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Pre-cachexia in patients with stages I–III non-small cell lung cancer: Systemic inflammation and functional impairment without activation of skeletal muscle ubiquitin proteasome system

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ABSTRACT

Cachexia is a prevalent phenomenon of non-small cell lung cancer (NSCLC) which is responsible for increased mortality and deterioration of physical performance. Preclinical research indicates that systemic inflammation induces cachexia-related muscle wasting through muscular Nuclear Factor-kappa B (NF-κB) signaling and subsequent ubiquitin proteasome system (UPS)-mediated proteolysis. As these pathways could be a target for early intervention strategies, it needs to be elucidated whether increased activation of these pathways is already present in early stage NSCLC cachexia. The aim of the present study was therefore to assess muscular NF-κB and UPS activation in patients with NSCLC pre-cachexia.

Sixteen patients with newly diagnosed stages I–III NSCLC having <10% weight loss and ten healthy controls were studied. Body composition, systemic inflammation and exercise capacity were assessed in all subjects and NF-κB and UPS activity in vastus lateralis muscle biopsies in a subset.

Patients showed increased plasma levels of C-reactive protein (CRP) ($P < 0.001$), soluble Tumor Necrosis Factor receptor 1 (sTNF-R1) ($P < 0.05$), fibrinogen ($P < 0.001$) and decreased levels of albumin ($P < 0.001$). No changes in fat free body mass or skeletal muscle NF-κB and UPS activity were observed, while peak oxygen consumption ($\dot{V}_{O_2}$ peak) was significantly decreased in patients compared with healthy controls.

In conclusion, this exploratory study demonstrates significantly reduced exercise capacity in NSCLC pre-cachexia despite maintenance of muscle mass and unaltered indices of UPS activation. The absence of muscular NF-κB-dependent inflammatory signaling supports the notion that transition of systemic to local inflammation is required to initiate UPS-dependent muscle wasting characteristic for (experimental) cachexia.

1. Introduction

Cachexia is a prevalent feature of non-small cell lung cancer (NSCLC) which is characterized by progressive body weight loss and peripheral tissue wasting [1]. The significance of cachexia in NSCLC is established by its negative impact on therapy responsiveness and survival [2–5]. Other important consequences are a decline in quality of life and progressive impairment of physical function [1,4]. Despite the importance, cachexia in NSCLC remains often unrecognized and no adequate management strategies are available [5,6].

Skeletal muscle atrophy is identified as the most important predictor of mortality and functional impairment in cancer cachexia [1]. Wasting of skeletal muscle tissue is considered to be induced by tumor-associated systemic inflammation, which subsequently triggers degradation of skeletal muscle proteins and thereby causing muscle atrophy [7,8]. Preclinical research has demonstrated that muscular Nuclear Factor-kappa B (NF-κB) integrates systemic inflammatory signals and is responsible for activation of the proteolytic ubiquitin (Ub) proteasome system (UPS) [9,10]. In the UPS, E1 enzymes and highly specific E2 Ub-conjugating and E3 Ub-ligating enzyme complexes attach a polyubiquitin chain to protein substrates. This marks the protein for degradation by the 26S proteasome 11. In experimental models of cancer cachexia, the muscle-specific E3 Ub-ligases Atrogin-1/MAFbx and muscle RING-finger protein-1 (MuRF1) have shown to be rate limiting in the degradation of skeletal muscle proteins [12]. Although increased NF-κB and UPS activation plays a predominant role in preclinical cancer cachexia, limited data is available on involvement of

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these pathways in human cancer cachexia and the onset of putative activation in particular.

Although it is evident that cancer cachexia occurs in progressive stages of severity, international experts have agreed upon a clear definition of distinct clinical stages only recently (*Lancet Oncology, May 2011*) [1]. In the present definition, three clinically relevant stages of cancer cachexia are distinguished, i.e. pre-cachexia, cachexia and refractory cachexia. Pre-cachexia is considered an initial phase in which metabolic changes, such as systemic inflammation, have resulted in minor body weight loss but not (yet) in significant depletion skeletal muscle mass or impairment of physical function. Pre-cachexia often progresses to (refractory) cachexia, with detrimental effects on survival and performance status. In the international consensus, it is specifically emphasized that pre-cachexia should be the focus of clinical and translational research, as preventive measures in pre-cachexia might delay or prevent progression to (refractory) cachexia [1,5]. To address this, we studied if skeletal muscle NF-κB and UPS activity is (already) increased in NSCLC pre-cachexia. As increased activity of these markers plays a pivotal role in advanced preclinical cachexia, these molecular markers might be important elements for early intervention strategies in pre-cachectic patients. We hypothesized that systemic inflammation already initiates muscular NF-κB and UPS activation in pre-cachexia but has not yet resulted in significant effects on body composition or exercise capacity.

2. Subjects and methods

2.1. Study population

Sixteen newly diagnosed pre-cachectic patients with locally (advanced) NSCLC consecutively admitted to the department of Respiratory Medicine of the Maastricht University Medical Centre+ and ten healthy controls were included. Pre-cachexia was defined as <10% loss of total body weight in the last six months as the cut-off of 10% weight loss was often used to distinguish pre-cachexia from clinical cachexia before the recent definition of pre-cachexia was published [1]. NSCLC was confirmed by pathological analysis and tumor stage was assessed using the tumor-node-metastasis (TNM) International Staging System for Lung Cancer [13]. Exclusion criteria were the presence of other malignancies or previous administration of anti-tumor treatment. Age-matched healthy control subjects were recruited from advertisements in local newspapers. The study was approved by the local medical ethical committee and written informed consent was obtained from all subjects.

2.2. Pulmonary function and body composition

Spirometry was performed in all subjects to determine the forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC). Dual energy X-ray absorptiometry (DXA; DPX-L, Lunar Radiation Corp., Madison, WI) was used to determine body composition, i.e. fat mass (FM) and fat free body mass (FFM) [14]. DXA measurements were performed in the fasted state.

2.3. Exercise capacity and physical activity

Exercise capacity testing was performed using an electrically braked cycle ergometer (Corival 400, Lode, Groningen, The Netherlands). The test started with 1 min of unloaded cycling, after which the load was increased by 10 W every minute in patients. For control subjects, the load was increased by 15–25 W every minute to achieve comparable test duration. None of the subjects knew the exercise load and all were encouraged to cycle at 60 rpm until exhaustion. Peak oxygen consumption ($\dot{V}_{O_2}$ peak) was measured at the moment of cessation of the exercise. Predictive values were calculated according to Jones (0.046 (Height) – 0.021 (Age) – 0.62 (Sex: 0, male; 1, female) – 4.31 l/min) [15]. Peak ventilatory (VE) reserve was calculated as 100% – (100 × peak VE)/(FEV_1 × 37.5) [16]. The level of physical activity during daily life was measured using a triaxial accelerometer (Tracmor; Philips Research, Eindhoven, The Netherlands), which measures body accelerations in anterioposterior, mediolateral, and vertical directions and expresses them in 'counts' per time interval (min) [17]. Tracmor data was obtained for 7 consecutive days during waking hours for at least 8 h. The time spent in each category of intensity (low, moderate and high) is presented as percentage of total wear time.

2.4. Plasma inflammatory markers

After an overnight fast, blood from an antecubital vein was collected in evacuated ethylenediaminetetraacetic acid (EDTA) blood collection tubes (Sherwood Medical, Ballymoney, Northern Ireland). Plasma was obtained by centrifugation of the blood at 3000 Relative Centrifugal Force (RCF) for 15 min at 4 °C. Blood samples were stored at −80 °C until sample analysis was performed. Plasma C-reactive protein (CRP) levels were assessed using turbidimetry. Soluble Tumor Necrosis Factor receptor 1 (sTNF-R1) levels were determined by sandwich ELISA as described elsewhere [18]. Recombinant human sTNF-R1 was used as standard. Albumin levels were measured using the Bromocresol Purple method with a Synchron CX-7 instrument (Beckman, Mijdrecht, The Netherlands).

2.5. Muscle biopsies

Of ten patients, adequate amounts of muscle tissue were available for molecular analysis. Percutaneous muscle biopsies of m. vastus lateralis were obtained under general anesthesia prior to thoracic surgery ($N = 5$) or local anesthesia ($N = 5$). Eight healthy control subjects underwent muscle biopsies under local anesthesia. The technique used for the muscle biopsies was described by Bergström [19]. Muscle biopsies were immediately frozen in liquid nitrogen and stored at −80 °C until sample analysis was performed. Muscle biopsies were homogenized using a Polytron PT1600E homogenizer (Kinematica, Littau-Lucerne, Switzerland).

2.6. Muscle mRNA analysis

Total RNA was isolated using the Totally RNA™ kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. 0.4 μg RNA was reverse transcribed to cDNA using the Reverse iT First Strand Synthesis kit (ABgene, Epsom, UK) with anchored oligo-dT primers. mRNA expression levels of NF-κB-dependent inflammatory signaling markers (IkappaBalpha (IκBα) and Tumor Necrosis Factor alpha (TNF-α)), UPS rate limiting E3 Ub-ligases (Atrogin-1/MAFbx and MuRF1) and housekeepers (β-actin, Cyclophilin, β_2-microglobulin) were determined by quantitative RT-PCR (Q-PCR). Q-PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and obtained from Sigma Genosys (Haverhill, UK). PCR reactions contained 1× Q-PCR MasterMix Plus for SYBR green I (ABgene, Epsom, UK) and 6 pmol of each primer (20 μl total volume) and were performed in a MyiQ thermocycler (Bio-Rad, Veenendaal, The Netherlands). Standard curves were made by performing serial dilutions of pooled cDNA aliquots. The expressions of the genes of interest were normalized by calculating an average value of the housekeeping genes β-actin, Cyclophilin and β_2-microglobulin using geNorm software (Primerdesign, Southampton, USA). Gene expression is expressed as arbitrary units (AU).

2.7. 26S proteasome activity assay

The method used for determining peptidase activity of the 20S subunit of the 26S proteasome was described previously [20,21]. The protocol was slightly modified to allow analysis of small human muscle biopsies. To isolate the 20S proteasome, muscle biopsies were homogenized in 10 volumes of ice-cold buffer (pH 7.5) containing 50 mM Tris, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT and protease inhibitors (10 μg/ml antipain, aprotinin, leupeptin and pepstatin A, 0.2 mM PMSF) using a Yellowline homogenizer (IKA Works, Wilmington, NC, USA). Proteasomes were isolated by sequential (ultra) centrifugation steps and the protein concentration in the proteasome fractions was measured with the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands), using bovine serum albumin as standard. The peptidase activities of the 20S proteasome were determined fluorometrically by measuring the hydrolysis of the fluorogenic substrates Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC, Sigma, Zwijndrecht, The Netherlands) and Benzyloxycarbonyl-Leu-Leu-Glu-7-amido-4-methylcoumarin (Z-LLE-AMC, BIOMOL, Exeter, UK). These substrates are preferentially hydrolyzed by the chymotrypsin-like and caspase-like peptidase activities of the 20S proteasome, respectively. Adding the proteasome inhibitor MG132 to the reaction resulted in complete inhibition of the proteasome peptidase activities.

2.8. Statistics

Because the study design was exploratory, no formal power calculation was performed. Data was analyzed using Statistical Package for the Social Sciences (SPSS version 15 for Windows, SPSS Inc., Chicago, IL, USA). Continuous variables were compared using an independent sample *t*-test. Pearson Chi-square test was used for comparing categorical variables. Correlations were evaluated using Pearson correlation test. Data is represented as mean ± SD. Significance was set at $P < 0.05$.

3. Results

3.1. Pre-cachexia in NSCLC is characterized by systemic inflammation without changes in body composition

Baseline characteristics of the study population are shown in Table 1. Patients had significantly more weight loss ($P = 0.008$) than healthy controls but the mean observed weight loss was limited, i.e. 3.1% of premorbid body weight (Table 1). Thirteen patients had <5% weight loss, while three patients had 5–10% weight loss. No changes were observed in body composition between the study groups (Table 1). Patients with NSCLC showed a profound pro-inflammatory status as illustrated by increased plasma sTNF-R1 levels ($P = 0.032$), as well as elevated plasma levels of the positive acute-phase reactants CRP ($P < 0.001$) and fibrinogen ($P < 0.001$). In addition, plasma levels of the negative acute phase reactant albumin were decreased in patients ($P < 0.001$) (Fig. 1). FEV_1 and FEV_1/FVC were lower in patients ($P < 0.001$) but obstruction was mild as only GOLD stages I–II of chronic obstructive pulmonary disease (COPD) were observed (Table 1).

3.2. Exercise capacity is decreased in pre-cachexia despite normal physical activity patterns

Incremental cycle ergometry testing revealed significantly reduced peak oxygen consumption in patients ($P = 0.010$) (Fig. 2). The $\dot{V}_{O_2}$ peak was not associated with FEV_1 (R: 0.41, $P = 0.075$) and patients nor healthy controls were restricted by their ventilatory capacity (VE reserve 44 ± 8% in patients and 18 ± 24% in controls)

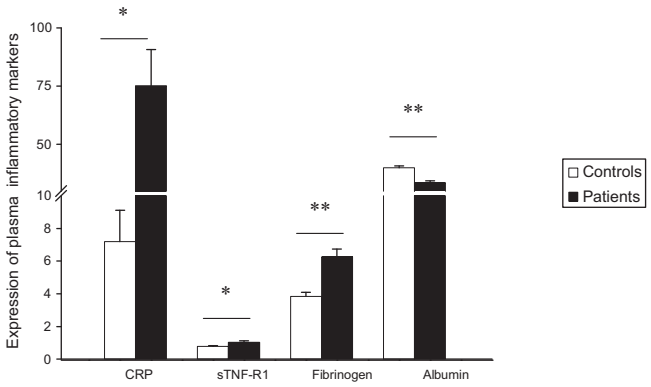

Fig. 1. Plasma levels of inflammatory mediators of healthy control subjects and NSCLC patients. Patient with pre-cachexia have an increased inflammatory profile compared with healthy controls. CRP: C-reactive protein (μg/ml); sTNF-R1: soluble Tumor Necrosis Factor receptor 1 (sTNF-R1) (μg/ml); fibrinogen (g/l) and albumin (mg/ml). $^{*}P < 0.05$ and $^{**}P < 0.001$.

(data not shown). Overall physical activity levels were not different and while the proportion of light and moderate activity was comparable in both groups, the proportion of high intensity activity was increased in patients ($P = 0.049$) (Table 1). No correlation were observed between $\dot{V}_{O_2}$ peak and fat free mass (R: −0.28, $P = 0.909$).

3.3. Muscular inflammatory signaling and UPS activity is not altered in patients with NSCLC pre-cachexia

To determine whether NF-κB-dependent inflammatory signaling and UPS activity were increased in NSCLC pre-cachexia, expression levels of NF-κB target genes IκBα and TNFα and E3 Ub-ligases Atrogin-1/MAFbx and MuRF1 were measured in skeletal muscle. Furthermore, in muscle homogenates containing the isolated 26S proteasome fraction, activity levels of two proteolytic enzymes of the 20S core subunit were assessed. In Table 2, it is shown that there were no differences in NF-κB, UPS E3-ligase or 26S proteasome activity in pre-cachectic patients compared with healthy controls (Table 2).

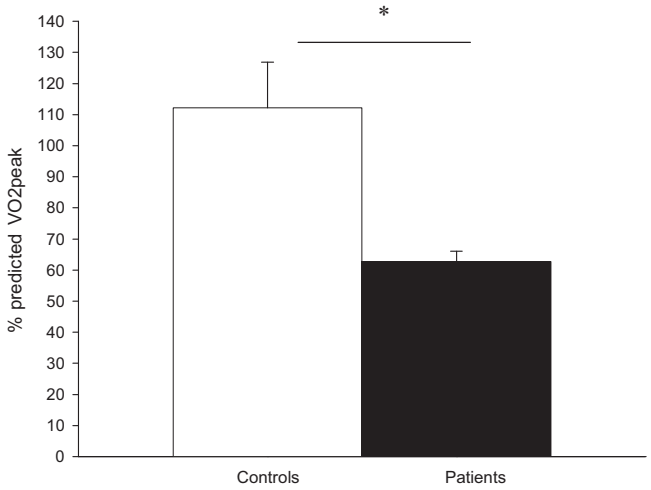

Fig. 2. Exercise capacity in healthy control subjects and NSCLC patients. The exercise capacity is significantly reduced in patients with NSCLC pre-cachexia compared with healthy control subjects. $^{*}P < 0.05$.

Table 1
Baseline characteristics of healthy control subjects and patients with NSCLC.

	Patients ($N = 16$)	Controls ($N = 10$)	P-Value
Gender (males (%))	93.8	70	0.102
Age (y)[a]	65.9 ± 7.5	63.7 ± 5.6	0.427
Weight loss (%)[b]	3.1 ± 4.4	−0.6 ± 2.0	0.008*
Body mass index (BMI) (kg/m^2)	24.2 ± 3.9	27.0 ± 3.6	0.104
Fat mass index (kg/m^2)	6.0 ± 2.7	8.1 ± 3.2	0.079
Fat free mass index (kg/m^2)	17.3 ± 1.7	17.8 ± 2.2	0.612
FEV_1 (% predicted)[c]	78 ± 18	115 ± 22	<0.001*
FEV_1/FVC[d] (%)	62 ± 17	78 ± 4	<0.001*
COPD[e] GOLD stage (0:I:II) (%)	38:31:31	90:10:0	0.026*
Smoking (current:former:never) (%)	10:70:20	19:81:0	0.165
Physical activity (counts/min)	674 ± 150	694 ± 239	0.831
Low intensity (% of time)	84 ± 10	89 ± 4.9	0.198
Moderate (% of time)	7 ± 4	7 ± 2	0.819
High (% of time)	9 ± 1	4 ± 1	0.049*
Stage, n (%)			
I–II	11 (69)		
IIIA	2 (12)		
IIIB	3 (19)		
Histology, n (%)			
Adenocarcinoma	2 (12)		
Squamous cell	8 (50)		
Large cell	6 (38)		

[a] Data are represented as means ± SD, all such values.
[b] Body weight loss in 6 months prior to diagnosis.
[c] Forced expiratory volume in 1 s.
[d] Forced vital capacity.
[e] Chronic obstructive pulmonary disease.
* $P < 0.05$.

4. Discussion

This exploratory study shows that pre-cachexia in NSCLC is associated with significantly decreased exercise capacity without changes in body composition, and that despite the presence of systemic inflammation, no inflammatory signaling or increased UPS proteolytic activity appears appreciable in skeletal muscle. A schematic representation of alterations and consequences of pre-cachexia and (preclinical) cachexia is depicted in Fig. 3.

Although several inflammatory factors produced by tumor and/or host tissues have shown to be sufficient to induce muscle wasting in experimental cancer cachexia [7,8], our data suggests that additional factors or prolonged exposure to systemic inflammation is required to translate systemic to local muscular inflammation in human cachexia. In addition to systemic and local inflammation, increased UPS-mediated proteolysis has convincingly been demonstrated in preclinical models of cancer cachexia [7–9,11,12]. In line with these findings, a number of studies in advanced stages of human cancer cachexia have also reported increased transcriptional activity of UPS markers in skeletal muscle [22,23]. However, a small number of studies show that transcriptional activity of UPS markers like E1–E2 enzymes and 26S proteasome subunits is not increased in patients with <10% weight loss [24,25]. Furthermore, Smith et al. recently reported no changes in E3-ligase Atrogin-1/MAFbx and MuRF1 mRNA expression in gastric cancer patients with limited weight loss [24]. These findings are in agreement with the absence of increased expression of Atrogin-1/MAFbx, MuRF1 and 26S proteasomal activity in the current pre-cachectic patient population with NSCLC. Together, this indicates that UPS-mediated proteolysis is not (yet) increased in NSCLC pre-cachexia.

The distinct decrease in $\dot{V}_{O_2}$ peak independent of fat free mass and without changes in daily physical activity indicates that wasting-independent intrinsic muscular alterations result in exercise intolerance in early stages of NSCLC cachexia. This is an important finding as it has been demonstrated that decreased $\dot{V}_{O_2}$ peak is a strong predictor of mortality and increases the risk of postoperative complications in early stages of NSCLC [26,27]. As the presence of COPD might have an effect on exercise capacity and COPD is often present in patients with NSCLC due to the

Table 2
Expression of skeletal muscle inflammatory and ubiquitin proteasome system markers.

Baseline characteristic	Patients ($N = 10$)	Controls ($N = 8$)	P-Value
Inflammatory signaling (mRNA expression)			
IκBα[a] (AU)[b]	0.24 ± 0.16	0.26 ± 0.16	0.767
TNF-α (AU)	0.21 ± 0.09	0.25 ± 0.14	0.538
E3 UPS ligases (mRNA expression)			
MuRF1 (AU)	0.23 ± 0.25	0.18 ± 0.06	0.599
Atrogin-1/MAFbx (AU)	0.21 ± 0.10	0.28 ± 0.07	0.154
26S proteasome activity			
Caspase-like (pmol/μg protein/min)	20.38 ± 5.79	20.72 ± 4.16	0.896
Chymotrypsin-like (pmol/μg protein/min)	6.80 ± 3.03	6.34 ± 2.87	0.758

[a] Data are represented as means ± SD.
[b] AU, arbitrary units.

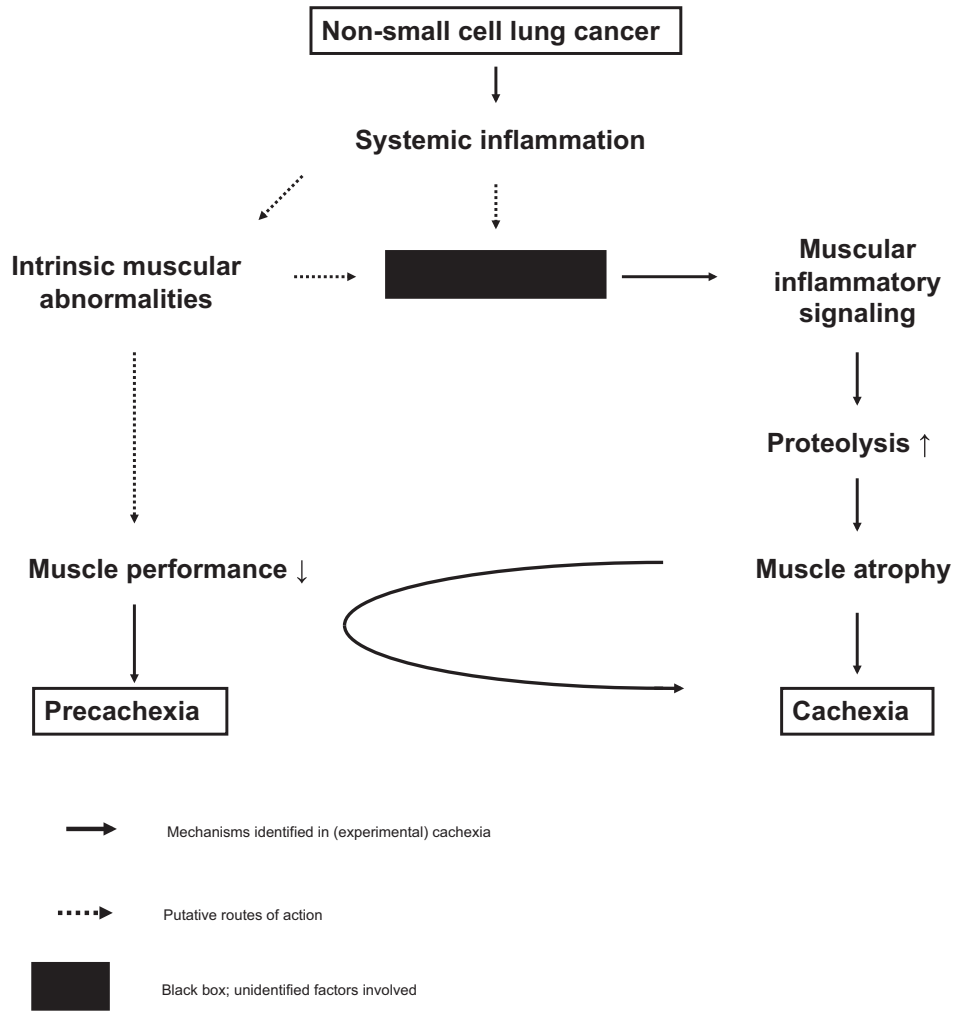

Fig. 3. Schematic representation of alterations and consequences in pre-cachexia and cachexia.

cigarette smoke history, the decreased exercise capacity could be a result of decreased lung function. Indeed, patients had decreased lung function capacity but only mild stages of COPD were observed, i.e. only one patient was diagnosed with chronic obstructive pulmonary disease (COPD) prior to inclusion in this study and only GOLD stages I–II were observed based on the current spirometry assessment. Furthermore, despite the presence of COPD, decreased exercise capacity was independent of lung function as illustrated by VE reserves in both groups and absence of correlations between FEV_1 and $\dot{V}_{O_2}$ peak. This indicates that other determinants are involved in exercise impairment in NSCLC pre-cachexia. The trigger and mechanism of decreased exercise capacity in NSCLC remains unidentified but as decreased exercise capacity is often observed in patients with systemic inflammation, the profound systemic inflammatory response in the current patient population is a promising lead [28,29]. It would be of interest to identify underlying mechanisms of reduced exercise capacity in patients with NSCLC pre-cachexia to minimize risk of postoperative complications and mortality. Furthermore, effectiveness of intervention strategies, possibly combined in a multimodal approach including exercise training and targeted pharmacological therapies specifically focused at improving physical performance should be studied in randomized controlled trials in patients with pre-cachexia.

In conclusion, this exploratory study shows that decreased exercise capacity independent of lung function (based on FEV_1) and systemic inflammation are present in pre-cachexia in NSCLC but have not (yet) resulted in increased inflammatory signaling, UPS-dependent protein degradation and subsequent wasting of skeletal muscle. These findings indicate that molecular profiles identified in experimental cancer cachexia models are not (yet) present in NSCLC pre-cachexia. As patients in different stages of cachexia could benefit from unique intervention strategies, it is of interest to identify underlying mechanisms of the decreased exercise capacity that is already observed in NSCLC pre-cachexia prior to muscle catabolism.

Conflict of interest statement

None declared.

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