC patients [5]. To determine changes in systemic circulating levels of IL6 and TNFα in NSCLC patients, protein concentration of both cytokines were examined in plasma samples using commercially available enzyme-linked immunosorbent assays (Arcus Biologicals, Italy). Samples were run in duplicate along with standards of known concentration according to the manufacturer's protocol. At the end of the assay, the absorbance at 450 nm was determined for each well of the ELISA plate using a spectrophotometer (Molecular devices, USA).

qPCR

Total RNA was extracted from 30 mg of muscle tissue using TRIzol reagent (Invitrogen, UK) and quantified on a nanoDrop spectrophotometer. cDNA was subsequently synthesised using the SuperScript III commercial cDNA synthesis kit (Invitrogen, UK) according to the manufacturer's protocol. Low-density microfluidic cards containing PCR primer pairs and Taqman probes for a number of regulatory genes associated with carbohydrate, fat and protein metabolism were utilised and analysed on a 7900 HT Real-Time PCR System (Applied Biosystems, USA). MAFbx and MuRF1, two ubiquitin ligases that appear central to muscle atrophy in a number of cachectic states [16] and myostatin, a negative regulator of muscle growth, were not represented on the microfluidic card and were instead analysed separately. In short, PCR primers and reporter probes labelled with a 6-carboxyfluorescein (FAM) fluorophore at the 3' end and a tetramethylrhodamine (TAMRA) quencher at the 5' end, were purchased for MAFbx (forward: 5' CAGCTCTGCAAACACTGTCACA 3'; reverse: 5' GAGCAGCTCTCTGGGTTATTGG 3'; probe: 5'

AAGGGCACTGACCATCCGTGCAC 3'), MuRF1 (forward: 5' GGAGCCACCTTCCTCTTGACT 3'; reverse: 5' CTCAAAGCCCTGCTCTGTCTTC 3'; probe: 5' AACTCATCAAAAGCATTGTGGAAGCTTCCAA 3'), myostatin (forward: 5' TGCTGTAACCTTCCCAGGAC 3'; reverse: 5' GGTGTGTCTGTTACCTTGACCTC 3'; probe: 5' AGGAGAAGATGGGCTGAATCCGTTTTT 3') and the housekeeping gene hydroxymethylbilane synthase (forward: 5' ACGATCCCGAGACTCTGCTTC 3'; reverse: 5' GCACGGCTACTGGCACACT 3' ; probe : 5' CAGCCTCCTTCCAGGTGCCTCAGG 3') from EuroFins, UK. Hydroxymethylbilane synthase was also represented on the microfluidic cards and was utilised as a housekeeping gene throughout the two qPCR approaches adopted. Relative quantification of MAFbx, MuRF1 and myostatin was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). Obtained data was analysed using the comparative Ct method (ΔΔCt) for the relative quantification of gene expression. No significant difference was observed between groups for cycle threshold values of the housekeeping gene (data not shown).

Western blotting

Given the essential role of mTOR signalling in the control of muscle protein synthesis, and the ubiquitin proteasome system in eliciting enhanced muscle proteolysis in a number of cachectic states, both were examined by western blot analysis in tandem to assess what impact they may be having on the regulation of muscle mass. In short, cytosolic proteins were extracted from 30 mg of muscle tissue, quantified, separated by molecular weight on a 4-12% Bis-Tris polyacrylamide gel by electrophoresis, and transferred to PVDF membranes (Amersham Biosciences, UK) using methods described in detail elsewhere [29].

Membranes were subsequently blocked with 5% (w/v) bovine serum albumin in 1X tris-buffered saline for 1 h, followed by incubation overnight with a primary polyclonal antibody for either AKT, mTOR, GSK3β, 4E-BP1, p70S6K (Cell Signaling Technology Inc., USA) or proteasome subunits α1-3; 5-7 (Biomol, USA). The following phospho-specific primary antibodies were also utilised: AKT Thr^{308}, mTOR Ser^{2448}, GSK3β Ser^{9}, 4E-BP1 Ser^{65} and p70S6K Thr^{389} (Cell Signaling Technology Inc., USA). Following incubation with the primary antibody and subsequent washing in TBS-T, the membranes were incubated with a fluorescently-labelled anti-mouse or anti-rabbit secondary antibody as appropriate (Dylight® conjugate, Cell Signaling Technology Inc., USA) prior to digital capture on an electronic image acquisition system (Odyssey CLx, Licor Biosciences, USA). Band densities were determined using the proprietary software provided with the Odyssey scanner and normalised to beta-actin (Sigma, UK) protein levels to control for loading.

26S Proteasomal activity

Proteasomal activity was measured using a method adapted from [30] and [31]. In detail, 20 mg of muscle tissue was homogenised for 2 mins with a glass-ground homogeniser in a 1:20 volume of extraction buffer (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT and 0.5 mM EDTA). Afterwards, the sample was centrifuged at 8,000g for 20 mins at 4°C. From the supernatant, 5 µl was assayed for proteasomal activity in 400 µl of assay buffer (50 mM Tris-HCl (pH 7.5), 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM dithiothreitol and 0.5 $mg.ml^{-1}$ bovine serum albumin) by the addition of 4 µl a 10 mM solution of the fluorogenic substrate Suc-LLVY-amc (Sigma, UK) and followed by incubation for 10 mins at 37°C. After the allotted time, the reaction was stopped via the addition of 1.6 ml of an 80 mM

potassium acetate solution (pH 4.3). Emission at 460 nm of the cleaved substrate was determined on the resultant solution by use of a fluorometer (F-2000, Hitachi, Japan) following excitation at 360 nm. Analysis of a duplicate sample treated in the same manner as above but including the proteasome-specific inhibitor epoxomicin (20 μM; Sigma, UK) in the reaction mixture completely prevented cleavage of the fluorogenic substrate, confirming specificity of the reaction for proteasomal-dependent proteolysis.

Pyruvate dehydrogenase complex activity

Approximately 5 mg of snap-frozen muscle that had remained stored under liquid nitrogen conditions was assayed for pyruvate dehydrogenase activity (PDCa) using an established radioisotopic assay [32]. PDCa was expressed as the rate of acetyl-CoA formation ($mmol.min^{-1}$ at 37°C) normalised for protein content in the biopsy specimens.

Assessment of intramyocellular lipid (IMCL) content

To establish if chronic and gross changes in muscle fat metabolism had occurred with NSCLC, the percentage area occupied by lipid droplets in muscle fibres was examined. Frozen muscle was sectioned (10 μm) and fixed in 4% paraformaldehyde phosphate buffered saline (pH 7.4) prior to assessment of intramyocellular lipid content using the lipophilic dye, LD540 (synthesised by the University of Nottingham School of Chemistry [33]). To accomplish this, sectioned samples were incubated at room temperature for 1 hour with 3 μM LD540 in dimethylsulfoxide, followed by 3 washes in PBS before being embedded in an antifade reagent (ProLong Gold, Life Technologies, Paisley, UK). LD540 stained lipid

droplets were visualised at x40 magnification as 1 μm z-stacks with the aid of a 561 nm laser coupled to a TCS SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany). The area of the fibre covered by fluorescence was calculated using the commercial Volocity software package (version 6.3; PerkinElmer, Cambridge, UK) and fibre-type identified via staining with an antibody raised against the human adult form of slow myosin heavy chain (A4.951, developed by Dr. Blau [34]). The Volocity software was used to calculate the intramuscular lipid content normalised to fibre area on a fibre-type specific basis.

Statistical analysis

Small sample sizes as employed in the current study are common in basic science studies requiring the use of invasive muscle sampling techniques in chronically ill small patient populations (e.g. [35, 36]). As such, between group comparisons were made using an unpaired t-test as it has been previously demonstrated that the t-test is a valid approach and outperforms the non-parametric Welch test when small sample sizes are used [37]. Similarly, given the number of patients recruited, correcting the qPCR data for multiple comparisons would lead to an increased incidence of type-II errors, masking genuine gene expression changes. As such, data is reported uncorrected and instead, potential type I errors are considered informally at the level of interpretation by looking at changes across the spectrum of genes analysed, a valid approach when performing multiple comparisons [38]. All statistical tests were performed using the Prism v6.02 statistical software package for Windows (GraphPad Software Inc., San Diego, USA) with data reported as means ± SEM and statistical significance accepted when $P \leq 0.05$.

Results

Demographic and other details

The characteristics of the patient and control subjects are described in Table 1. Patients with NSCLC satisfied recommended criteria for cachexia [2] and sarcopenia [24] with lumbar and/or appendicular muscle mass below normal reference ranges (L3 CT mass: males < 52.4 $cm^2.m^{-2}$, females < 38.5 $cm^2.m^{-2}$; appendicular lean mass: males < 7.26 $kg.m^{-2}$, females < 5.45 $kg.m^{-2}$; [2, 24]). Moreover, patients had lost a mean of 9 ± 2% of their body weight over the preceding 6-months. Compared to healthy volunteers, patients with NSCLC had lower BMI and activity levels but this was not statistically significant. The latter is perhaps not surprising given the well-reported issues faced with obtaining accurate self-reported measures of physical activity [39] Analysis of dietary intake revealed patients with NSCLC to have a positive protein balance (38 ± 21 $g.day^{-1}$) and a calorie intake marginally under predicted requirements (-72 ± 241 $kJ.day^{-1}$). Given that a notable proportion of late-stage lung cancer patients fail to experience cancer anorexia (>35%)[40], and that these patients were fit enough to undergo subsequent palliative chemotherapy, the general ability of the NSCLC patients to mostly maintain protein and calorie intake isn't perhaps surprising. The median survival time of patients from time of their study visit was 23 weeks (range 15-35 weeks).

Systemic cytokine and muscle inflammatory marker levels

Compared to healthy volunteers, patients with NSCLC had a significantly higher plasma IL6 concentration and exhibited a trend towards increased plasma TNFα concentrations (P = 0.07) (Fig. 1A and 1B). Because skeletal muscle can also

be a significant source of IL6 and TNFα production, either from invading neutrophil and macrophage cell populations or the muscle cells themselves (27), we also determined the expression of IL6 and TNFα mRNA within muscle biopsy specimens. Compared to healthy control subjects, patients with NSCLC had a statistically significant 2.6-fold increase in IL6 muscle mRNA expression (Fig. 1C), but TNFα mRNA levels were equivalent between groups. NSCLC was also associated with significantly higher mRNA levels for examined components of the NFκB pro-inflammatory signalling pathway, NFκB1 and NFκB3 (RELA), while mRNA levels of the NFκB signalling pathway inhibitor, IκK-α, was also greater in muscle of NSCLC patients.

Muscle protein metabolism and the regulation of muscle size

Compared to the healthy volunteers lower mTOR protein and Ser^{2448} phosphorylation levels were observed in the patients with NSCLC but this was not statistically significant (Fig. 2A), with no difference in phosphorylation state (phosphorylation:total protein) observed between groups. In contrast, the phosphorylation state of residue Ser^{65} of the downstream protein 4E-BP1 was significantly lower in the muscle of NSCLC patients (Fig. 2B) together with a trend towards lower p70 S6K Thr^{389} phosphorylation ($P = 0.07$; Fig. 2C). There were no significant differences in 4E-BP1 and p70 S6K muscle protein levels between NSCLC patients and healthy controls (Fig. 2B and 2C respectively).

Messenger RNA levels of MAFbx, MuRF1 and myostatin did not differ between NSCLC patients and control subjects (Fig. 2D). In keeping with the failure of NSCLC to enhance MAFbx and MuRF1 expression, protein levels of α1-3 and 5-7

subunits of the proteasome, and chymotrypsin-like activity of the 26S proteasome, were unaltered in the muscles of patients with NSCLC (Fig. 2E and 2F respectively).

Insulin signalling and carbohydrate metabolism

Examination of mRNA levels for several components of the insulin signalling pathway, revealed NSCLC was associated with significantly enhanced levels of both AKT and GSK-3β in the muscle of affected patients (Fig. 3A). However, this did not translate into changes at the total protein level. Muscle samples from NSCLC patients displayed significantly lower levels of AKT protein compared to healthy volunteers (Fig. 3B), while absolute phosphorylation levels of AKT at residue Thr^{308} did not differ between groups; as a result, the phosphorylation state of AKT was significantly increased. Similarly, the protein level of the downstream kinase GSK3β was equivalent between NSCLC patients and control subjects (Fig. 3C), but on determination, a significant increase in the phosphorylation state of GSK3β in NSCLC patients was observed despite similar degrees of absolute phosphorylation between groups. Similarly, no differences were observed for mRNA levels of several genes intimately associated with carbohydrate metabolism (Fig. 4A) or oxidative metabolism (Fig. 4B). In further support, rates of muscle pyruvate decarboxylation activity (PDCa), a pivotal regulator of acetyl-CoA formation from pyruvate for utilisation by the Kreb's cycle, was comparable between groups (Fig. 4C).

Fat metabolism

NSCLC was associated with a lower area of the muscle fibre occupied with lipid in slow- but not fast-twitch muscle fibres (Fig. 5A; $P<0.05$). However, with the notable exception of Long-chain acyl CoA synthetase where increased mRNA levels

were observed (Fig. 5A), late-stage NSCLC was not associated with alterations in the expression of multiple key genes associated with the regulation of fat metabolism (Fig. 5D).

Discussion

The impact of late-stage cancer on muscle metabolism in patients remains largely unknown. Here we demonstrate for the first time that in stage IV NSCLC patients with cachexia and increased plasma IL6 concentrations, only modest changes in key transcriptional and signalling events underpinning carbohydrate, fat and protein metabolism are observed. Moreover, they do not explain the pronounced cachexia or decreased muscle lipid accumulation seen in these patients reinforcing the essential need to initiate treatments aimed at preventing cachexia early in the disease process before cachexia becomes evident.

It has been suggested that hypermetabolism and activation of muscle proteolytic pathways are confined to the early stages of the disease [41], and resolve on removal of tumour burden [3, 4]. Our results are in agreement with the proposed dynamic nature of cancer cachexia and provide the first insight that latter stages of NSCLC do not appear defined by substantial changes in muscle metabolism despite the prevalence of systemic and localised markers of inflammation. Our findings thus demonstrate the complex interplay that exists between tumour burden and metabolism in distal tissues and which likely changes over time.

While myostatin acts as a negative regulator of muscle mass [42], we could find no evidence that its expression was altered in the muscle samples of late-stage NSCLC patients; a finding that is in keeping with the reports of others from studies performed in both lung [43] and gastric cancer patients [43, 44]. Likewise, our inability to detect any indication of increased ubiquitin proteasome system-mediated proteolysis corroborates recent reports by others that MuRF1 and MAFbx are not elevated in late stage NSCLC cachectic patients [19]. It is interesting to note that multiple reports

have failed to identify any traditional indicator of increased muscle proteasome-mediated proteolysis in NSCLC patients at various stages of cachexia [5, 18]. In contrast, while gastric cancer patients undergoing tumour resection show basal levels of atrophy gene expression compared to patients undergoing abdominal surgery for benign disease [44, 45], markers of increased ubiquitin proteasome system activity appear increased and moreover, dependent on the degree of cachexia present [12]. Collectively, this evidence suggests that the recruitment of proteasome-mediated proteolysis may be specific to certain cancer-types or clinical scenarios (e.g. when cancer-induced anorexia is evident). While ubiquitin proteasome-mediated proteolysis is generally considered the principal mechanism by which muscle atrophy proceeds in cachectic states (see [46]), our findings do not preclude proteolysis occurring via an alternative route. Indeed, previous observations of increased transcription of proteases involved in lysosomal-mediated protein breakdown in the muscle of lung cancer patients [5], highlights the need to consider more widely the processes by which atrophy is occurring in NSCLC patients.

An alternative mechanism that may contribute to the loss of muscle mass during NSCLC is a suppression of muscle protein synthesis via inhibition of mTOR signalling, where mTOR acts as a nutrient sensor activating muscle protein synthesis during periods of nutrient availability [47]. In patients with NSCLC, reductions in mTOR protein and phosphorylation levels were observed in addition to reduced p70 S6K and 4E-BP1 phosphorylation, albeit only for the latter was the difference statistically significant. The collective consequences of reduced p70 S6K and 4E-BP1 phosphorylation would be a reduced drive for muscle protein synthesis [48]. However, reports that acute mTOR inhibition via rapamycin administration does not impact on postabsorptive rates of muscle protein synthesis in healthy humans [49], questions the

impact of the observed reduction in mTOR signalling on the induction of cachexia. In contrast, given the essential role of mTOR in eliciting increases in muscle protein synthesis during hyperaminoacidemia [49] or following contractile activity [50], if reductions in mTOR activity persisted following meal consumption or physical activity it would be anticipated to lead to pronounced blunting of the protein synthetic response in NSCLC patients. Recent evidence from a feeding study utilising patients with operable colorectal cancer, provide credence to this suggestion, where the provision of amino acids was seen to stimulate muscle protein synthesis in healthy age-matched controls but not in the patient population [4]. Likewise, similar observations have been seen in cachectic cancer patients during intravenous protein provision [51]. Collectively, this highlights the pressing need to understand the metabolic response seen to feeding and physical activity in NSCLC patients and whether it persists into the late stages of the disease.

In many cancers, the catabolism of body fat reserves is common and occurs in response to potential declines in food intake concomitant to hypermetabolism [52]. Despite the high occurrence of fat catabolism in cancer patient populations, the impact of cancer on human muscle fat metabolism or muscle lipid accumulation is poorly understood. Here we show that late-stage NSCLC is associated with a significant reduction in lipid accumulation in slow-twitch fibres, where oxidative metabolism of fatty acids is likely to dominate. Moreover, in the absence of a robust induction of transcriptional events previously associated with altered muscle fat metabolism in humans [53], it reinforces the notion that gross changes in fat metabolism occurred early in the disease process and had rectified at time of study participation, with decreased lipid accumulation acting as a remnant of earlier changes. Given the low subject numbers employed in the current study, some

caution must be applied to this finding; previous reports of increased intramyocellular droplets in rectus abdominis muscle being associated with cancer cachexia [54], contrast with the observations reported here. However, their use of gastrointestinal cancer patients in conjunction with patients undergoing elective surgery as control subjects, use of a non-ambulatory muscle for examination, in addition to their failure to control for age (it is well known that IMCL increases with age) between subject groups, makes any direct comparison difficult. Nevertheless, there was a clear relationship between loss of fat mass and increased IMCL [54] suggesting a lipolysis-mediated imbalance between skeletal muscle fatty acid delivery and oxidation. The later stage cancer, lower BMI and reduced IMCL in the patients of the present study would thus suggest that skeletal muscle fatty acid oxidation has outweighed delivery and the muscle is in a state of overt undernutrition. Interestingly, it appeared that PDCa was lower in the face of greater PDK4 mRNA expression, which would fit with a skeletal muscle undernutrition and insulin resistance, although this failed to reach statistical significance [55]. In common with a number of disease states where chronic inflammation and IMCL accumulation is evident, cancer has been associated with the development of insulin resistance [20], thereby impacting on carbohydrate metabolism in peripheral tissues. To date, despite the potential contribution to muscle fatigue, a comprehensive evaluation of the impact of late-stage NSCLC on muscle carbohydrate metabolism remains unexplored. However, while no index of insulin sensitivity was assessed in the current study and subject numbers were small increasing the probability of type II errors, NSCLC failed to have a consistent effect synonymous of insulin resistance on insulin signalling or transcriptional regulation of key genes involved in glycolysis or oxidative metabolism, which would perhaps fit with the lower IMCL. Notably, these observations were made in the fasted state and

further research is required to establish if NSCLC has an impact when subjects are in the postprandial state to determine if insulin resistance coincides with anabolic resistance.

Limitations

While the data reported provides a unique insight into the metabolic consequences of late-stage NSCLC on muscle, they are the result of observations obtained from a limited number of patients. Nonetheless our findings highlight important features of NSCLC-related cachexia worthy of further investigation and identify several gaps in our understanding. Given our findings, future work should consider examining NSCLC patients with and without cachexia and incorporate detailed measures of muscle metabolism utilising robust stable-isotope methodologies even though the additional invasive nature of the procedures and the prolonged laboratory visits required, may represent additional challenges to the recruitment of patients with advanced cancer.

Conclusions

Our findings provide insight into the metabolic changes evident in skeletal muscle during late-stage NSCLC and contributes to the mounting evidence that the ubiquitin-proteasome system does not play a primary role in the loss of muscle mass in NSCLC patients. Moreover, it highlights our need to greater understand the contribution of impaired fat metabolism and muscle protein synthesis in the aetiology of cancer cachexia.

Clinical Practice Points

The loss of muscle mass is a common consequence of non-small cell lung cancer (NSCLC), which can substantially impact on a patient's quality of life and their ability to adhere to prescribed chemotherapy treatments. Moreover, a significant number of individuals are diagnosed in the later stages of disease where weight loss and muscle atrophy are already established. Despite this, the impact of late-stage NSCLC on muscle metabolism and associated mechanisms are largely unknown but represent a crucial prerequisite for the development of effective treatments against cachexia in these patients. Based on work performed in animal models of cancer cachexia, it would be anticipated that the loss of muscle mass during NSCLC is largely a consequence of enhanced rates at which muscle proteins are broken down via increased ubiquitin-proteasome system activity. In stark contrast, our current report demonstrates that blunted mTOR signalling, an essential pathway for driving the synthesis of new muscle protein, is reduced in the muscle of NSCLC patients and a likely culprit for the loss of muscle mass in these patients, while markers of ubiquitin-proteasome system activity remain unaltered. Furthermore, we provide the first evidence that late-stage NSCLC is associated with reduced intramyocellular lipid content, the consequences of which remain to be characterised. Collectively, these observations reinforce the notion that treatments aimed at reducing cachexia during late-stage NSCLC should be focussed on restoring cellular processes responsible for promoting the synthesis of muscle proteins. Speculatively, combined resistance exercise and protein supplementation may represent one such strategy.

Acknowledgements

We are particularly grateful to the volunteers and patients who generously gave up their free time to participate in the study, Dr Vanessa Halliday for analysis of the food diaries, and Dr Chris Shannon for performing the PDCa measurements. Author's contributions: AJM, MM and AW conceived and designed the study; AJM, MM, KM and RE carried out participant study visits; AJM and FBS performed laboratory analytical work; AJM, MM, FBS and AW analysed and interpreted collected data; All authors contributed to drafting and revising the manuscript.

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Table 1: Subject characteristics

	Control subjects (n=4)	**NSCLC patients (n=4)**
Age	71 ± 2	73 ± 3
Male:Female	2:2	1:3
BMI ($kg.m^{-2}$)	26.6 ± 2.1	21.7 ± 1.9
Diagnosis	-	Adenocarcinoma (n=2) Squamous cell (n=2)
Stage	-	Stage IV (n=4)
% weight loss over 6 months	0 (†)	9 ± 2
Lumbar CT mass ($cm^2.m^{-2}$)	-	35.5 ± 2.8
Appendicular mass ($kg.m^{-2}$)	-	5.44 ± 0.34
C-reactive protein ($mg.L^{-1}$)	-	68.3 ± 34.5
Albumin ($g.L^{-1}$)	-	32.5 ± 3.2
Energy balance ($kJ.day^{-1}$)	-	-72 ± 241
Protein balance ($g.day^{-1}$)	-	38 ± 21
12-item Modified Baecke Questionnaire (median(range))	5 (4-12)	2 (2-6)

All values are means ± SEM unless otherwise stated. (†) Self-reported as no weight loss over the previous 6 months.

Figure 1: Circulating concentrations and localised mRNA levels of proteins associated with inflammation in cancer cachexia. Plasma concentrations of TNFα and IL6 (A and B respectively) in late-stage NSCLC patients and age-matched healthy controls as determined by ELISA. Localised mRNA levels of transcripts encoding the two cytokines and components of NFκB signalling were determined in muscle specimens obtained from the same subjects (panel C) with expression in cancer patients normalised to healthy control values. Bars represent average values ± SEM with * denoting significantly different from control (P≤0.05).

Figure 2: Effect of late-stage NSCLC on regulators of muscle protein metabolism. Total protein, absolute degree of phosphorylation, and phosphorylation status (phosphorylated:total protein) for A) mTOR Ser^{2448}, B) 4E-BP1 Thr^{389}, and C) p70 S6K S^{65}, determined by western blot. D) mRNA levels of MAFbx, MuRF1 and myostatin in NSCLC patients relative to healthy control subjects. E) Protein levels of proteasome subunits alpha 1-3 and 5-7 and F) ex vivo assessment of chymotrypsin-like activity of 26S proteasome in muscle extracts. Values represent mean ± SEM. * denotes significantly different from healthy controls (P≤0.05). Where appropriate, representative blots obtained from western blot analysis are provided above their respective bars.

Figure 3: Impact of NSCLC on muscle insulin signalling. A) muscle mRNA levels of key proteins associated with insulin signalling in NSCLC patients relative to healthy control subjects. Total protein, absolute degree of phosphorylation, and phosphorylation status (phosphorylated:total protein) for B) AKT T^{308} and C) GSK3β S^{9}. Bars represent average values expressed in arbitrary units ± SEM. * denotes

statistical significance where P≤0.05. Representative blots obtained from western blot analysis are provided above their respective bars.

Figure 4: Consequences of NSCLC on transcription of genes associated with carbohydrate metabolism. Muscle mRNA levels of genes associated with A) glycolysis and B) oxidative metabolism in NSCLC patients expressed relative to healthy control subjects. To assess carbohydrate oxidation rates, the activity of the pyruvate dehydrogenase complex was determined ex vivo (panel C). Values expressed as means ± SEM. No significant differences were observed for any of the parameters determined (P<0.05).

Figure 5: Impact of NSCLC on indices of muscle fat metabolism. A) Proportion of muscle fibre occupied by lipid droplets reported on a fibre-type specific basis. B) Representative confocal microscope images obtained from muscle sections of healthy individuals and NSCLC patients stained with LD540 to identify lipid droplets (represented in red). Images obtained at 40X magnification, with slow-twitch fibres stained green via the use of an antibody that identifies the slow isoform of human myosin heavy chain. C) Muscle mRNA levels of genes implicated in the regulation of fat metabolism in NSCLC subjects, expressed relative to healthy control subjects. Values represent mean ± SEM. * denotes significantly different from healthy controls.