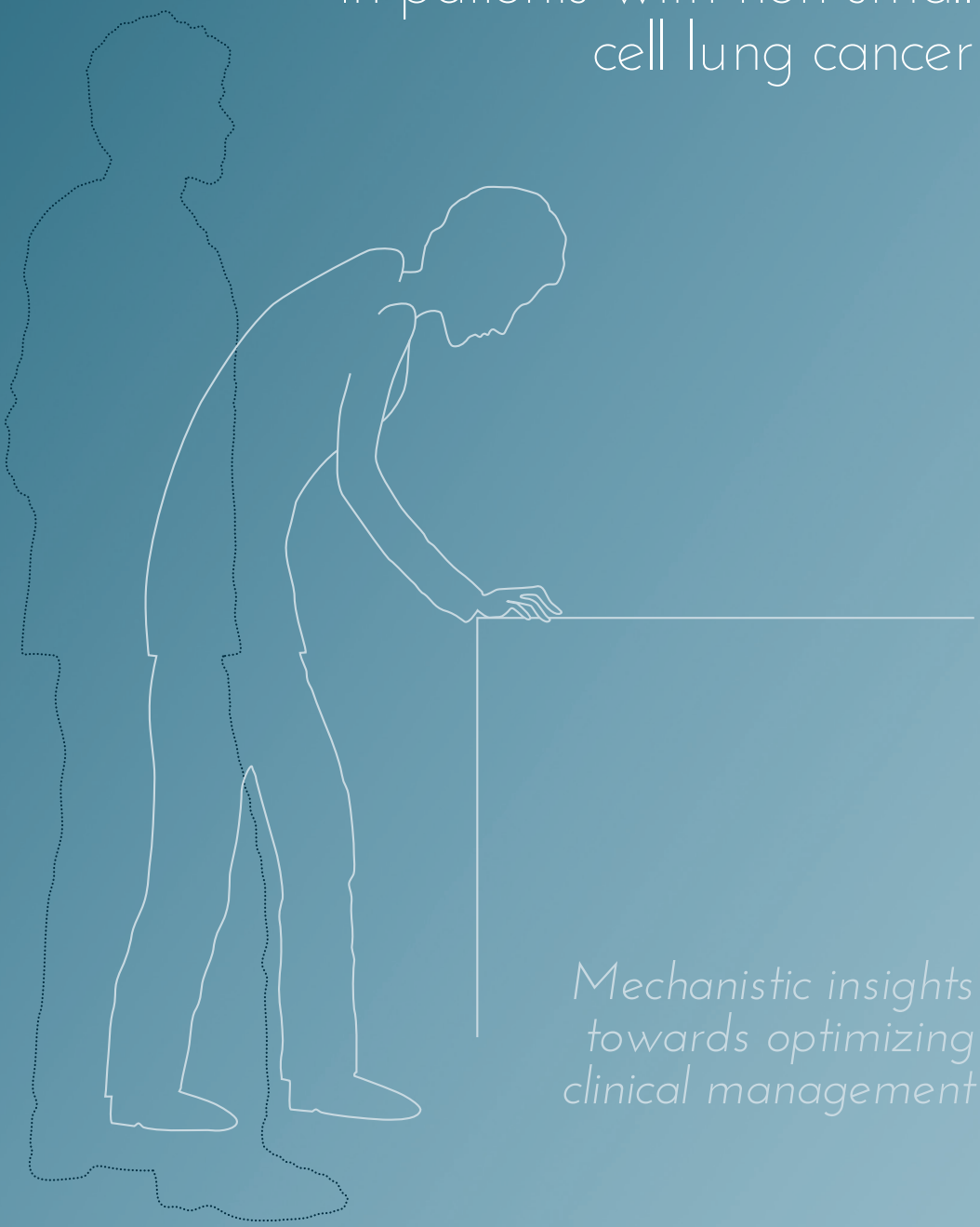
Cachexia in patients with non-small cell lung cancer : mechanistic insights towards optimizing clinical management

Céline Op den Kamp

Cachexia

in patients with non-small cell lung cancer



Mechanistic insights towards optimizing clinical management

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Cachexia in patients with non-small cell lung cancer

Mechanistic insights towards optimizing clinical management

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In memory of all the patients who participated in the studies in this thesis…

"...the shoulders, clavicles, chest and thighs melt away. This illness is fatal..."

—Hippocrates (460 –370 BC)

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Chapter 1

General introduction

General introduction

Cancer cachexia is a debilitating paraneoplastic feature that accompanies many types of cancer[1]. The term cachexia is derived from the Greek words “kakós” meaning “bad” and “hexis” meaning “condition”, which illustrates the negative consequences of the syndrome[2]. The most distinct characteristics of cancer cachexia are progressive body weight loss and wasting of skeletal muscle[1]. The latter is disproportionately high in cancer cachexia and differentiates the syndrome from (semi-) starvation, in which wasting of adipose tissue is more prominent[1, 3].

Weight loss and muscle wasting have major negative implications in cancer patients[4, 5]. Muscle wasting is an important determinant of muscle weakness, which negatively influences performance status and quality of life[1, 6-8]. Furthermore, decreased responses to anti-tumor treatments have been reported in cachectic cancer patients, as is illustrated by decreased post-operative survival, increased toxicity of chemotherapy, and increased mortality [1, 4, 9-11]. Although it is difficult to determine the exact contribution of cancer cachexia to mortality, it is estimated that 20-30% of cancer-related deaths are a direct consequence of cancer cachexia[12, 13]. Particularly, loss of skeletal muscle appears an important determinant of decreased survival in cancer patients, even independent of total body and fat mass[11, 14]. Unfortunately, a low or decreasing muscle mass is often not recognized in patients suffering from cancer[14]. Due to an increasing prevalence of obesity in developed countries, loss of muscle mass may occur in normal to overweight cancer patients (often referred to as sarcopenic obesity), which visually do not appear cachectic[14]. Increased attention for weight loss in patients with malignant disease is therefore essential, preferably in combination with assessment of body composition[14].

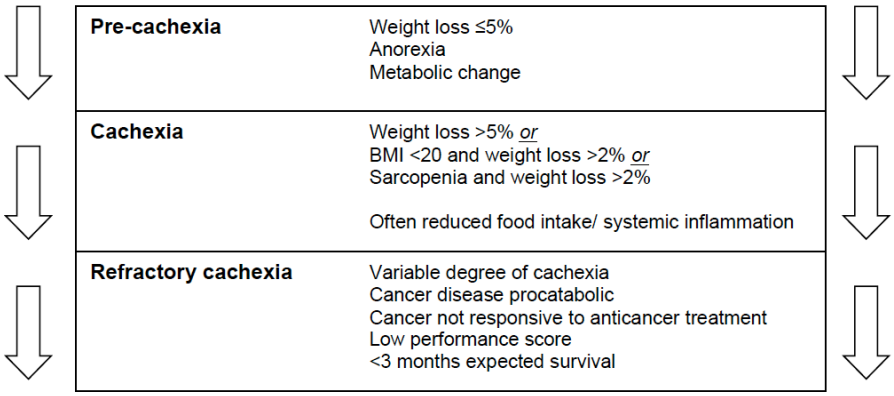
Definition and classification of cancer cachexia

The summation of these negative consequences demonstrates the negative impact of cancer cachexia on prognosis and quality of life, and emphasizes the need for adequate clinical recognition and management. However, while already known for centuries, this devastating syndrome is still not adequately acknowledged and managed in current clinical cancer care[1]. Although research on mechanistic cues lying at the basis of cancer cachexia has increased our understanding of cancer

cachexia, the absence of a uniform definition and classification system has hampered clinical advancement and development of strategic clinical management[1]. To aid advancement in clinical and therapeutic care of cancer cachexia, experts in the field of cancer cachexia recently proposed a uniform and operational definition and classification system for cancer cachexia[1]. In a formal consensus process the following definition was formulated: *"Cancer cachexia is a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment"* [1]. Cancer cachexia is defined as *"a continuum with three stages of clinical relevance, i.e. pre-cachexia, cachexia, and refractory cachexia, which can all be traversed in one patient".* Pre-cachexia is defined as *"a condition in which weight loss and depletion of adipose and muscle tissue are not yet distinctive (weight loss is ≤5 percent) but early clinical and metabolic signs can precede substantial involuntary weight loss. The presence of factors, such as systemic inflammation, cancer type and stage or responses to therapy, determines the risk of progression".* The consensus report emphasizes that especially in pre-cachexia, intervention strategies aiming to prevent or attenuate advancement into cancer cachexia could be effective, conceivably because molecular mechanisms that cause cancer cachexia might already be activated at this point but have not yet resulted in severe wasting of muscle mass and deterioration of physical performance. In cancer cachexia *"weight loss exceeds 5% of total body weight, or >2% when body mass index (BMI) is <20 kg/m^2 or patients exhibit signs of sarcopenia"*. Further clarified, this stage is characterized by substantial weight loss, disproportionate skeletal muscle wasting and functional deconditioning, and is often accompanied by reduced dietary intake and systemic inflammation. Ultimately, cancer cachexia can progress to refractory cachexia, i.e. *"a clinically refractory stage as a result of very advanced cancer (pre-terminal) or the presence of rapidly progressive cancer unresponsive to anticancer therapy"*[1].

Figure 1

Cancer cachexia classification[1]



Cachexia associated with lung cancer

Lung cancer imposes an important health care problem. A worldwide incidence of >1.6 million new cases per year illustrates that lung cancer is one of the most commonly diagnosed types of cancer[15, 16]. Malignant lung disease is currently accountable for the highest number of cancer-related deaths worldwide and it is expected that lung cancer will continue to be a leading cause of cancer-related mortality in the next decades[15, 16]. Lung cancer is subdivided in two major subtypes, i.e. non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for 75-85% of new lung cancer cases[17, 18]. At diagnosis, NSCLC is staged according to Tumor, Node, Metastasis (TNM) system in which dissemination of the malignant process is systematically evaluated using tumor characteristics (T), lymph node spreading (N) and presence of distant metastasis (M) to predict prognosis. In addition, TNM staging of NSCLC is used as a guideline for management of NSCLC[19]. In the TNM staging system, four stages are distinguished. Stage I-II represents early-stage "localized" disease, stage III "locoregionally advanced" disease and stage IV "metastatic" NSCLC[19-22]. The preferred treatment for stage I-II NSCLC is surgical resection, followed by platinum-based adjuvant chemotherapy in stage II NSCLC[20]. In stage III (N2 and N3), concurrent chemoradiotherapy (CT-RT) administered with curative intent is the treatment of choice when patients are eligible for this intense treatment regimen. In case of low performance status (Eastern

European Oncology Group (ECOG) >1) or substantial weight loss (>10% of body weight), careful consideration of the balance between risks and benefits is recommended[21]. In addition, the presence of comorbidities (especially pulmonary and cardiac dysfunction) should be taken into account when considering administration of concurrent CT-RT[21, 23]. In patients suffering from stage III NSCLC with limited lymph node dissemination (discrete N2 involvement), induction therapy followed by surgical resection can be considered as an alternative for definitive concurrent CT-RT[21]. In stage IV NSCLC, treatment with curative intent is not achievable but prolongation of survival can be accomplished by administration of platinum containing chemotherapy or, when driver mutations are present, with targeted therapies[22]. Furthermore, early initiation of palliative care is recommended to improve quality of life and extend survival in patients with non-curable NSCLC[22].

Cancer cachexia is an important entity of lung cancer disease due to its high prevalence in this malignant condition[12]. The fact that up to 58% of patients with lung cancer already report some weight loss at diagnosis and 30-36% of newly diagnosed lung cancer patients have already lost more than 5% of their habitual body weight, indicates that many patients with lung cancer exhibit signs of cancer cachexia[4, 12, 24, 25]. Only one small study of 40 patients with stage III NSCLC has evaluated the incidence of pre-cachexia and cancer cachexia using the recent international consensus guidelines. This study revealed that 23% of patients met the criteria for pre-cachexia and 18% of patients met the criteria for cancer cachexia at baseline (before anti-tumor treatment was started)[26]. The risk of weight loss subsequently increases during the disease course, which further enhances the negative effects of weight loss on performance status, treatment tolerance and survival[1].

Etiology of cancer cachexia in lung cancer

The underlying mechanisms responsible for cancer cachexia in lung cancer are considered complex and multifactorial but presumably include alterations in host metabolism and dietary intake (e.g. anorexia or secondary causes like pain, dyspnea, constipation) that lead to negative energy and protein balances[1, 27]. Host-tumor responses like systemic inflammation[28-30] and putatively tumor-derived factors, such as proteolysis inducing factor (PIF)[31], are considered important in inducing

these disturbances in energy and protein metabolism, which result in a net loss of muscle protein.

The exact role of increased systemic inflammation in cancer pathophysiology is yet to be determined but either involves a host immune response raised against components of the malignant process[32, 33] or conversely, a tumor-induced reaction to attract pro-inflammatory mediators, i.e. some reports indicate increased proliferation of malignant cells in inflammatory micro-environments[34, 35]. Independent of the underlying mechanisms, the inflammatory response observed in lung cancer can be associated with increased energy requirements as a result of the energy-dependent formation of inflammatory mediators such as cytokines and acute phase proteins (as part of an acute phase response)[30, 36]. As protein released by skeletal muscle can be processed for energy fuel as well as building blocks for formation of inflammatory mediators, systemic inflammation could be a potential driver of protein catabolism from skeletal muscle (muscle wasting)[37]. In addition, the presence of pro-inflammatory cytokines has been linked to anorexia by altering neuro-hormonal and neuropeptide signaling, which could further contribute to weight loss[29, 38-40].

In addition to systemic inflammation, the tumor-derived glycoprotein PIF has been identified as a factor that can induce proteolysis of muscle proteins and thereby, disturbing balances in muscle maintenance in experimental cancer cachexia[41-43]. While PIF was expressed in little over half (56%) of tumor samples of patients with NSCLC[44], a causal relation between PIF expression and weight loss or survival in has not yet been identified in human cancer cachexia [44-47].

In addition to dietary and metabolic derangements induced by the presence of a malignant process, the full spectrum of anti-tumor treatment toxicities, i.e. surgery, chemotherapy (CT) and radiotherapy (RT), can induce anorexia, nausea, mucositis and postoperative metabolic alterations, which could further contribute to deterioration of nutritional and functional status in cancer patients by reducing dietary intake or alternating host metabolism[48-50]. Combining treatment modalities, like concurrent CT-RT in stage III NSCLC, increases toxicities like radiation esophagitis[21, 51]. The presence of esophagitis could lead to a dysphagia-induced decrease in dietary intake and subsequent weight. Indeed, weight loss is commonly observed in concurrent CT-RT but it is currently unknown whether this occurs via direct toxicity of therapy (e.g. esophagitis-related dysphagia, nausea, vomiting) or indirect host responses to therapy that alter metabolic energy requirements and appetite regulation (e.g. systemic inflammatory responses).

The role for nutrition in attenuating tumor- and treatment-induced weight loss

Currently, treatment options for cachexia in lung cancer are both limited and insufficient[1, 52]. Considering the multifactorial aspect of cancer cachexia, clinical experts advise a multimodal approach in which management of decreased dietary intake, systemic inflammation, muscle wasting and physical dysfunction are addressed simultaneously[1, 53] but thus far limited clinical trials have investigated this approach. A multimodal approach would presumably involve nutritional supplementation to compensate reduces in dietary intake and increases in energy requirements[54]. Accordingly, recent guidelines for management of lung cancer include an advice on nutritional supplementation in patients with NSCLC: "*addition of high caloric and protein supplements (1.5 kcal/kg) are suggested to achieve weight stabilization in patients that are undergoing treatment for NSCLC and have experienced weight loss*"[55]. However, the evidence for this suggestion is currently limited and the recommendation only involves advice on total protein intake, whereas evidence in experimental research indicates that attenuation of muscle wasting and stimulation of muscle anabolism in cachexia might be optimal when supplementing specific amino acids, such as branched chain amino acids (BCAAs)[56]. To determine the optimal nutritional advice in patients with cancer cachexia, currently available (pre-) clinical data needs to be evaluated. Furthermore, increasing insight in muscular targets of specific dietary formulas would further elucidate the mechanistic potential of (muscle) mass maintenance by tailored nutritional support as single therapy or as integrated part of a multimodal approach.

Molecular mechanisms of skeletal muscle wasting in cancer cachexia

Because muscle content predominantly comprises protein, the balance between protein synthesis and protein degradation is essential to muscle mass regulation[57, 58]. Moreover, myonuclear turn-over constitutes an additional mechanism of muscle mass regulation by the accretion (regeneration) and loss of myonuclei (apoptosis)[27, 58-61]. Maintenance of muscle mass is an intricate and dynamic process that is strictly regulated under physiologic and pathophysiologic conditions, including cancer cachexia[57, 58].

Regulation of protein synthesis and degradation in skeletal muscle

The PI3K/Akt signaling pathway is considered a major regulator of muscle protein synthesis[62]. A central mediator in the PI3K/Akt pathway is the serine-theorine protein kinase Akt, which is a potent inducer of muscle protein synthesis and an inhibitor of proteolytic cues[62]. Via phosphorylation of downstream substrates, Akt influences the activity of important mediators involved in protein synthesis, e.g. mammalian target of rapamycin (mTOR) and Glycogen synthase kinase 3 beta (GSK-3β). When phosphorylated by Akt, mTOR subsequently orchestrates activation of eukaryotic translation Initiation Factor 4E **(**eIF-4E) and p70S6 kinase (p70S6K), target molecules that are important in the initiation and elongation phases of mRNA translation[59]. Conversely, GSK-3β phosphorylation by Akt suppresses GSK-3b enzymatic activity, resulting in de-repression of translation initiator eIF2B, which facilitates protein synthesis[59, 62].

In addition to the stimulatory effects on muscle protein synthesis, Akt also functions as a regulator of proteolytic signaling[62]. When activated, Akt can inhibit proteolytic signaling by suppressing the family of Forkhead box (FOXO) transcription factors[62]. FOXO proteins are involved in the regulation of two major proteolytic systems: the ubiquitin 26S proteasome system (UPS) and autophagy lysosomal pathway (ALP)[63-65].

In the UPS, individual proteins are modified by covalent attachment of poly-ubiquitin-chains[66, 67]. This process, termed ubiquitin-conjugation, includes a number of steps relying on E1 ubiquitin-activating enzymes (activates ubiquitin), E2 ubiquitin conjugases (conjugates ubiquitin molecules to substrates or polymer chains) and E3 ubiquitin ligases (covalently binds ubiquitin chains to specific protein substrates)[67]. Conjugation of poly-ubiquitin chains marks the substrate protein for degradation by the 26S-proteasome, where the actual protein breakdown occurs[67]. Specifically, E3-ubiquitin ligases Muscle-specific RING finger 1 (MuRF1) and MAFbx/Atrogin-1 (Atrogin-1) but also Tripartite motif-containing protein 32 (TRIM32), Neuronal precursor cell-expressed developmentally down regulated 4 (Nedd4) are considered important for targeting of specific substrate proteins in skeletal muscle[68-71].

In the lysosomal pathway, formation of the autophagosome, i.e. the process in which cellular compartments are sequestered, is followed by fusion with lysosomal structures and subsequent degradation of its content[72]. Autophagy is considered a

bulk degradation system but while it was long considered a non-selective degradation system, recent literature suggests selective elimination of cytoplasmic components by the autophagy process[72-74].

Figure 2 illustrates a schematic representation of muscle protein synthesis and degradation pathways.

Protein synthesis and degradation in cancer cachexia

The general concept of cachexia-related tissue wasting is that the balance between synthesis and degradation, which preserves muscle mass in physiologic conditions, is disturbed[27, 75]. The general consensus is that proteolytic activity is disproportionately increased and exceeds muscle protein synthesis[27, 75]. This subsequently results in net loss of muscle proteins and consequently, loss muscle of mass[27].

With respect to muscle protein synthesis, data in cancer cachexia (models) is scarce and inconsistent. In experimental models of cancer cachexia, both stable and decreased phosphorylation of the anabolic integrator Akt has been observed[76, 77]. In the clinical setting, one study showed a decrease in expression of muscle Akt and phosphorylation of several of its downstream targets of cachectic patients with pancreatic cancer in comparison with non-cachectic patients[78]. Conversely, no changes were found in Akt signaling between colorectal cancer patients exhibiting lean mass atrophy and healthy controls[79].

Proteolytic signaling has been studied more frequently in cancer cachexia, as it is generally believed that increased proteolysis plays an important role in cancer cachexia-associated muscle wasting[27]. Experimental research has predominantly provided evidence for increased proteolytic signaling via activity of the UPS in cancer-induced muscle atrophy[75]. Especially muscle-specific E3 ubiquitin-ligases MuRF1 and Atrogin-1 are considered essential in muscle protein degradation in cancer cachexia models[68, 75]. Recent experimental studies also indicated that autophagy contributes to protein degradation in cancer cachexia[80]. However, while increased proteolytic UPS and autophagy signaling appears to be linked to muscle wasting in experimental models of cancer cachexia, its exact involvement in clinical cancer cachexia has yet to be revealed, as current data has mainly been obtained

from small and heterogeneous studies, which demonstrate contradicting results[79, 81-84].

Figure 2 illustrates a schematic representation of the muscle protein synthesis and degradation pathways presumably involved in cancer cachexia.

Myonuclear turn-over in skeletal muscle

An important concept in postnatal myogenic adaptation is the myonuclear domain theory, i.e. in a multinucleated muscle fiber a single myonucleus controls cellular processes of a specified amount of sarcoplasm[85]. Alterations in myonuclear turn-over could therefore also affect muscle size by a disturbed balance in accretion or loss of myonuclei[85].

Essential for myonuclear accretion is the activation and proliferation of quiescent, mononuclear muscle precursor cells named satellite cells[86]. Subsequent myogenic differentiation of these cells and fusion provides new myonuclei to myofibers (myogenesis)[86]. The important regulating role for myogenic regulatory factors (MRF's) including MyoD and Myogenin in myogenesis is well recognized and involves activation of muscle-specific gene transcription (**Figure 2**)[87, 88]. Apoptosis is a process of controlled cell death, which can be activated by intracellular or systemic stimuli and involves several executive steps including activation of highly specific caspases[89, 90]. Although it remains to be conclusively demonstrated, apoptotic mechanisms have been postulated in selective removal of myonuclei from multinucleated muscle fibers, which may contribute to myonuclear turn-over and control of muscle mass[90, 91].

Figure 2 illustrates a schematic representation of myonuclear mediators in skeletal muscle.

Myonuclear turn-over in cancer cachexia

Especially downregulation of Myogenic Regulatory Factors (MRFs) such as MyoD and Myogenin has been implicated in cancer cachexia by studies in experimental models[92-94]. Furthermore, decreased MyoD protein content was observed in a study of weight-losing patients with gastro-intestinal cancer compared with controls[95],

which is in contrast to increased MyoD mRNA expression in early stage and stable MyoD expression in advanced stage weight-losing gastric cancer patients[96].

Though some reports describe increased apoptotic activity in experimental cancer cachexia[97-99], alterations in the rate of apoptosis remains contradicting in the few human studies that are published[95, 100].

As can be appreciated from these results, myonuclear turn-over seems to be affected in experimental models of cancer cachexia, while studies investing these mechanisms in patients with cancer cachexia are scarce and inconclusive.

Figure 2 illustrates a schematic representation of the myonuclear mediators presumably involved in cancer cachexia.

Transduction and integration of extra-muscular cues to protein- and myonuclear turn-over regulation in cancer cachexia

At the muscle level, several signaling modules that integrate systemic signals and subsequently control pathways involved in protein and myonuclear turn-over have been implicated in cancer cachexia[27].

An important integrator of systemic pro-inflammatory stimuli is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[101, 102]. Upon activation by inflammatory mediators, IκBα, a member of the Inhibitory κB family (IκB's), is degraded via an UPS-dependent mechanism[103]. This allows the NF-κB complex to translocate to the nucleus and convey its action on a variety of cellular processes, which in skeletal muscle includes activation of proteolytic UPS activity and inhibition of myogenic regulators[101, 103-105]. Experimental research has indicated that muscle wasting in cancer cachexia depends, at least partly, on NF-κB activation [104-107]. However, NF-κB activity has scarcely been investigated in patients suffering from cancer cachexia and therefore, needs further exploration[106].

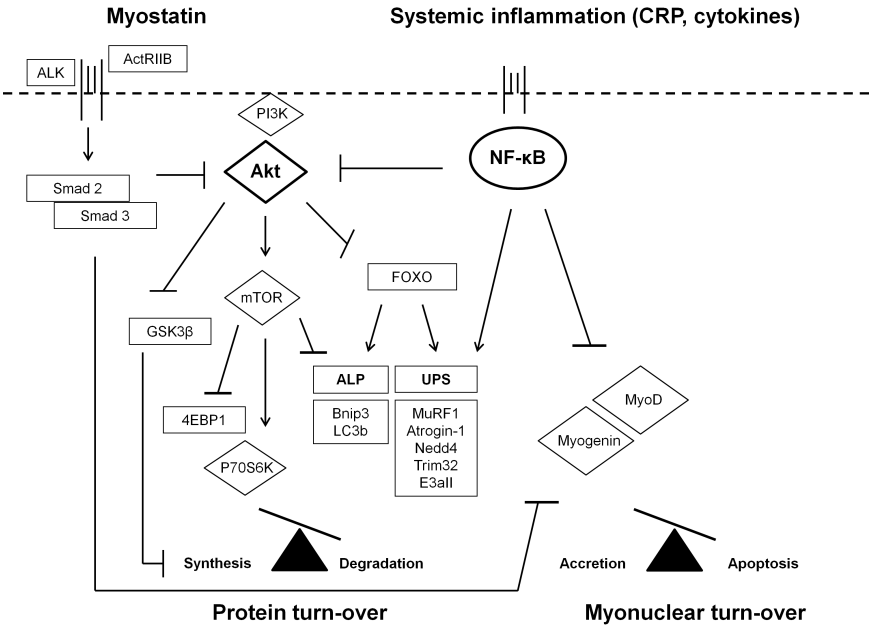
A more recently identified molecule involved in skeletal muscle protein and nuclear turn-over mediator is myostatin, a TGF-beta (TGFβ) super family member[108]. Its potential in muscle mass regulation can be readily appreciated from myostatin null animals, which display an exceptionally hypertrophic phenotype[108]. The intracellular signaling cascade involved in myostatin signaling is initiated by binding of a myostatin molecule to its muscular receptor, typically ActRIIB as this is the

predominant receptor in skeletal muscle (in comparison with ActRIIA) [109]. Myostatin binding to the ActRIIB receptors stimulates recruitment of activin receptor like kinases (ALK's), which induce an intracellular activation cascade including phosphorylation of Smad 2 and 3 proteins[110]. These Smad transcription factors subsequently form complexes and transfer to the nucleus to stimulate selected gene transcription[110-112]. Due to the relative recent identification of myostatin in regulation of muscle mass, the exact role of myostatin activation in cancer cachexia has not been studied in detail yet. However, while experimental research has indicated that myostatin activation leads to induction of proteolytic systems, inhibition of the anabolic PI3K/Akt pathway and decreased activity of MRF's[27, 110, 113, 114], early reports on myostatin in human cancer cachexia have shown contradicting results[79, 115].

Figure 2 illustrates a schematic representation of these regulatory mediators and their putative role in protein and myonuclear turn-over during cancer cachexia.

Figure 2

Schematic representation of signaling molecules putatively involved in muscle mass turn-over in cancer cachexia



Inhibitory signal

Activating signal

ActRIIB: activin receptor type II B, ALK: activin receptor like kinase, CRP: C reactive protein, mTOR: mammalian target of rapamycin, NF-κB: Nuclear Factor kappa B, PI3K: Phosphatidylinositide 3-kinases, GSK-3β: Glycogen synthase kinase 3 beta, 4EBP1: 4E-binding protein 1, P70S6K: P70S6 kinase, FOXO 1: Forkhead box protein 1, FOXO 3a; Forkhead box protein 3a, UPS: ubiquitin 26S-proteasome system, MuRF1: Muscle-specific RING finger 1 (MuRF1), Atrogin-1: MAFbx/Atrogin-1, TRIM32: Tripartite motif-containing protein 32, Nedd4: Neuronal precursor cell-expressed developmentally down regulated 4, E3aII: E3alpha-II, LC3B: Microtubule-associated proteins 1A/1B light chain 3B, BNIP-3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3.

Muscle dysfunction in cancer cachexia

Fatigue and muscle dysfunction are common denominators of decreased performance status in cancer cachexia[1]. The impact of these symptoms are significant, e.g. the presence of a low performance status might result in the choice for a secondary anti-tumor treatment regimen because the expected risks of the first choice treatment might not outweigh treatment benefits[20-22]. Conceivably, this can negatively influence prognosis. Increased fatigue and decreased muscle dysfunction might (partly) be explained by decreased muscle strength, which largely results from the loss of contractile units due to muscle wasting[1, 6, 7]. However, alterations in muscle energy metabolism could also affect muscle function. In COPD, a chronic lung disorder that has a high prevalence of cachexia and is also associated with muscle dysfunction and fatigue, alterations in muscle energy metabolism have been linked to decreased muscle endurance. In patients suffering from COPD, a shift from oxidative to glycolytic metabolism, decreased oxidative capacity and mitochondrial dysfunction is observed [116-119]. Combined theses processes can be referred to as "loss of oxidative phenotype (Oxphen)". Findings in experimental models of cancer cachexia suggest that in parallel to the findings in COPD, decreases in oxidative metabolism occur in muscle during inflammation-induced cancer cachexia, i.e. a shift from oxidative to glycolytic metabolism and mitochondrial dysfunction[120-123]. Based on these observations, altered muscle energy metabolism might also play a role in clinical cancer cachexia. However, oxidative and glycolytic metabolism has never been studied in muscle of patients with lung cancer cachexia.

Aims & outline of the thesis

The overall aim of this thesis was to provide new insight in putative triggers and mechanisms involved in initiation and progression of weight loss and muscle wasting in patients with lung cancer cachexia. Two specific aims were addressed:

- To investigate whether early weight loss during concurrent administration of chemotherapy and radiotherapy for stage III NSCLC is associated with decreased dietary intake as a result of therapy-induced esophagitis

- To investigate whether alterations in muscle protein and myonuclear turnover, and energy metabolism are present in skeletal muscle of patients with progressive stages of NSCLC cachexia, i.e. pre-cachexia and cancer cachexia.

In **chapter 2**, body weight alterations in patients with NSCLC treated with concurrent CT-RT were studied in a retrospective and prospective study. Patients treated with concurrent CT-RT were evaluated throughout the course of treatment to identify the time of onset of body weight loss, and to determine whether or not this coincided with dysphagia and decreased dietary intake associated with radiation esophagitis. Based on clinical observations it was hypothesized that body weight loss precedes significant esophagitis.

In **chapter 3**, a literature study was conducted on availability of (clinical) data regarding the efficacy of dietary protein supplementation in counterbalancing muscle atrophy during cachexia. Characteristics of diet formulations as well as the time of administration were considered. Furthermore, the putative molecular mechanisms by which proteins or specific amino acids could attenuate muscle mass loss were evaluated.

From **chapter 4** on, alterations in skeletal muscle protein and myonuclear turnover, as wells as energy metabolism were investigated in patients with progressive stages of NSCLC cachexia. The patient groups of which findings are presented in **chapter 4 and 6** were stratified according to the recent international cancer cachexia consensus. Consequently, this dissertation contributes to the validation of this definition as an instrument for standardizing cancer cachexia classification and

management.

In **chapter 4**, a comprehensive verification of protein and myonuclear turnover markers identified in experimental cancer cachexia was conducted in patients with NSCLC pre-cachexia and cachexia. In addition, it was studied whether systemic mediators are causally linked to muscular activation of the inflammatory integrator NF-κB.

In **chapter 5**, exercise endurance was assessed to investigate muscle function in comprehensively phenotyped pre-cachectic patients with NSCLC. In addition, muscle biopsy analyses were performed to evaluate activation of the proteolytic ubiquitin proteasome system.

In **chapter 6**, we investigated whether alterations in muscle oxidative phenotype are present in patients with NSCLC cachexia based on experimental evidence in models of cancer cachexia. Muscle energy metabolism was also studied in pre-cachectic patients with NSCLC because the data in chapter 5 showed that decreased exercise capacity is already present in pre-cachectic patients with NSCLC.

Chapter 7 provides a critical evaluation of the data obtained in this dissertation and directions for future research, and includes presentation of recent preliminary data.

References

1. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489-495 (2011).
2. Delano, M.J. & Moldawer, L.L. The origins of cachexia in acute and chronic inflammatory diseases. *Nutr Clin Pract* **21**, 68-81 (2006).
3. Evans, W.J. *et al.* Cachexia: a new definition. *Clin Nutr* **27**, 793-799 (2008).
4. Dewys, W.D. *et al.* Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. *Am J Med* **69**, 491-497 (1980).
5. Fearon, K.C., Voss, A.C., Hustead, D.S. & Cancer Cachexia Study, G. Definition of cancer cachexia: effect of weight loss, reduced food intake, and systemic inflammation on functional status and prognosis. *Am J Clin Nutr* **83**, 1345-1350 (2006).
6. Jubrias, S.A., Odderson, I.R., Esselman, P.C. & Conley, K.E. Decline in isokinetic force with age: muscle cross-sectional area and specific force. *Pflugers Arch* **434**, 246-253 (1997).
7. Larsson, L., Grimby, G. & Karlsson, J. Muscle strength and speed of movement in relation to age and muscle morphology. *J Appl Physiol* **46**, 451-456 (1979).
8. Weber, M.A. *et al.* Morphology, metabolism, microcirculation, and strength of skeletal muscles in cancer-related cachexia. *Acta Oncol* **48**, 116-124 (2009).
9. Pausch, T. *et al.* Cachexia but not obesity worsens the postoperative outcome after pancreatoduodenectomy in pancreatic cancer. *Surgery* **152**, S81-88 (2012).
10. Prado, C.M. *et al.* Body composition as an independent determinant of 5-fluorouracil-based chemotherapy toxicity. *Clin Cancer Res* **13**, 3264-3268 (2007).
11. Tsai, S. Importance of lean body mass in the oncologic patient. *Nutr Clin Pract* **27**, 593-598 (2012).
12. von Haehling, S. Cachexia as major underestimated and unmet medical need: facts and numbers. *J Cachex Sarcopenia Muscle* **1**, 1-5 (2010).
13. Tisdale, M.J. Cachexia in cancer patients. *Nat Rev Cancer* **2**, 862-871 (2002).
14. Martin, L. *et al.* Cancer cachexia in the age of obesity: skeletal muscle depletion is a powerful prognostic factor, independent of body mass index. *J Clin Oncol* **31**, 1539-1547 (2013).
15. Jemal, A. *et al.* Global cancer statistics. *CA Cancer J Clin* **61**, 69-90 (2011).
16. Alberg, A.J., Brock, M.V., Ford, J.G., Samet, J.M. & Spivack, S.D. Epidemiology of lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e1S-29S (2013).
17. Rivera, M.P., Mehta, A.C. & Wahidi, M.M. Establishing the diagnosis of lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e142S-165S (2013).
18. Molina, J.R., Yang, P., Cassivi, S.D., Schild, S.E. & Adjei, A.A. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. *Mayo Clin Proc* **83**, 584-594 (2008).

19. Mirsadraee, S., Oswal, D., Alizadeh, Y., Caulo, A. & van Beek, E., Jr. The 7th lung cancer TNM classification and staging system: Review of the changes and implications. *World J Radiol* **4**, 128-134 (2012).
20. Howington, J.A., Blum, M.G., Chang, A.C., Balekian, A.A. & Murthy, S.C. Treatment of stage I and II non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e278S-313S (2013).
21. Ramnath, N. *et al.* Treatment of stage III non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e314S-340S (2013).
22. Socinski, M.A. *et al.* Treatment of stage IV non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e341S-368S (2013).
23. Semrau, S., Klautke, G., Virchow, J.C., Kundt, G. & Fietkau, R. Impact of comorbidity and age on the outcome of patients with inoperable NSCLC treated with concurrent chemoradiotherapy. *Respir Med* **102**, 210-218 (2008).
24. Staal-van den Brekel, A.J., Schols, A.M., ten Velde, G.P., Buurman, W.A. & Wouters, E.F. Analysis of the energy balance in lung cancer patients. *Cancer Res* **54**, 6430-6433 (1994).
25. Ross, P.J. *et al.* Do patients with weight loss have a worse outcome when undergoing chemotherapy for lung cancers? *Br J Cancer* **90**, 1905-1911 (2004).
26. van der Meij, B.S. *et al.* Pre-cachexia and cachexia at diagnosis of stage III non-small-cell lung carcinoma: an exploratory study comparing two consensus-based frameworks. *Br J Nutr* **109**, 2231-2239 (2013).
27. Fearon, K.C., Glass, D.J. & Guttridge, D.C. Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell Metab* **16**, 153-166 (2012).
28. Argiles, J.M., Busquets, S. & Lopez-Soriano, F.J. Anti-inflammatory therapies in cancer cachexia. *Eur J Pharmacol* **668 Suppl 1**, S81-86 (2011).
29. Braun, T.P. & Marks, D.L. Pathophysiology and treatment of inflammatory anorexia in chronic disease. *J Cachexia Sarcopenia Muscle* **1**, 135-145 (2010).
30. Deans, C. & Wigmore, S.J. Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr Opin Clin Nutr Metab Care* **8**, 265-269 (2005).
31. Tisdale, M.J. The ubiquitin-proteasome pathway as a therapeutic target for muscle wasting. *J Support Oncol* **3**, 209-217 (2005).
32. Smyth, M.J., Dunn, G.P. & Schreiber, R.D. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol* **90**, 1-50 (2006).
33. Dunn, G.P., Old, L.J. & Schreiber, R.D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21**, 137-148 (2004).
34. Keibel, A., Singh, V. & Sharma, M.C. Inflammation, microenvironment, and the immune system in cancer progression. *Curr Pharm Des* **15**, 1949-1955 (2009).
35. Allavena, P., Sica, A., Solinas, G., Porta, C. & Mantovani, A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol* **66**, 1-9 (2008).

36. Falconer, J.S., Fearon, K.C., Plester, C.E., Ross, J.A. & Carter, D.C. Cytokines, the acute-phase response, and resting energy expenditure in cachectic patients with pancreatic cancer. *Ann Surg* **219**, 325-331 (1994).
37. Donohoe, C.L., Ryan, A.M. & Reynolds, J.V. Cancer cachexia: mechanisms and clinical implications. *Gastroenterol Res Pract* **2011**, 601434 (2011).
38. Suzuki, H., Asakawa, A., Amitani, H., Nakamura, N. & Inui, A. Cancer cachexia--pathophysiology and management. *J Gastroenterol* **48**, 574-594 (2013).
39. Krasnow, S.M. & Marks, D.L. Neuropeptides in the pathophysiology and treatment of cachexia. *Curr Opin Support Palliat Care* **4**, 266-271 (2010).
40. Ramos, E.J. *et al.* Cancer anorexia-cachexia syndrome: cytokines and neuropeptides. *Curr Opin Clin Nutr Metab Care* **7**, 427-434 (2004).
41. Whitehouse, A.S. & Tisdale, M.J. Increased expression of the ubiquitin-proteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the transcription factor NF-kappaB. *Br J Cancer* **89**, 1116-1122 (2003).
42. Tisdale, M.J. Tumor-host interactions. *J Cell Biochem* **93**, 871-877 (2004).
43. Tisdale, M.J. The 'cancer cachectic factor'. *Support Care Cancer* **11**, 73-78 (2003).
44. Wang, Q., Lu, J.B., Wu, B. & Hao, L.Y. Expression and clinicopathologic significance of proteolysis-inducing factor in non-small-cell lung cancer: an immunohistochemical analysis. *Clin Lung Cancer* **11**, 346-351 (2010).
45. Monitto, C.L., Dong, S.M., Jen, J. & Sidransky, D. Characterization of a human homologue of proteolysis-inducing factor and its role in cancer cachexia. *Clin Cancer Res* **10**, 5862-5869 (2004).
46. Teich, N. *et al.* The presence of the proteolysis-inducing factor in urine does not predict the malignancy of a pancreatic tumour. *BMC Gastroenterol* **5**, 20 (2005).
47. Jatoi, A. *et al.* The proteolysis-inducing factor: in search of its clinical relevance in patients with metastatic gastric/esophageal cancer. *Dis Esophagus* **19**, 241-247 (2006).
48. McGuire, M. Nutritional care of surgical oncology patients. *Seminars in oncology nursing* **16**, 128-134 (2000).
49. Donaldson, S.S. Nutritional consequences of radiotherapy. *Cancer Res* **37**, 2407-2413 (1977).
50. Nicolini, A. *et al.* Malnutrition, anorexia and cachexia in cancer patients: A mini-review on pathogenesis and treatment. *Biomed Pharmacother* **67**, 807-817 (2013).
51. De Ruysscher, D. *et al.* Maximal neutropenia during chemotherapy and radiotherapy is significantly associated with the development of acute radiation-induced dysphagia in lung cancer patients. *Ann Oncol* **18**, 909-916 (2007).
52. Senior, K. Why is progress in treatment of cancer cachexia so slow? *Lancet Oncol* **8**, 671-672 (2007).
53. Madeddu, C., Maccio, A. & Mantovani, G. Multitargeted treatment of cancer cachexia. *Crit Rev Oncog* **17**, 305-314 (2012).
54. Bosaeus, I. Nutritional support in multimodal therapy for cancer cachexia. *Support Care Cancer* **16**, 447-451 (2008).
55. Deng, G.E. *et al.* Complementary therapies and integrative medicine in lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e420S-436S (2013).

56. Vary, T.C. & Lynch, C.J. Nutrient signaling components controlling protein synthesis in striated muscle. *J Nutr* **137**, 1835-1843 (2007).
57. Glass, D.J. Signaling pathways perturbing muscle mass. *Curr Opin Clin Nutr Metab Care* **13**, 225-229 (2010).
58. Banerjee, A. & Guttridge, D.C. Mechanisms for maintaining muscle. *Curr Opin Support Palliat Care* **6**, 451-456 (2012).
59. Glass, D.J. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* **37**, 1974-1984 (2005).
60. Langen, R.C., Gosker, H.R., Remels, A.H. & Schols, A.M. Triggers and mechanisms of skeletal muscle wasting in chronic obstructive pulmonary disease. *Int J Biochem Cell Biol* (2013).
61. Remels, A.H., Gosker, H.R., Langen, R.C. & Schols, A.M. The mechanisms of cachexia underlying muscle dysfunction in COPD. *J Appl Physiol* **114**, 1253-1262 (2013).
62. Glass, D.J. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. *Curr Top Microbiol Immunol* **346**, 267-278 (2010).
63. Mammucari, C., Schiaffino, S. & Sandri, M. Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. *Autophagy* **4**, 524-526 (2008).
64. Zhao, J. *et al.* FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab* **6**, 472-483 (2007).
65. Mammucari, C. *et al.* FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**, 458-471 (2007).
66. Schwartz, A.L. & Ciechanover, A. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med* **50**, 57-74 (1999).
67. Ciechanover, A. The ubiquitin-mediated system for intracellular protein degradation. *J Basic Clin Physiol Pharmacol* **2**, 141-159 (1991).
68. Bodine, S.C. *et al.* Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-1708 (2001).
69. Nagpal, P. *et al.* The ubiquitin ligase Nedd4-1 participates in denervation-induced skeletal muscle atrophy in mice. *PLoS One* **7**, e46427 (2012).
70. Plant, P.J. *et al.* Cellular markers of muscle atrophy in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **42**, 461-471 (2010).
71. Cohen, S., Zhai, B., Gygi, S.P. & Goldberg, A.L. Ubiquitylation by Trim32 causes coupled loss of desmin, Z-bands, and thin filaments in muscle atrophy. *J Cell Biol* **198**, 575-589 (2012).
72. Klionsky, D.J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4**, 151-175 (2008).
73. Mizushima, N. Methods for monitoring autophagy. *Int J Biochem Cell Biol* **36**, 2491-2502 (2004).
74. Dengjel, J., Kristensen, A.R. & Andersen, J.S. Ordered bulk degradation via autophagy. *Autophagy* **4**, 1057-1059 (2008).
75. Acharyya, S. & Guttridge, D.C. Cancer cachexia signaling pathways continue to emerge yet much still points to the proteasome. *Clin Cancer Res* **13**, 1356-1361 (2007).
76. Asp, M.L., Tian, M., Wendel, A.A. & Belury, M.A. Evidence for the contribution of insulin resistance to the development of cachexia in tumor-bearing mice. *Int J Cancer* **126**, 756-763 (2010).
77. Penna, F. *et al.* Muscle atrophy in experimental cancer cachexia: is the IGF-1 signaling pathway involved? *Int J Cancer* **127**, 1706-1717 (2010).

78. Schmitt, T.L. *et al.* Activity of the Akt-dependent anabolic and catabolic pathways in muscle and liver samples in cancer-related cachexia. *J Mol Med (Berl)* **85**, 647-654 (2007).
79. Williams, J.P. *et al.* Effect of tumor burden and subsequent surgical resection on skeletal muscle mass and protein turnover in colorectal cancer patients. *Am J Clin Nutr* **96**, 1064-1070 (2012).
80. Penna, F. *et al.* Autophagic degradation contributes to muscle wasting in cancer cachexia. *Am J Pathol* **182**, 1367-1378 (2013).
81. Bossola, M. *et al.* Increased muscle ubiquitin mRNA levels in gastric cancer patients. *Am J Physiol Regul Integr Comp Physiol* **280**, R1518-1523 (2001).
82. Bossola, M. *et al.* Increased muscle proteasome activity correlates with disease severity in gastric cancer patients. *Ann Surg* **237**, 384-389 (2003).
83. Jagoe, R.T., Redfern, C.P., Roberts, R.G., Gibson, G.J. & Goodship, T.H. Skeletal muscle mRNA levels for cathepsin B, but not components of the ubiquitin-proteasome pathway, are increased in patients with lung cancer referred for thoracotomy. *Clin Sci (Lond)* **102**, 353-361 (2002).
84. Williams, A., Sun, X., Fischer, J.E. & Hasselgren, P.O. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery* **126**, 744-749; discussion 749-750 (1999).
85. Allen, D.L., Roy, R.R. & Edgerton, V.R. Myonuclear domains in muscle adaptation and disease. *Muscle Nerve* **22**, 1350-1360 (1999).
86. Montarras, D., L'Honore, A. & Buckingham, M. Lying low but ready for action: the quiescent muscle satellite cell. *FEBS J* **280**, 4036-4050 (2013).
87. Rescan, P.Y. Regulation and functions of myogenic regulatory factors in lower vertebrates. *Comp Biochem Physiol B Biochem Mol Biol* **130**, 1-12 (2001).
88. Mok, G.F. & Sweetman, D. Many routes to the same destination: lessons from skeletal muscle development. *Reproduction* **141**, 301-312 (2011).
89. Primeau, A.J., Adhihetty, P.J. & Hood, D.A. Apoptosis in heart and skeletal muscle. *Can J Appl Physiol* **27**, 349-395 (2002).
90. Otrocka-Domagala, I. Sensitivity of skeletal muscle to pro-apoptotic factors. *Pol J Vet Sci* **14**, 683-694 (2011).
91. Dupont-Versteegden, E.E. Apoptosis in skeletal muscle and its relevance to atrophy. *World J Gastroenterol* **12**, 7463-7466 (2006).
92. Acharyya, S. *et al.* Cancer cachexia is regulated by selective targeting of skeletal muscle gene products. *J Clin Invest* **114**, 370-378 (2004).
93. Costelli, P. *et al.* Skeletal muscle wasting in tumor-bearing rats is associated with MyoD down-regulation. *Int J Oncol* **26**, 1663-1668 (2005).
94. Penna, F. *et al.* Muscle wasting and impaired myogenesis in tumor bearing mice are prevented by ERK inhibition. *PLoS One* **5**, e13604 (2010).
95. Busquets, S. *et al.* Apoptosis is present in skeletal muscle of cachectic gastro-intestinal cancer patients. *Clin Nutr* **26**, 614-618 (2007).
96. Pessina, P. *et al.* Skeletal muscle of gastric cancer patients expresses genes involved in muscle regeneration. *Oncol Rep* **24**, 741-745 (2010).
97. Smith, H.J. & Tisdale, M.J. Induction of apoptosis by a cachectic-factor in murine myotubes and inhibition by eicosapentaenoic acid. *Apoptosis* **8**, 161-169 (2003).
98. Figueras, M. *et al.* Interleukin-15 is able to suppress the increased DNA fragmentation associated with muscle wasting in tumour-bearing rats. *FEBS Lett* **569**, 201-206 (2004).

99. Irminger-Finger, I., Busquets, S., Calabrio, F., Lopez-Soriano, F.J. & Argiles, J.M. BARD1 content correlates with increased DNA fragmentation associated with muscle wasting in tumour-bearing rats. *Oncol Rep* **15**, 1425-1428 (2006).
100. Bossola, M. *et al.* Skeletal muscle apoptosis is not increased in gastric cancer patients with mild-moderate weight loss. *Int J Biochem Cell Biol* **38**, 1561-1570 (2006).
101. Guttridge, D.C., Mayo, M.W., Madrid, L.V., Wang, C.Y. & Baldwin, A.S., Jr. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science* **289**, 2363-2366 (2000).
102. Li, Y.P. & Reid, M.B. NF-kappaB mediates the protein loss induced by TNF-alpha in differentiated skeletal muscle myotubes. *Am J Physiol Regul Integr Comp Physiol* **279**, R1165-1170 (2000).
103. Jackman, R.W., Cornwell, E.W., Wu, C.L. & Kandarian, S.C. Nuclear factor-kappaB signalling and transcriptional regulation in skeletal muscle atrophy. *Exp Physiol* **98**, 19-24 (2013).
104. Cai, D. *et al.* IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* **119**, 285-298 (2004).
105. Wyke, S.M. & Tisdale, M.J. NF-kappaB mediates proteolysis-inducing factor induced protein degradation and expression of the ubiquitin-proteasome system in skeletal muscle. *Br J Cancer* **92**, 711-721 (2005).
106. Rhoads, M.G., Kandarian, S.C., Pacelli, F., Doglietto, G.B. & Bossola, M. Expression of NF-kappaB and IkappaB proteins in skeletal muscle of gastric cancer patients. *Eur J Cancer* **46**, 191-197 (2010).
107. Zhou, W. *et al.* Role of NF-kappaB and cytokine in experimental cancer cachexia. *World J Gastroenterol* **9**, 1567-1570 (2003).
108. McPherron, A.C., Lawler, A.M. & Lee, S.J. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90 (1997).
109. Lee, S.J. & McPherron, A.C. Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci U S A* **98**, 9306-9311 (2001).
110. Han, H.Q., Zhou, X., Mitch, W.E. & Goldberg, A.L. Myostatin/activin pathway antagonism: Molecular basis and therapeutic potential. *Int J Biochem Cell Biol* (2013).
111. Bradley, L., Yaworsky, P.J. & Walsh, F.S. Myostatin as a therapeutic target for musculoskeletal disease. *Cell Mol Life Sci* **65**, 2119-2124 (2008).
112. Tsuchida, K., Nakatani, M., Uezumi, A., Murakami, T. & Cui, X. Signal transduction pathway through activin receptors as a therapeutic target of musculoskeletal diseases and cancer. *Endocr J* **55**, 11-21 (2008).
113. Sakuma, K. & Yamaguchi, A. Sarcopenia and cachexia: the adaptations of negative regulators of skeletal muscle mass. *J Cachexia Sarcopenia Muscle* **3**, 77-94 (2012).
114. Busquets, S. *et al.* Myostatin blockage using actRIIB antagonism in mice bearing the Lewis lung carcinoma results in the improvement of muscle wasting and physical performance. *J Cachexia Sarcopenia Muscle* **3**, 37-43 (2012).
115. Aversa, Z. *et al.* Changes in myostatin signaling in non-weight-losing cancer patients. *Ann Surg Oncol* **19**, 1350-1356 (2012).
116. Gosker, H.R. *et al.* Striking similarities in systemic factors contributing to decreased exercise capacity in patients with severe chronic heart failure or COPD. *Chest* **123**, 1416-1424 (2003).

117. Gosker, H.R., Wouters, E.F., van der Vusse, G.J. & Schols, A.M. Skeletal muscle dysfunction in chronic obstructive pulmonary disease and chronic heart failure: underlying mechanisms and therapy perspectives. *Am J Clin Nutr* **71**, 1033-1047 (2000).
118. Allaire, J. *et al.* Peripheral muscle endurance and the oxidative profile of the quadriceps in patients with COPD. *Thorax* **59**, 673-678 (2004).
119. van den Borst, B. *et al.* Loss of quadriceps muscle oxidative phenotype and decreased endurance in patients with mild-to-moderate COPD. *J Appl Physiol* **114**, 1319-1328 (2013).
120. White, J.P. *et al.* Muscle oxidative capacity during IL-6-dependent cancer cachexia. *Am J Physiol Regul Integr Comp Physiol* **300**, R201-211 (2011).
121. White, J.P. *et al.* IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse. *Skelet Muscle* **2**, 14 (2012).
122. Constantinou, C. *et al.* Nuclear magnetic resonance in conjunction with functional genomics suggests mitochondrial dysfunction in a murine model of cancer cachexia. *Int J Mol Med* **27**, 15-24 (2011).
123. Diffee, G.M., Kalfas, K., Al-Majid, S. & McCarthy, D.O. Altered expression of skeletal muscle myosin isoforms in cancer cachexia. *Am J Physiol Cell Physiol* **283**, C1376-1382 (2002).

Chapter 2

Early body weight loss during concurrent chemo-radiotherapy for non-small cell lung cancer

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Abstract

Radiation-esophagitis and weight loss are frequently observed toxicities in patients treated with concurrent chemo-radiotherapy (CT-RT) for non-small cell lung cancer (NSCLC) and might be related. The purpose was to investigate whether weight loss already starts early after initiation of CT-RT and precedes radiation-esophagitis. In a retrospective cohort, weight and esophagitis grade ≥2 were assessed during the first weeks of (CT-)RT in patients treated with concurrent (n=102) or sequential (n=92) therapy. In a prospective validation study, data on body weight, esophagitis grade ≥2, nutritional intake and muscle strength were obtained before, during and following CT-RT. In the retrospective cohort, early weight loss was observed in concurrently treated patients ($p=0.002$), independent of esophagitis ≥ grade 2. Early weight loss was also observed in the prospective cohort ($p=0.003$) and was not accompanied by decreases in nutritional intake. In addition lower limb muscle strength rapidly declined ($p=0.042$). In the later weeks of treatment, further body weight loss occurred ($p<0.001$) despite increased nutritional supplementation and body weight was only partly recovered after 4 weeks post CT-RT ($p=0.003$). In conclusion, weight loss during concurrent CT-RT for NSCLC starts early and prior to onset of esophagitis, requiring timely and intense nutritional rehabilitation.

Introduction

Concurrent administration of chemotherapy and radiotherapy (CT-RT) is the treatment of choice for many patients with locally advanced non-small cell lung cancer (NSCLC). It has been demonstrated that this intensive multimodal treatment regimen results in significantly longer disease free and overall survival[1-4]. However, concurrent administration of CT-RT is associated with a higher incidence of severe esophagitis[5, 6]. Therefore, according to current treatment guidelines, only patients with minimal comorbidity and with a good performance status are considered eligible for concurrent CT-RT[7].

Concurrent CT-RT in NSCLC generally consists of one induction cycle of chemotherapy, which is followed by a 5-week period of concurrent CT-RT. Acute esophagitis develops during the administration of RT. Patients experience dysphagia, which continues to get worse up to 2 weeks after the end of CT-RT, with healing within 4–8 weeks. Severe (grade 3 or more) dysphagia is observed in about 25 % of patients treated with concurrent CT-RT and when taken any grade into account, over 80 % experience difficulties in swallowing[8]. Intuitively, the frequently observed body weight loss during concurrent CT-RT is a result of impaired dietary intake due to dysphagia[9]. However, alternatively, induction of systemic metabolic alterations by the intense treatment regimen could deplete body mass by altering neuroendocrine regulation of dietary intake and/or wasting of fat and muscle body compartments[10]. Because these alternative mechanisms might require different (co)interventions, the exact time course and etiology of body weight loss during CT-RT should be identified to optimize patient care[11, 12].

Based on clinical observations, we hypothesized that body weight loss starts early after initiation of therapy and precedes the presence of esophagitis. Since no published data on this clinically relevant problem is to the best of our knowledge available, we assessed weekly body weight changes during concurrent and sequential CT-RT for NSCLC and correlated this with esophagitis scores in a retrospective cohort. The findings in the retrospective cohort were validated in a prospective study design, in which body weight and esophagitis scores were assessed over a longer time period, i.e. prior, during and following concurrent CT-RT for NSCLC, and additional data on nutritional intake, muscle strength and quality of life was collected.

Patients and Methods

Study population

Outcome parameters were determined in a retrospective cohort and prospective study (**Figure 1**). A schematic representation of the study points in the retrospective cohort is depicted in **Figure 2A** and of the prospective study in **Figure 2B**. Please see the supplementary data for details on inclusion criteria, study population characteristics and ethical guidelines of the study.

Figure 1

Flowchart of inclusion in retrospective and prospective analysis

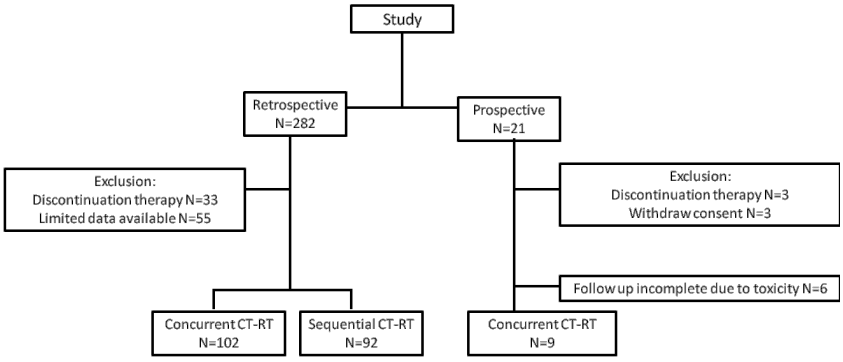
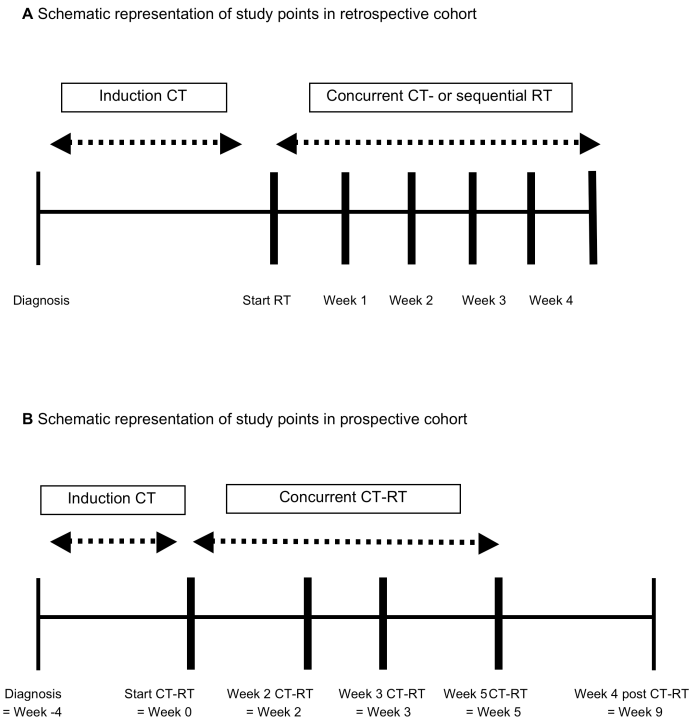


Figure 2

Study points in cohorts



Chemotherapy

Chemotherapy was administered according to national and international guidelines. Generally, patients received one or more cycles of induction therapy followed by RT alone, i.e. sequential CT-RT, or RT in combination with CT, i.e. concurrent CT-RT. Please see **Table 2** for an overview of the respective CT types used. Standard dose reduction rules were applied for all CT schemes if indicated. Cycles were repeated every 21 days.

Radiotherapy

RT techniques have been described previously[13, 14]. Please see the supplementary data for a short description. Patients in both the retrospective and prospective cohort

treated with concurrent CT-RT received on the same target volumes first 45 Gy, delivered in twice-daily fractions of 1.5 Gy with at least 8 h of inter-fraction interval, followed by once-daily fractions of 2 Gy until a pre-defined normal tissue constraint was reached, being a mean lung dose (MLD) of 19 ± 1 Gy or a spinal cord maximal dose of 54 Gy. The maximal allowed dose was 71 Gy. Patients treated with sequential therapy received twice-daily 1.8 Gy until the normal tissue constraints reported in the supplementary data were reached.

Toxicity scoring

Toxicity of treatment was assessed by a radiation oncologist using the Common Terminology Criteria for Adverse Events version 3.0 (CTCAEv3.0). According to the CTCAEv3.0, treatment-induced dysphagia and esophagitis are almost similar. For consistency, the problems with swallowing and dietary intake associated with concurrent or sequential CT-RT are referred to as esophagitis. According to the CTCAEv3.0, esophagitis causes changes in dietary intake from grade 2 on. In order to study effects of esophagitis on dietary intake, esophagitis scores were therefore further referred to as grade <2 or ≥2. Esophagitis scores were collected at the time points depicted in **Figure 2**.

Nutritional intake

In the prospective study, dietary intake was calculated using a 24 h dietary recall assessment at the time points indicated in **Figure 2B**. Energy and macronutrient intake were calculated according to Netherlands Nutrition Centre guidelines (www.voedingscentrum.nl).

Muscle strength

In the prospective study, hand and quadriceps muscle strength was assessed at the time points indicated in **Figure 2B** (when physical condition of patients allowed it). Hand muscle strength was assessed using a hand grip meter. Isometric and isokinetic strength of quadriceps muscle was measured by Biodex dynamometer (Biodex system version 3.3). Please see the supplementary data for a description of the muscle strength assessment procedure.

Quality of life

For the evaluation of the quality of life in patients in the prospective cohort, the European Organisation for Research and Treatment of Cancer (EORTC) quality of life questionnaire C30 (QLQ-C30) was used. Data on quality of life was obtained at the time points indicated in **Figure 2B**.

Statistical analysis

Data was analysed using SPSS version 15.0. For descriptive statistics, results are expressed as means ± standard deviation (SD). *P* values lower than 0.05 were considered statistically significant.

In the retrospective cohort, continuous variables were compared using an independent samples *T* test. The Pearson chi-square test was used for comparing categorical variables. Associations between body mass index (BMI; body weight corrected for height) changes during concurrent or sequential CT-RT and a number of clinical and treatment parameters (age, gender, World Health Organization (WHO) performance status, Charlson comorbidity index[15], smoking incidence, duration of CT-RT (in weeks), mean lung dose (MLD), mean esophageal dose (MED), maximum spinal cord dose and grade esophagitis ≥2) were analysed with longitudinal data analysis by means of a linear mixed regression model.

In the prospective data, paired sample *T* test was used for statistical analysis for outcome parameter comparison between specific time points.

Results

Retrospective cohort
Study population characteristics

Baseline characteristics of the study population are shown in **Table 1**. No differences were observed between patients treated with sequential and concurrent CT-RT with respect to gender, height, body weight at diagnosis or at start of (CT)-RT, histology, TNM stage, Charlson Comorbidity Index and smoking incidence, whereas the mean age of patients treated with concurrent CT-RT was significantly lower than the age of

sequentially treated patients ($p = 0.001$).

Treatment characteristics

Treatment characteristics are depicted in **Table 2**. Patients treated with concurrent CT-RT received one or two cycles of induction CT in 84.7 % of the cases. Induction CT in this group consisted in 82.2 % of cases of carboplatin or cisplatin combined with gemcitabine. The majority of patients (84.7 %) of concurrently treated patients received two cycles of cisplatin-based concurrent CT, combined with either vinorelbine or etoposide. Patients treated with sequential CT-RT received three cycles of induction CT in 89.8 % of cases. This induction chemotherapy consisted in 84.7 % of patients of carboplatin or cisplatin combined with gemcitabine (**Table 2**).

The total radiation dose was 61.4 ± 6.7 Gy (range 45–69 Gy) in the concurrent group and 59.2 ± 10.8 Gy (range 20–79 Gy) in sequentially treated patients, which was not significantly different (**Table 2**). Also, the mean lung, mean esophagus and maximal spinal cord dose did not differ between the two groups (**Table 2**).

Body weigth loss during concurrent and sequential CT-RT

A significantly higher number of sequentially than concurrently treated patients showed a decrease in body weight during the period between diagnosis and start of (CT-)RT, which represents the induction chemotherapy period ($p = 0.012$; **Table 3**). However, the mean loss of body weight during induction chemotherapy was not significantly different between both study groups ($p = 0.736$). From week 2 of (CT-) RT on, body weight loss was significantly more frequent in concurrently treated patients than in sequentially treated patients ($p = 0.005$; **Table 3**). Also, the mean body weight loss, both in absolute values (**Table 3**) as well as calculated as percentage of total body weight (**Figure 3a**), was significantly higher in concurrently treated patients in week 2 ($p = 0.002$), week 3 ($p = 0.003$) and week 4 ($p = 0.001$) of (CT-)RT when compared to start of (CT-)RT.

Table 1

Study population characteristics at baseline

	Retrospective		Prospective
	Sequential CT-RT	**Concurrent CT-RT**	**Concurrent CT-RT**
Number of patients	92	102	9
Age (years)			
Mean ± SD[1]	65.8 ± 9.4*	61.5 ± 8.6*	56.9 ± 10.3
Range	42 - 83	40 – 80	38 – 73
Gender (*n* (%))			
Male	61 (66%)	64 (63%)	6 (67%)
Female	31 (34%)	38 (37%)	3 (33%)
Body weight at diagnosis (kg)			
Mean ± SD	73.7 ± 15.4	72.8 ± 13.6	70.9 ± 14.1
Reported body weight loss in 6 months prior to diagnosis (% of total body weight)			
Mean ± SD	6.42 ± 7.34	4.65 ± 5.95	3.4 ± 5.7
Histology/ cytology (*n* (%))			
Adenocarcinoma	10 (10.9%)	22 (21.6%)	4 (44.4%)
Squamous cell	25 (27.2%)	22 (21.6%)	3 (33.3%)
Large cell	44 (47.8%)	39 (38.2%)	0 (00.0%)
Not otherwise specified	13 (14.1%)	19 (18.6%)	2 (22.2%)
Stage TNM[2] (*n* (%))			
IIIA	31 (33.7%)	31 (30.4%)	5 (55.6%)
IIIB	61 (66.3%)	71 (69.6%)	3 (33.3%)
IV	00 (00.0%)	00 (00.0%)	1 (11.1%)
Smoking (*n* (%))			
Current cigarette smoker	35 (38.0%)	43 (42.2%)	2 (22.2%)
Former cigarette smoker	49 (53.3%)	55 (53.9%)	7 (77.8%)
Never smoker	8 (8.7%)	4 (4.0%)	0 (00.0%)

[1] Mean ± standard deviation (SD)

[2] According to tumor-node-metastasis (TNM) International Staging System for Lung Cancer

** P<0.05 retrospective data, significant difference between sequential and concurrent treated patients (Independent sample T-test or Pearson Chi-Square test)*

Table 2

Treatment characteristics

	Retrospective		Prospective
	Sequential CT-RT	**Concurrent CT-RT**	**Concurrent CT-RT**
Number induction chemotherapy cycles (*n* (%))	***n* = 88**	***n* = 85**	***n* = 9**
0	0 (0.0%)*	0 (0.00%)*	3 (33.3%)
1	4 (4.5%)*	51 (60.0%)*	0 (0.00%)
2	4 (4.5%)*	21 (24.7%)*	6 (66.7%)
3	79 (89.8%)*	12 (14.1%)*	0 (0.00%)
4	1 (1.1%)*	1 (1.2%)*	0 (0.00%)
Type induction chemotherapy (*n* (%))	***n* = 85**	***n* = 84**	***n* = 9**
Carboplatin – gemcitabine	49 (57.6%)	44 (52.4%)	0 (0.0%)
Carboplatin – paclitaxel	1 (1.2%)	0 (0%)	0 (0.0%)
Carboplatin – docetaxel	3 (3.5%)	0 (0%)	0 (0.0%)
Carboplatin – etoposide	0 (0%)	1 (1.2%)	0 (0.0%)
Cisplatin – gemcitabine	23 (27.1%)	25 (29.8%)	0 (00.0%)
Cisplatin – vinorelbine	6 (7.1%)	2 (2.4%)	4 (44.4%)
Cisplatin – paclitaxel	1 (1.2%)	4 (4.8%)	0 (00.0%)
Cisplatin – etoposide	2 (2.4%)	5 (6.0%)	5 (55.6%)
Number of concurrent chemotherapy cycles (*n* %))		***n* = 87**	***n* = 9**
1		51 (60.0%)	1 (6.7%)
2		21 (24.7%)	14 (93.3%)
3		12 (14.1%)	0 (0.0%)
4		1 (1.2%)	0 (0.0%)
Type concurrent chemotherapy (*n* (%))		***n* = 87**	***n* = 9**
Cisplatin – vinorelbine		54 (62.1%)	4 (44.4%)
Cisplatin – etoposide		23 (26.4%)	5 (55.6%)
Cisplatin – vinorelbine – cetuximab		6 (6.9%)	0 (0.00%)
Carboplatin – etoposide		3 (3.4%)	0 (0.0%)
Carboplatin – paclitaxel		1 (1.1%)	0 (0.0%)
Treatment time RT (days)			
Mean ± SD	23 ± 6	31 ± 7*	33 ± 5
Range	7 - 41	14 - 52	26 - 41
Total dose RT (Gy)			
Mean ± SD	59.2 ± 10.8	61.4 ± 6.7	64.3 ± 6.6
Range	20 - 79	45 - 69	53 – 69
Mean lung dose (Gy)			
Mean ± SD	15.3 ± 3.8	15.6 ± 4.6	19.2 ± 1.2
Range	5 - 21	4 - 29	26 - 41
Mean esophageal dose (Gy)			
Mean ± SD	24.8 ± 10.1	24.7 ± 9.2	30.6 ± 9.5
Range	5 – 49	4 - 43	14.6 – 45.90
Max spinal cord dose (Gy)			
Mean ± SD	45.6 ± 11.0	44.0 ± 12.1	44.4 ± 9.6
Range	16 – 55	9 - 56	22.0 – 53.3

** P<0.05 retrospective data, significant difference between sequential and concurrent treated patients (independent sample T-test or Pearson Chi-Square test)*

Table 3

Body weight changes and grade esophagitis prior and during (CT-) RT

	Retrospective		Prospective
	Sequential CT-RT	**Concurrent CT-RT**	**Concurrent CT-RT**
Weight loss diagnosis–start (CT-)RT (kg)	***n* = 83**	***n* = 94**	***n* = 6**
Mean ± SD[1]	-1.31 ± 10.78	-0.50 ± 3.03	-0.13 ± 1.97
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight	51 (61.4%)*	75 (81.5%)*	4 (66.7%)
0-5% loss of total body weight	17 (20.5%)*	8 (8.7 %)*	2 (33.3 %)
≥ 5% loss of total body weight	15 (18.1%)*	9 (9.8%)*	0 (0.00%)
Weight loss week 1 (CT-)RT (kg)	***n* = 68**	***n* = 91**	
Mean ± SD[3]	0.38 ± 2.48	0.61 ± 1.69	
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight	39 (57.4%)	42 (46.2%)	
0-5% loss of total body weight	26 (38.2%)	45 (49.5%)	
≥ 5% loss of total body weight	3 (4.4%)	4 (4.4%)	
Weight loss week 2 (CT-)RT[3] (kg)	***n* = 71**	***n* = 94**	***n* = 9**
Mean ± SD	0.36 ± 2.27*	1.58 ± 2.2*	2.53 ± 1.75†
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight	37 (52.1%)*	27 (28.7%)	1 (11.1%)
0-5% loss of total body weight	29 (40.8%)*	50 (53.2%)	6 (66.7%)
≥ 5% loss of total body weight	5 (7.0%)*	17 (18.1%)	2 (22.2%)
Weight loss week 3 (CT-)RT (kg)	***n* = 65**	***n* = 87**	***n* = 9**
Mean ± SD	0.54 ± 2.21*	1.95 ± 2.67*	4.07 ± 3.07†
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight	31(47.7%)*	23 (26.4%)*	1 (11.1%)
0-5% loss of total body weight	29 (44.6%)*	47 (54.0%)*	3 (33.3%)
≥ 5% loss of total body weight	5 (7.7%)*	17 (19.5%)*	5 (55.6%)
Weight loss week 4 (CT-)RT (kg)	***n* = 29**	***n* = 71**	
Mean ± SD	0.22 ± 2.02*	2.56 ± 3.36*	
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight	17 (60.7%)*	17 (23.9%)*	
0-5% loss of total body weight	9 (32.1%)*	33 (46.5%)*	
≥ 5% loss of total body weight	2 (7.1%)*	21(29.6%)*	

Weight loss week 5 (CT-)RT (kg)			***n* = 9**
Mean ± SD			5.62 ± 2.43†
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight			2 (22.2%)
0-5% loss of total body weight			0 (0.00%)
≥ 5% loss of total body weight			7 (77.8%)
Weight loss week 4 post (CT-)RT (kg)			***n* = 9**
Mean ± SD			4.41 ± 3.11†
Number of patients losing weight (*n* (%)):			
≤ 0% loss of total body weight			1 (11.1%)
0-5% loss of total body weight			2 (22.2%)
≥ 5% loss of total body weight			6 (66.7%)
Grade esophagitis week 1 RT (n (%))	***n = 74***	***n = 86***	
< 2	73 (98.7%)	81 (94.2%)	
≥ 2	1 (1.4%)	5 (5.8%)	
Grade esophagitis week 2 RT (n (%))	***n = 79***	***n = 85***	***n* = 9**
< 2	71(89.9%)	77 (90.6%)	7 (77.8%)
≥ 2	8 (10.1%)	8 (9.4%)	2 (22.2%)
Grade esophagitis week 3 RT (n (%))	***n* = 72**	***n* = 81**	***n* = 9**
< 2	48 (66.6%)	53 (65.4%)	5 (55.6%)
≥ 2	24(33.3%)	28(34.5%)	4 (44.4%)
Grade esophagitis week 4 RT (n (%))	***n* = 31**	***n* = 66**	
< 2	22 (71.0%)	43 (65.2%)	
≥ 2	9 (29.0%)	23 (34.8%)	
Grade esophagitis week 5 RT (n (%))			***n* = 8**
< 2			2 (25%)
≥ 2			6 (75%)
Grade esophagitis week 4 post RT (n (%))			***n* = 12**
< 2			8 (88.9%)
≥ 2			1 (11.1%)

[1] *Mean ± standard deviation (SD)*

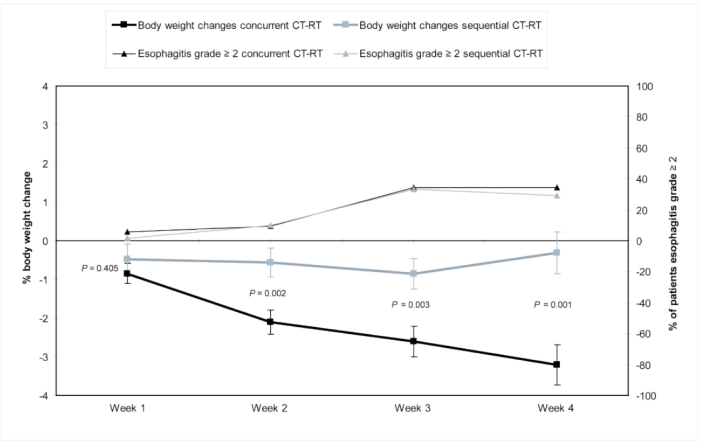
[2] *In the retrospective cohort, body weight loss during (CT-)RT is depicted relative to body weight at start of (CT-)RT. In the prospective cohort, the body weight is depicted relative to diagnosis.*

** P<0.05 in retrospective study, comparison between sequential and concurrent treated patients (independent sample T-test or Pearson Chi-Square test).*

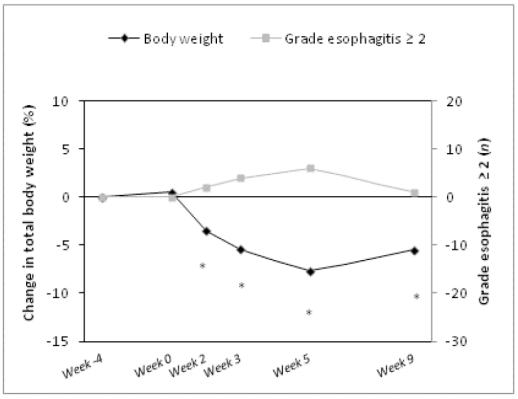
† P<0.05 in prospective study, comparison between body weight at a specific time point compared to body weight at diagnosis (paired sample T-test).

Figure 3
Body weight changes and grade dysphagia

A



B



A *Body weight changes and grade dysphagia during concurrent and sequential CT-RT in the retrospective cohort. Left Y axis: body weight loss as percentage of total body weight during concurrent and sequential CT-RT. Right Y axis: percentage of patients with esophagitis grade ≥ 2 during concurrent and sequential CT-RT.*

B *Body weight changes and grade dysphagia during concurrent CT-RT in the prospective cohort. Left Y axis: body weight loss as percentage of total body weight. Right Y axis: number of patients with esophagitis grade ≥ 2 during concurrent and sequential CT-RT. Week 4: diagnosis, Week 0: start of concurrent CT-RT, Week 2: week 2 of concurrent CT-RT, Week 3: week 3 of concurrent CT-RT, Week 5: week 5 of concurrent CT-RT, Week 9: week 4 post CT-RT. *Significant difference between indicated time point and diagnosis (P < 0.05)*

Grade esophagitis during concurrent and sequential CT-RT

No significant differences were observed in the frequency of esophagitis symptoms during the first weeks of (CT-)RT between patients treated with sequential CT-RT vs. patients treated with concurrent CT-RT (**Table 3** and **Figure 1**). Because esophagitis grade ≥2 is associated with altered nutritional intake according to the CTCAE 3.0, longitudinal data analysis by means of a linear mixed model was performed to assess whether or not the observed body weight loss during concurrent CT-RT was associated with esophagitis grade ≥2. Associations between body weight loss and grade ≥2 esophagitis were tested in two separate models, one for patients treated with sequential and one for patients treated with concurrent CT-RT. BMI was used in these models to calculate associations between grade ≥2 esophagitis and body weight loss independent of height. No significant associations were observed between body weight loss and esophagitis grade ≥2 in the first 3 weeks of concurrent CT-RT (estimated effect = 0.47, 95 % C.I. = −0.03–0.49, $p = 0.096$), while significant associations were observed between body weight loss and esophagitis grade ≥2 in patients treated with sequential therapy (estimated effect = 0.55, 95 % C.I. = 0.28–0.82, $p < 0.001$). In concurrently treated patients, significant associations were observed between decreases in BMI and duration of treatment (estimated effect week 2 = 0.28, 95 % C.I. = 0.14–0.43, $p < 0.001$; estimated effect week 3 = 0.39, 95 % C.I. = 0.16–0.61, $p = 0.001$).

Prospective study

Study population characteristics

Baseline characteristics of the prospective validation group are shown in **Table 1**.

Treatment characteristics

Treatment characteristics are depicted in **Table 2**. Most patients ($n = 6$) received one cycle of induction CT. The other patients did not receive any induction therapy ($n = 3$). The type induction chemotherapy as well as the concurrent CT consisted of cisplatin combined with either vinorelbine (44 %) or etoposide (56 %). Patients received two cycles of cisplatin-based concurrent CT. Total concurrent radiation dose

was 64.3 ± 6.6 Gy (range 53–69).

Body weight loss during concurrent CT-RT

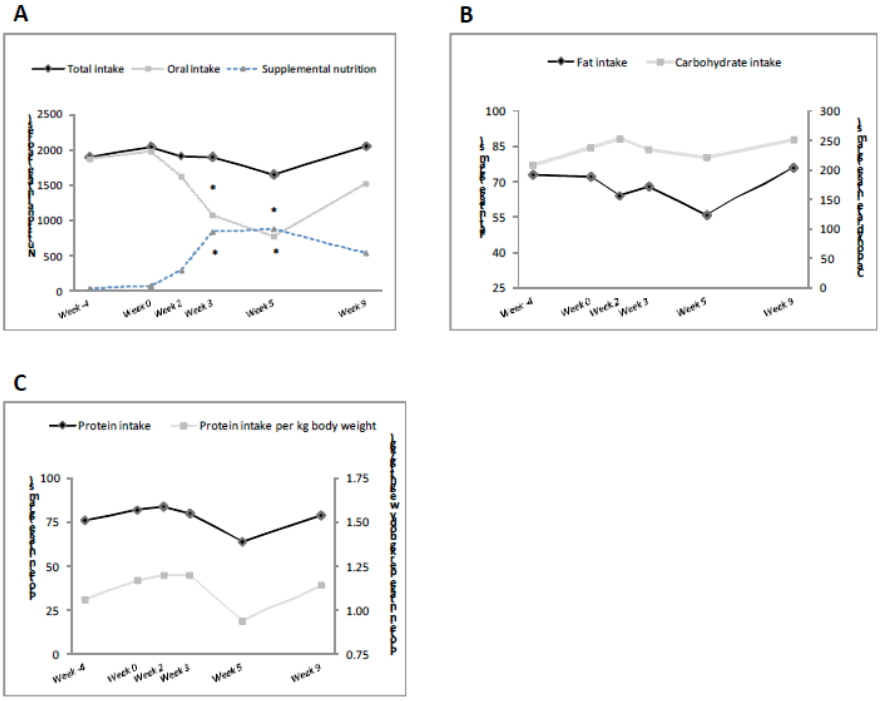
Data on body weight in the prospective cohort are shown in **Figure 3B** and **Table 3**. Body weight remained relatively stable between diagnosis and start of concurrent CT-RT. As **Figure 3B** indicates, body weight loss started early after initiation of therapy ($p = 0.002$) and the body weight reached its lowest level at the end of concurrent CT-RT ($p < 0.001$). In the 4 weeks following CT-RT, body weight partly recovered but was still significantly lower than the body weight at diagnosis ($p = 0.003$).

Grade esophagitis and nutritional intake during concurrent CT-RT

In **Table 3**, it can be observed that the number of patients in the prospective analysis suffering from grade esophagitis that interferes with nutritional intake (grade ≥2) is low at 2 weeks after initiation of concurrent CT-RT (~20 %) and increases in the later weeks of treatment (44 % in week 3 and 75 % in week 5). As in the retrospective analysis, the number of patients losing weight was consistently higher than the percentage of patients having grade esophagitis ≥2 (**Table 3**).

Total dietary intake remained relatively stable until week 3 of CT-RT, albeit that the proportion of calories consumed from nutritional support was already significantly increased and the proportion of calorie consumption from regular diet was already decreased in week 3 of CT-RT (**Figure 4A**). Despite continuation of nutritional support, total calorie consumption further decreased to about -15 % at week 5 of CT-RT (statistically not significant from start of treatment; **Figure 4A**). After 4 weeks of follow up, total calorie intake was comparable to the dietary intake at diagnosis, though some patients still partly relied on supplemental nutrition. The findings in **Figure 4B and C** indicated that macronutrient intake did not significantly alter during concurrent CT-RT, i.e. total carbohydrate, fat and protein intake (per kg body weight) was not significantly decreased during concurrent CT-RT (**Figure 4C**).

Figure 4



A *Caloric intake. The solid line represent total calorie intake, which consists of oral intake (grey line) and supportive nutrition (drink supplementation or tube feeding).*
B *Changes in dietary carbohydrate (grams) and fat (grams) intake (oral intake and supportive nutrition).*
C *Changes in total dietary protein (grams) and protein intake per kg body weight (grams/kilograms).*

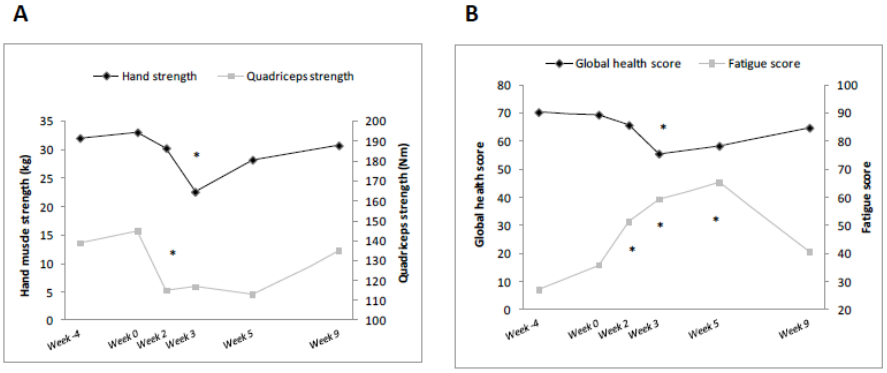
Week -4: diagnosis, Week 0: start of concurrent CT-RT, Week 2: week 2 of concurrent CT-RT, Week 3: week 3 of concurrent CT-RT, Week 5: week 5 of concurrent CT-RT, Week 9: week 4 post CT-RT.

**Significant difference between indicated time point and diagnosis ($P < 0.05$)*

Physical performance during concurrent CT-RT

Patients demonstrated a significant decline in muscle strength in the first weeks of concurrent CT-RT (**Figure 5A**). For quadriceps muscle, the strength declined immediately after initiation of concurrent CT-RT and reached its minimum at week 2 of concurrent CT-RT ($p = 0.042$; **Figure 5A**). For handgrip strength, the decline also started at initiation of concurrent CT-RT but the lowest muscle strength was observed at week 3 of CT-RT ($p = 0.002$; **Figure 5A**). Both hand and quadriceps muscle strength improved in the 4 weeks following concurrent CT-RT and nearly reached the level of muscle strength at diagnosis at that time point.

Figure 5



A *Muscle strength dominant hand (kilograms) and quadriceps (Nm). Quadriceps strength measurements were not obtained in some patients due to overall weakness, indicating even lower numbers.*

B *Quality of life scores (global health score and fatigue score) assessed using QLQ-C30 questionnaire.*

*Week -4: diagnosis, Week 0: start of concurrent CT-RT, Week 2: week 2 of concurrent CT-RT, Week 3: week 3 of concurrent CT-RT, Week 5: week 5 of concurrent CT-RT, Week 9: week 4 post CT-RT. *Significant difference between indicated time point and diagnosis ($P < 0.05$)*

In a similar pattern, global health score was decreased ($p = 0.024$) and fatigue score was increased ($p = 0.012$) in the first weeks of concurrent CT-RT. Both global health and fatigue score improved in the 4 weeks following concurrent CT-RT (global health: $p = 0.438$, fatigue score: $p = 0.200$; **Figure 5B**).

Discussion

In order to provide optimal care to patients being treated with concurrent CT-RT for NSCLC, the course of treatment-induced weight loss and its dependence on the most important acute dose-limiting side effect, i.e. esophagitis, needs to be determined. It is believed that weight loss and radiation-esophagitis are causally linked because esophagitis can lead to decreased dietary intake and subsequently, to loss of body weight[9]. However, no data on the incidence and pattern of weight loss or its association with treatment-induced esophagitis is available in patients treated with concurrent CT-RT for locally advanced NSCLC.

In a large retrospective cohort in which patients treated with concurrent CT-RT for locally advanced NSCLC were compared to patients treated with sequential CT-RT, we show that loss of body weight is a common systemic complication of concurrent CT-RT, starts early after initiation of treatment and surprisingly, is independent of treatment-induced esophagitis in the first weeks of CT-RT. These data on early body weight loss were confirmed in a prospective validation study, i.e. body weight loss was also observed during the first 3 weeks of concurrent CT-RT in the prospective study population, while the number of patients suffering from grade ≥2 esophagitis was still low at that time and importantly, total caloric intake was not decreased. In addition, no correlations were found between weight loss and esophagitis until week 3 of concurrent CT-RT in the prospective cohort (data not shown). Combined, these findings suggest that processes different from esophagitis-induced nutritional intake problems may contribute to “early” body weight loss during concurrent CT-RT. The loss of body weight despite stable energy intake suggests that energy needs are increased, i.e. dysbalance between energy intake and energy expenditure leads to an energy deficit and subsequently, weight loss.

The observations in the prospective dataset further indicate that body weight progressively deteriorates from week 3 to 5 of concurrent CT-RT, together with an increase in patients exhibiting esophagitis grade ≥2. This ‘late’ body weight loss is

accompanied by a significant decrease of spontaneous oral intake and increased reliance on supplemental nutrition. Although administration of supplemental nutrition prevents the total calorie intake to decline significantly, administration of supplemental nutrition is not sufficient to prevent further progression of body weight loss.

Another important finding in the current study is that after 4 weeks of completion of concurrent CT-RT, the body weight has not completely returned to the level at diagnosis. This, despite the fact that some of the patients are still using supplemental nutrition, indicates that body weight loss is a problem that extends over at least 2 months. Since significant body weight loss in cancer patients has been associated with negative effects on therapy response, survival as well physical as emotional wellbeing[16], the weight loss during and following concurrent CT-RT requires intense management to prevent these negative consequences.

Our observations that body weight loss occurs frequently during concurrent administration of CT-RT are consistent with literature on body weight changes in concurrent CT-RT treatment regimens in other malignancies. Several studies in head and neck cancer show a decrease in body weight during concurrent CT-RT starting from the first week on and continuing in the weeks after concurrent CT-RT[17, 18]. These changes in body weight are also addressed in the Clinical Practice Guideline of the American Society of Clinical Oncology (ASCO) for Laryngeal Cancer[19]. In addition, comparison between radiation treatment alone and concurrent CT-RT in cervical cancer patients showed increased body weight loss during concurrent CT-RT[20]. However, none of these studies have addressed the etiology or systemic effects of this treatment-related body weight loss.

An observation in the current study that could be of importance for optimization of patient care is that the ‘early’ weight loss during concurrent CT-RT is accompanied by a significant decline in muscle strength. As muscle strength strongly correlates with muscle mass, it is possible that the early weight loss originates from a disturbed muscle protein turnover and subsequent loss of muscle mass, i.e. the balance between muscle protein synthesis and degradation is disturbed in favor of protein degradation. Undergoing the aggressive concurrent CT-RT treatment regimen may increase energy needs, which could induce catabolism and deplete muscle mass. Therefore, increased energy needs require adequate balancing by nutritional intake to maintain muscle mass. Yet, not only energy balance should be maintained but also specific attention is warranted to the role of dietary protein intake, as this can

maintain muscle mass by stimulation of protein synthesis. However, the recommended protein intake between 1.2 and 1.5 g/kg body weight was not reached during the concurrent treatment regimen[21, 22], which indicates that more aggressive supplementation of dietary protein is needed. More precisely, provision of branched chain amino acids (BCAA) might by indicated, since BCAA's can stimulate muscle protein synthesis downstream of muscle anabolic integrator Akt[23] and we recently reported an impairment in protein synthesis signaling at the level of Akt (as part of the anabolic PI3K/Akt/mTOR pathway) in muscle of cachectic cancer patients with NSCLC[24].

Continuation of aggressive nutritional support during the 'late', esophagitis-associated weight loss (weeks 3–5 of concurrent CT-RT) seems plausible, as patients are at an even higher risk of dietary uncompensated muscle catabolism due to decreased esophagitis-related energy intake. Therefore, it seems conceivable to initiate aggressive nutritional support, possibly with specific amino acid formulation from the start of concurrent CT-RT to prevent or attenuate 'early' and 'late' weight loss associated with concurrent CT-RT. As simultaneous administration of exercise training or neuromuscular electrical stimulation significantly enhances the positive effects of nutritional support on muscle synthesis, these physical interventions should be considered as co-intervention[25].

In conclusion, the current study shows that 'early' body weight loss is a common complication of concurrent CT-RT in patients with locally advanced NSCLC and is independent of decreased intake due to esophagitis. This suggests that other treatment-dependent metabolic alterations contribute to this 'early' weight loss. A further decline in body weight is observed during the later weeks of concurrent CT-RT, when the incidence of esophagitis increases, and body weight is still not totally recovered after 4 weeks post treatment. Since the 'early' body weight loss is accompanied by a significant decline in muscle strength, which may implicate active catabolism, more supportive and early initiated nutritional intervention, preferably combined with tailored exercise could be suggested to optimize concurrent CT-RT management. The efficacy of such interventions needs to be explored in adequately designed clinical trials.

Chapter 2

Supplemental Data

Early body weight loss during concurrent chemo-radiotherapy for non-small cell lung cancer

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J Cachexia Sarcopenia Muscle, 2014 Jan 23

Supplemental data

Study population

For the retrospective cohort, all patients with locally advanced NSCLC consecutively referred to the department of radiotherapy of the Maastricht University Medical Centre + (MAASTRO clinic) from the 1st of January 2006 till the 31st of December 2008 for sequential or concurrent CT-RT with curative intent were enrolled (*n*=282). A consort diagram is depicted in **Figure 1**. Clinical data was obtained by reviewing the clinical charts. Patients visited the outpatient clinic weekly during radiation treatment, where body weight and toxicity of treatment was recorded by a radiation oncologist. A schematic representation of the study points in the retrospective cohort is depicted in **Figure 2A**.

For prospective data collection, patients with locally advanced NSCLC consecutively referred to the department of Respiratory Medicine of the Maastricht University Medical Centre + (MAASTRO clinic) from the 1st of October 2010 till the 30th of September 2011 were eligible for inclusion. A total of 21 were included. As can be observed in the consort diagram in **Figure 1**, body weight and toxicity scoring of a proportion of the group was missing at some time points (due to toxicity and/or physical fatigue). Therefore, only data of the 9 patients of which data on body weight was present at all study points is presented here. Data analysis was also performed on all patients of whom at least 3 points were present (n=15) and this revealed the same results (data not shown).

Due to the non-interfering design of the study, evaluation of a medical ethical committee and trial registration was not required. However, the study was nonetheless submitted to review by the local medical ethics committee and it was confirmed that the study followed all standard medical ethical guidelines. In addition, all patients signed informed consent. A schematic representation of study points in the prospective can be observed in **Figure 2B**.

Tumor stage was recorded in accordance with the 7th tumor-node-metastasis (TNM) International Staging System for Lung Cancer[26]. Comorbidity of all patients was scored using the Charlson Comorbidity Index[15]. Patients were classified as never,

former or current smoker. Never smokers were defined as those having used less than 100 cigarettes in their life-time, former smokers as those who did not smoke at the time of initiation of radiotherapy, and current smokers as those individuals who kept on smoking at the start of treatment.

Radiotherapy

In short, in all patients a treatment planning ^{18}F-deoxyglucose (FDG)-PET-CT scan was performed in radiotherapy position on a dedicated PET-CT-simulator. The CT scan performed was a spiral CT scan of the whole thorax, with intravenous contrast. Subsequently, a 4D-CT scan was performed. Gross Tumor Volumes (GTV) were delineated on a mid-ventilation CT scan.
The GTV was defined as the primary tumor on CT and lymph nodes positive on PET scan or proven to be positive on mediastinoscopy or transesophageal/transbronchial biopsy. If the patient received induction chemotherapy the pre-chemotherapy PET scan was used to decide which nodal regions needed to be included in the GTV. No elective hilar of mediastinal irradiation was carried out. The Clinical Target Volume (CTV) was defined as the GTV with a margin of 5 mm, whereas an individual non-isotropic margin (Amplitudo/4) with an extra 2 mm for set-up margins was added to define the Planning Target Volume (PTV).
For the calculation of the mean lung dose (MLD), the volume of both lungs minus the GTV was considered. The outer contour of the esophagus was contoured from the cricoid to the esophago-gastric junction.

Muscle strength

For hand muscle strength assessment, patients sat upright on a chair with the shoulder placed in 0° abduction and the elbow in 90° flexion. The lower arm was placed on the table for support. Patients were instructed to press the hand grip meter (Jamer, Sammons Preston INC, Maastricht) as hard as possible for 5 seconds. The hand grip strength was calculated as the mean of three measurements (kg).
For quadriceps muscle strength, subjects were seated on the Biodex dynamometer (Biodex system version 3.3) chair with belts attached at the level the thigh and ankle for stability. Isometric muscle strength was assessed by 3 maximal voluntary

contractions (MVCs) at an angle of 60°. Muscle strength was defined as the highest muscular force output (peak torque) in Newton meters (Nm).

References

1. Auperin, A. *et al.* Meta-analysis of concomitant versus sequential radiochemotherapy in locally advanced non-small-cell lung cancer. *J Clin Oncol* **28**, 2181-2190 (2010).
2. Fournel, P. *et al.* Randomized phase III trial of sequential chemoradiotherapy compared with concurrent chemoradiotherapy in locally advanced non-small-cell lung cancer: Groupe Lyon-Saint-Etienne d'Oncologie Thoracique-Groupe Francais de Pneumo-Cancerologie NPC 95-01 Study. *J Clin Oncol* **23**, 5910-5917 (2005).
3. Furuse, K. *et al.* Phase III study of concurrent versus sequential thoracic radiotherapy in combination with mitomycin, vindesine, and cisplatin in unresectable stage III non-small-cell lung cancer. *J Clin Oncol* **17**, 2692-2699 (1999).
4. Rowell, N.P. & O'Rourke N, P. Concurrent chemoradiotherapy in non-small cell lung cancer. *Cochrane Database Syst Rev*, CD002140 (2004).
5. Jain, A.K. *et al.* A phase II study of concurrent chemoradiation with weekly docetaxel, carboplatin, and radiation therapy followed by consolidation chemotherapy with docetaxel and carboplatin for locally advanced inoperable non-small cell lung cancer (NSCLC). *J Thorac Oncol* **4**, 722-727 (2009).
6. Price, K.A., Azzoli, C.G. & Gaspar, L.E. Chemoradiation for unresectable stage III non-small cell lung cancer. *Seminars in thoracic and cardiovascular surgery* **20**, 204-209 (2008).
7. De Ruysscher, D. *et al.* Eligibility for concurrent chemotherapy and radiotherapy of locally advanced lung cancer patients: a prospective, population-based study. *Ann Oncol* **20**, 98-102 (2009).
8. De Ruysscher, D. *et al.* Maximal neutropenia during chemotherapy and radiotherapy is significantly associated with the development of acute radiation-induced dysphagia in lung cancer patients. *Ann Oncol* **18**, 909-916 (2007).
9. Werner-Wasik, M. Treatment-related esophagitis. *Seminars in oncology* **32**, S60-66 (2005).
10. Johnke, R.M. *et al.* Circulating cytokine levels in prostate cancer patients undergoing radiation therapy: influence of neoadjuvant total androgen suppression. *In Vivo* **23**, 827-833 (2009).
11. Tan, B.H. & Fearon, K.C. Cachexia: prevalence and impact in medicine. *Curr Opin Clin Nutr Metab Care* **11**, 400-407 (2008).
12. Tisdale, M.J. Mechanisms of cancer cachexia. *Physiological reviews* **89**, 381-410 (2009).
13. De Ruysscher, D. *et al.* European Organisation for Research and Treatment of Cancer recommendations for planning and delivery of high-dose, high-precision radiotherapy for lung cancer. *J Clin Oncol* **28**, 5301-5310 (2010).
14. van Baardwijk, A. *et al.* Mature results of an individualized radiation dose prescription study based on normal tissue constraints in stages I to III non-small-cell lung cancer. *J Clin Oncol* **28**, 1380-1386.
15. Charlson, M., Szatrowski, T.P., Peterson, J. & Gold, J. Validation of a combined comorbidity index. *J Clin Epidemiol* **47**, 1245-1251 (1994).
16. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489-495 (2011).

17. McRackan, T.R. *et al.* Effect of body mass index on chemoradiation outcomes in head and neck cancer. *Laryngoscope* **118**, 1180-1185 (2008).
18. Silver, H.J., Dietrich, M.S. & Murphy, B.A. Changes in body mass, energy balance, physical function, and inflammatory state in patients with locally advanced head and neck cancer treated with concurrent chemoradiation after low-dose induction chemotherapy. *Head Neck* **29**, 893-900 (2007).
19. Pfister DG SAL, W.G., Mendenhall WM, Adelstein DJ, Ang KK, Clayman GL, Fisher SG, Forastiere AA, Harrison LB, Lefebvre J-L, Leupold N, List MA, O'Malley BO, Patel S, Marshall RP, Schwartz MA, and Wolf GT American Society of Clinical Oncology Clinical Practice Guideline for the Use of Larynx-Preservation Strategies in the Treatment of Laryngeal Cancer. *J Clin Oncol* **24**, 3693–3704 (2006).
20. Ohno, T. *et al.* Incidence and temporal pattern of anorexia, diarrhea, weight loss, and leukopenia in patients with cervical cancer treated with concurrent radiation therapy and weekly cisplatin: comparison with radiation therapy alone. *Gynecol Oncol* **103**, 94-99 (2006).
21. Op den Kamp, C.M., Langen, R.C., Haegens, A. & Schols, A.M. Muscle atrophy in cachexia: can dietary protein tip the balance? *Curr Opin Clin Nutr Metab Care* **12**, 611-616 (2009).
22. Wolfe, R.R., Miller, S.L. & Miller, K.B. Optimal protein intake in the elderly. *Clin Nutr* **27**, 675-684 (2008).
23. Vary, T.C. & Lynch, C.J. Nutrient signaling components controlling protein synthesis in striated muscle. *J Nutr* **137**, 1835-1843 (2007).
24. Op den Kamp, C.M. *et al.* Nuclear transcription factor kappaB activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. *Am J Clin Nutr* (2013).
25. Cermak, N.M., Res, P.T., de Groot, L.C., Saris, W.H. & van Loon, L.J. Protein supplementation augments the adaptive response of skeletal muscle to resistance-type exercise training: a meta-analysis. *Am J Clin Nutr* **96**, 1454-1464 (2012).
26. Mirsadraee, S., Oswal, D., Alizadeh, Y., Caulo, A. & van Beek, E., Jr. The 7th lung cancer TNM classification and staging system: Review of the changes and implications. *World J Radiol* **4**, 128-134 (2012).

Chapter 3

Muscle atrophy in cachexia: can dietary protein tip the balance?

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Abstract

The purpose of this study was to review the efficacy of dietary protein supplementation in attenuating muscle atrophy in cachexia. Only very few recent randomized controlled trials have studied the effects of protein supplementation in clinical cachexia. It appears that supplementation of dietary protein (>1.5 g/kg per day) alone or in combination with other anabolic stimuli such as exercise training maintains or even improves muscle mass, but results on muscle function are controversial and no clinical studies have yet directly linked alterations in cellular signaling or metabolic signatures of protein intake-induced muscle anabolism to muscle weight gain.

To elucidate the role of dietary protein supplementation in attenuating muscle atrophy in cachectic patients, randomized clinical trials are needed in adequately phenotyped patients using sensitive measures of muscle mass and function.

Introduction

Cachexia is a complex debilitating metabolic syndrome associated with underlying illness[1] that may not yet be cured but benefit from a multidimensional therapeutic approach consisting of nutritional support, exercise training and/or pharmacological treatment. Although muscle protein wasting is the most distinctive feature of cachexia, the relative influence on muscle protein turn-over of common denominators of chronic disease progression, that is, adaptive low physical activity level, compulsory inactivity during acute hospitalization or advancing age versus disease-specific factors such as inflammation and hypoxia, remains unclear. In addition, the relative contribution of dietary protein intake in maintenance or accretion of muscle mass in the different stages of chronic wasting diseases remains unidentified and receives scarce attention in current clinical practice. Efficacy of dietary protein supplementation may not only depend on protein quantity and the specific amino acid formulation but also on the underlying disease and clinical condition, as well as on presence of other intervention strategies targeted at muscle maintenance. These issues can only be addressed by a translational research approach including relevant in-vitro and in-vivo experimental models and controlled clinical trials with adequately phenotyped patients and appropriate outcome measures. In this review, we will elaborate the efficacy of dietary protein supplementation in counter-acting muscle atrophy in cachexia by discussing recent findings of intervention studies in muscle wasting patients and by reviewing the putative molecular mechanisms by which dietary protein regulates muscle protein turnover.

Optimal protein intake in cachexia

Maintenance of muscle is determined by the balance between muscle protein synthesis and breakdown, and therefore, to accomplish maintenance or even restoration of muscle mass in cachexia, maximal stimulation of protein synthesis and inhibition of degradation is desired. This requires optimal dietary protein intake, which may very well exceed the Recommended Dietary Allowance (RDA) of 0.8 grams (g) of protein per kilogram (kg) of body weight per day, the amount of protein that

adequately maintains nitrogen balance in healthy individuals, including the elderly[2]. Current estimations of protein requirements are mainly derived from studies on non-cachectic individuals, although some study populations shared characteristics of cachexia such as disuse. For instance, 45g of essential amino acids per day, in addition to the RDA for protein, preserved muscle strength outcome during compulsory bed rest in elderly volunteers[3]. Optimal protein requirements are difficult to estimate as they may depend on the protocol, that is, higher values are obtained in short-term studies and estimates may be 40 – 50% higher using tracer methodology than using nitrogen balance studies[4]. Despite these difficulties and the possible variations in protein requirements in different diseases and clinical conditions, a minimum of 1.5 g/kg of body weight per day or 15 – 20% of total caloric intake appears justified for cachexia, considering that this amount was determined as optimal protein intake in sarcopenia[5], which is also characterized by muscle depletion. Furthermore, experimental evidence and recent clinical studies indicate that in contrast to sarcopenia, in which a decreased muscle protein synthetic response has been identified, active cachexia is also characterized by increased muscle protein degradation[6, 7].

For optimal dietary supplementation in cachexia, protein source and meal composition also need to be considered, as in the elderly, muscle protein synthesis was hypothesized to be blunted when protein and carbohydrate are co-ingested or when the quantity of protein is less than 20g/meal[8]. Finally, timing of protein intake may be important in cachectic patients to avoid adverse effects of high protein intake on overall dietary intake in view of recently described dose-dependent satiating effects of protein in healthy volunteers[9].

Effects of protein and amino acids on muscle mass

There are few recent randomized controlled trials (RCTs) investigating the effect of protein in muscle wasted patients, but these trials do provide new insights into the positioning of nutritional intervention in attenuating muscle atrophy. A recent RCT, which included 59 outpatients with advanced chronic obstructive pulmonary disorder (COPD), reported a positive effect of dietary counseling and food fortification (using

milk powder) on muscle mass[10]. The intervention group consumed more energy and protein (±1.47 g/kg body weight; mean difference 11.8 g/day), resulting in weight gain and maintenance of muscle mass during 6 months of intervention period and weight maintenance during 6 months of follow-up. Although control COPD patients lost weight and muscle mass throughout the study, significant differences were observed between the groups in quality of life, but not in skeletal muscle function. Campbell et al.[11] performed a RCT of individualized nutritional counseling in 56 patients with chronic kidney disease. Intervention aimed at a protein and energy intake of 0.8 – 1.0 g/kg and at least 125 kJ/kg, respectively, per day. During 12 weeks, the decrease in body cell mass was reduced (3.5%) by the intervention, with a greater increase in energy intake, but no difference in measurable overall protein intake showing that a protein intake around the RDA may attenuate muscle atrophy, but not stimulate muscle regrowth. The effect of increasing protein intake was also investigated in 38 muscle-wasted patients with stable chronic heart failure[12]. Oral supplementation of essential amino acids (8 g/day for 8 weeks in addition to habitual dietary intake ≥125 kJ/kg and protein intake >1.1 g/kg) resulted in a greater increase in body weight compared with non-supplemented patients (80% of supplemented patients versus 30% of controls >1kg of body weight gain). Changes in arm muscle size and nitrogen balance were similar but only supplemented patients improved exercise output, peak oxygen consumption and walking distance, illustrating an apparent dissociation between effects of nutritional supplementation on muscle mass versus muscle endurance. These studies confirm the argumentation that a protein intake of at least 1.5 g/kg body weight is needed to induce slight muscle hypertrophy.

Limited studies have yet compared muscle anabolic effects of selective proteins or focused on specific amino acids in patients at risk or suffering from cachexia. In a short-term tracer experiment, Engelen et al.[13] reported that supplementation of branched-chain amino acids (BCAAs) to a soy protein meal resulted in a significant acute increase in whole body protein synthesis in weight stable COPD patients with borderline muscle mass but not in age-matched healthy controls. In a recent RCT, the effect of supplementation of a combination of b-hydroxyl b-methyl butyrate (a metabolite of the essential amino acid leucine), glutamine and arginine was studied in weight losing cancer patients with stage III or IV solid tumors or currently metastatic cancer of any initial stage. Administration of this supplement for 8 weeks did not result in significant changes in lean body mass[14]. Because the experimental

conditions in both studies were different and no information on protein balance was available in both groups, no definite conclusions can be drawn and further research should be conducted to clarify the role of specific amino acid supplementation in maintaining muscle mass or preventing muscle wasting during acute weight loss.

Because the cause of cachexia is considered multifactorial, numerous studies have indicated that next to isolated nutritional supplementation, other intervention strategies could be beneficial in attenuating muscle atrophy in cachexia. The beneficial effects of resistance training on maintenance of muscle mass and function in sarcopenia have recently been highlighted[15], but limited studies have specifically addressed the additive effects of nutritional supplementation to (resistance) exercise in cachexia. Several studies on patients with COPD showed that protein/carbohydrate-rich supplements as integrated part of a pulmonary rehabilitation program were effective in inducing (muscle) weight gain and improving physical performance[16-18], but only one study[19] was yet able to disentangle the effect of nutritional support as adjunct to exercise training. In this study, cachectic patients receiving exercise only did not gain weight or increase muscle mass in contrast to those receiving exercise, nutritional supplementation and anabolic steroids. Agin et al.[20] studied in a RCT the effect of 14 weeks of intervention consisting of whey protein (1g/kg body weight) in addition to consumption of energy and protein above RDA. Treatment with whey protein promoted weight and fat gain with little effect on physical function and quality of life, whereas resistance training increased muscle mass, muscle strength and quality of life, leading to functional improvement. Combining whey protein and resistance exercise did not further promote accretion of muscle mass achieved by exercise alone. Potential enhancing effect of anabolic pharmacological agents as single therapy or as precursor or co-treatment of the muscle growth response to rehabilitation is currently a hot topic. The efficacy of anabolics is affected by protein intake, but no clinical data are available regarding the response to anabolics in cachexia during normal and high protein intake (0.8 – 1.0 versus >1.5 g/kg body weight, respectively).

Molecular regulation of muscle protein metabolism by dietary protein in cachexia

Despite the potential for dietary protein in attenuating muscle atrophy in cachexia, no clinical studies are available in which the putative molecular mechanisms by which dietary protein influences muscle protein turnover have been addressed. This could, however, be instrumental in optimizing and fine-tuning the dose, composition and timing of dietary protein as well as potential combined treatments. To this end, the following paragraphs will discuss the molecular mechanisms of protein synthesis and breakdown, which could be involved in beneficial effects of dietary protein in attenuating muscle atrophy.

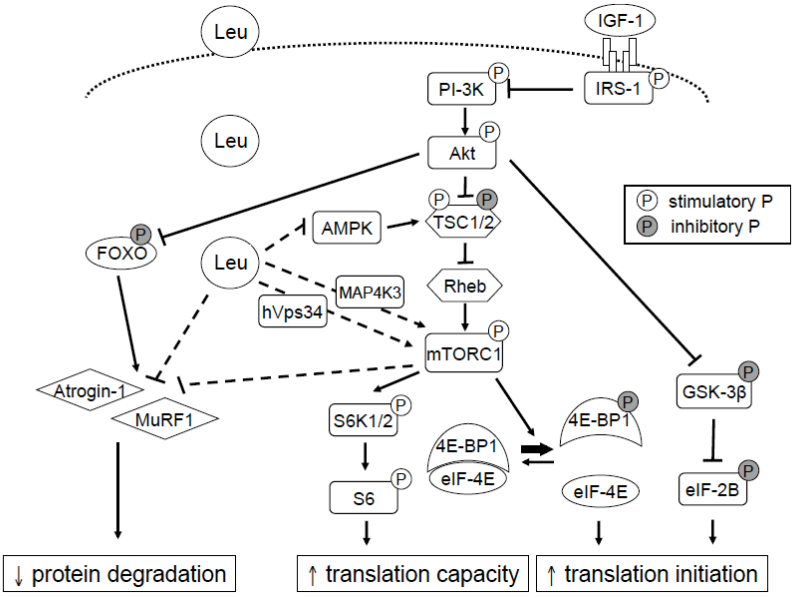
Regulation of muscle protein synthesis by dietary protein

The regulation of muscle protein synthesis is very similar to that of other cell types, whereas some protein degradation routes unique to striated muscle have been identified. In non-pathological conditions, modulation of muscle protein turnover relies on the postprandial availability of nutrients like amino acids, which directly, and indirectly via the actions of or in combination with insulin, stimulate muscle protein synthesis and decrease degradation (**Figure 1**). Anabolic effect of amino acids may partly be attributed to increased substrate availability; however, a mixture of the BCAAs or leucine alone stimulates protein synthesis to the same extent as a complete mixture of amino acids, indicative of a signaling role of these particular amino acids[21-23]. In contrast to protein synthesis signaling by insulin[24] or insulin-like growth factor-I (IGF-I)[25], these anabolic actions of BCAAs do not appear to be receptor-mediated[26], or require insulin receptor substrate (IRS-1) phosphatidyl-inositol-3 kinase (PI3K), and PKB/Akt activation. Sub- sequent signaling to increase protein synthesis by insulin/ IGF-I involves stimulation of mRNA translation via activation of eukaryotic initiation factors (eIFs) by inhibition of glycogen synthase kinase (GSK)-3 and activation of mammalian target of rapamycin (mTOR; reviewed in detail by Glass[25] and Proud[24]). Inhibition of GSK-3 abrogates its suppressive effect on eIF2B. Currently, there is no evidence to support regulation of GSK-3 activity by amino acids in the control of translation initiation. mTOR, on the other hand, is

activated indirectly by Akt signaling – as this suppresses TSC1/ 2-inhibition of mTOR – resulting in increased activity of eukaryotic translation initiation factor 4E (eIF4E) as a consequence of the dissociation of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) following mTOR-mediated phosphorylation. In addition to stimulatory effects on eIF activity, insulin/IGF-I signaling also promotes protein synthesis by increasing translation capacity and elongation. This occurs via phosphorylation of S6K1/2 and eIF-4E by the mTORC1 complex (mTOR associated with raptor and GbL)[27]. Supplementation of rats, which were starved for 18 h, with dietary protein results in decreased binding of 4E-BP1 to eIF4E[28], whereas long-term amino acid supplementation attenuated aging-induced decrease in muscle sarcomers[29]. However, dietary protein supplementation does not always result in differences on muscle protein metabolism as is indicated by unchanged ^{13}C-valine enrichment in muscle tissue[30], or muscle mass in tumor- bearing mice[31].

Leucine alone is sufficient to increase protein synthesis, which is mediated via the phosphorylation of 4E-BP1 and S6K1 as a result of phosphorylation and activation of mTOR in the mTORC1 complex. Of all amino acids, leucine appears the most potent stimulator of mTORC1 phosphorylation[21]. Glutamine even has inhibiting effects on mTORC1 signaling in cultured muscle cells[32, 33]. The mechanism by which leucine stimulates mTORC1 phosphorylation is not fully understood, but human vacuolar protein sorting-34 (hVps34) and mitogen-activated protein kinase-3 (MAP4K3) have recently been described to be involved[34, 35]. Leucine also stimulates protein synthesis by inhibiting adenosine monophosphate-activated protein kinase (AMPK)-mediated phosphorylation of TSC2, which negatively controls mTORC1, linking energy availability to mTORC1 signaling[23]. Indeed, supplementation of dietary leucine increased phosphorylation of mTOR, S6K1 and 4E-BP1 and formation of active eIF4E complex in 7-day-old piglets[36]. In contrast, addition of leucine had no effect on muscle or carcass weight in cachectic mice[31]. This could be explained by the recent finding that dietary leucine influences peak activation but not duration of skeletal muscle protein synthesis and mTOR activation[37]. Discrepancies between anabolic signaling signatures and muscle maintenance have also been described in weight stable COPD patients with muscle atrophy, as increased phosphorylation of Akt and downstream mediators (i.e., 4E-BP1, S6K1 and GSK-3b) were explained as a failed attempt to maintain muscle mass[38].

Figure 1



AMPK, adenosine monophosphate-activated protein kinase; eIF, eukaryotic initiation factor; GSK, glycogen synthase kinase; IGF, insulin-like growth factor; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin.

Regulation of protein degradation by dietary protein

Maintenance of muscle mass by insulin/IGF-I signaling also involves suppression of protein degradation. Proteolytic systems known to be suppressed by insulin/IGF-I signaling include the 26S-ubiquitin (Ub) proteasome pathway (UPP)[25] and lysosomal protein degradation (autophagy)[39]. In muscle, increased protein degradation by the UPP has been shown to be of great importance in muscle atrophy in experimental models[25] and humans[40]. Although various components of the UPP have been reported to be increased during active atrophy, the most consistent are the increased expressions of striated muscle-specific E3 Ub-ligases MuRF1 and atrogin-1/MAFbx. The expression of these so-called 'atrogenes' is negatively regulated by insulin/IGF-I signaling[41, 42]. The molecular basis for this control of UPP-mediated protein

degradation has to a large extent been clarified and relies on the negative regulation of the transcription factors FOXO1/3[42, 43]. In the presence of insulin/IGF-I, Akt-mediated phosphorylation inhibits FOXO1/3 nuclear translocation, suppressing FOXO-dependent transcription of atrogin-1 and MuRF1. Interestingly, the expression of Bnip3 – an essential protein in lysosomal protein degradation – is also regulated by FOXO3, demonstrating how insulin signaling controls multiple proteolytic systems in skeletal muscle[44].

In a recent study, it was shown that dietary leucine supplementation inhibits muscle protein breakdown in rats[45]. In cultured muscle cells, insulin and leucine were found to act additively in downregulating 14kDa E2 Ub-conjugating enzyme expression[46], whereas BCAA reduced atrogin-1 and MuRF1 expression[47]. The signaling route employed by leucine to inhibit atrogin-1 and MuRF1 expression has not been identified. However, as IGF-I-mediated inhibition of atrogin-1 and MuRF1 expression was shown to require mTOR activity[48], it is tempting to speculate that leucine may decrease expression of the atrogenes by activation of mTOR.

Despite our increasing understanding based on experimental studies of the mechanisms by which protein/ amino acids can directly or indirectly stimulate anabolic or anti-catabolic signaling, much of this awaits confirmation in cachexia models and clinical cachexia.

Conclusion

Recent in-vitro studies provide convincing evidence for pro-anabolic and anti-catabolic effects of amino acids, but limited data are available in experimental animal models and in patients suffering from cachexia. Recent studies on weight stable patients with muscle atrophy show that enhancing protein intake may result in maintenance or accretion of muscle mass depending on the amount of protein. Efficacy in terms of muscle regain and physical functioning appears enhanced when patients receive an additional anabolic stimulus such as exercise training, but more RCTs are needed to disentangle the effects of dietary protein and resistance exercise on muscle mass and functional improvement in acute and chronic wasting.

In contrast to sarcopenia, relatively little attention has been given to potential differential effects of different types of protein or of specific amino acids on muscle wasting in cachectic patients. Surprisingly, the efficacy of protein intake in attenuation of muscle atrophy in cancer (pre)cachexia or as adjunct to cancer specific therapy is still rarely investigated in RCTs. The variable disease course of many chronic wasting conditions may also demand fine tuning of protein supplementation, but no data are yet available on, for example, protein requirements in specific diseases during clinically and weight stable conditions, in comparison to acute exacerbations or in the recovery phase. Therefore, to optimize the application of dietary protein in cachexia management, expansion of supplementation studies in experimental cachexia models is required, as well as increased efforts to conduct proof-of-concept controlled clinical trials, which combine molecular signatures of protein turnover regulation in skeletal muscle biopsies and sensitive measures of muscle mass and function.

References

1. Evans, W.J. *et al.* Cachexia: a new definition. *Clin Nutr* **27**, 793-799 (2008).
2. Campbell, W.W., Johnson, C.A., McCabe, G.P. & Carnell, N.S. Dietary protein requirements of younger and older adults. *Am J Clin Nutr* **88**, 1322-1329 (2008).
3. Ferrando, A.A. *et al.* EAA supplementation to increase nitrogen intake improves muscle function during bed rest in the elderly. *Clin Nutr* (2009).
4. Roth, E. Skeletal muscle gain: how much can be achieved by protein and amino acid administration? *Curr Opin Clin Nutr Metab Care* **11**, 32-33 (2008).
5. Wolfe, R.R., Miller, S.L. & Miller, K.B. Optimal protein intake in the elderly. *Clin Nutr* **27**, 675-684 (2008).
6. Rutten, E.P. *et al.* Greater whole-body myofibrillar protein breakdown in cachectic patients with chronic obstructive pulmonary disease. *Am J Clin Nutr* **83**, 829-834 (2006).
7. Tisdale, M.J. Mechanisms of cancer cachexia. *Physiological reviews* **89**, 381-410 (2009).
8. Paddon-Jones, D. & Rasmussen, B.B. Dietary protein recommendations and the prevention of sarcopenia. *Curr Opin Clin Nutr Metab Care* **12**, 86-90 (2009).
9. Veldhorst, M.A. *et al.* Dose-dependent satiating effect of whey relative to casein or soy. *Physiol Behav* **96**, 675-682 (2009).
10. Weekes, C.E., Emery, P.W. & Elia, M. Dietary counselling and food fortification in stable COPD: a randomised trial. *Thorax* **64**, 326-331 (2009).
11. Campbell, K.L., Ash, S., Davies, P.S. & Bauer, J.D. Randomized controlled trial of nutritional counseling on body composition and dietary intake in severe CKD. *Am J Kidney Dis* **51**, 748-758 (2008).
12. Aquilani, R. *et al.* Adequate energy-protein intake is not enough to improve nutritional and metabolic status in muscle-depleted patients with chronic heart failure. *Eur J Heart Fail* **10**, 1127-1135 (2008).
13. Engelen, M.P. *et al.* Supplementation of soy protein with branched-chain amino acids alters protein metabolism in healthy elderly and even more in patients with chronic obstructive pulmonary disease. *Am J Clin Nutr* **85**, 431-439 (2007).
14. Berk, L. *et al.* A randomized, double-blind, placebo-controlled trial of a beta-hydroxyl beta-methyl butyrate, glutamine, and arginine mixture for the treatment of cancer cachexia (RTOG 0122). *Support Care Cancer* **16**, 1179-1188 (2008).
15. Adamo, M.L. & Farrar, R.P. Resistance training, and IGF involvement in the maintenance of muscle mass during the aging process. *Ageing research reviews* **5**, 310-331 (2006).
16. Broekhuizen, R., Creutzberg, E.C., Weling-Scheepers, C.A., Wouters, E.F. & Schols, A.M. Optimizing oral nutritional drink supplementation in patients with chronic obstructive pulmonary disease. *Br J Nutr* **93**, 965-971 (2005).
17. Creutzberg, E.C., Wouters, E.F., Mostert, R., Weling-Scheepers, C.A. & Schols, A.M. Efficacy of nutritional supplementation therapy in depleted patients with chronic obstructive pulmonary disease. *Nutrition* **19**, 120-127 (2003).

18. Steiner, M.C., Barton, R.L., Singh, S.J. & Morgan, M.D. Nutritional enhancement of exercise performance in chronic obstructive pulmonary disease: a randomised controlled trial. *Thorax* **58**, 745-751 (2003).
19. Schols, A.M., Soeters, P.B., Mostert, R., Pluymers, R.J. & Wouters, E.F. Physiologic effects of nutritional support and anabolic steroids in patients with chronic obstructive pulmonary disease. A placebo-controlled randomized trial. *Am J Respir Crit Care Med* **152**, 1268-1274 (1995).
20. Agin, D. *et al.* Effects of whey protein and resistance exercise on body cell mass, muscle strength, and quality of life in women with HIV. *Aids* **15**, 2431-2440 (2001).
21. Anthony, J.C. *et al.* Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr* **130**, 2413-2419 (2000).
22. Dreyer, H.C. *et al.* Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *Am J Physiol Endocrinol Metab* **294**, E392-400 (2008).
23. Du, M., Shen, Q.W., Zhu, M.J. & Ford, S.P. Leucine stimulates mammalian target of rapamycin signaling in C2C12 myoblasts in part through inhibition of adenosine monophosphate-activated protein kinase. *J Anim Sci* **85**, 919-927 (2007).
24. Proud, C.G. Regulation of protein synthesis by insulin. *Biochemical Society transactions* **34**, 213-216 (2006).
25. Glass, D.J. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* **37**, 1974-1984 (2005).
26. Vary, T.C. & Lynch, C.J. Nutrient signaling components controlling protein synthesis in striated muscle. *J Nutr* **137**, 1835-1843 (2007).
27. Kim, D.H. *et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002).
28. Yoshizawa, F., Kimball, S.R., Vary, T.C. & Jefferson, L.S. Effect of dietary protein on translation initiation in rat skeletal muscle and liver. *Am J Physiol* **275**, E814-820 (1998).
29. Corsetti, G. *et al.* Morphometric changes induced by amino acid supplementation in skeletal and cardiac muscles of old mice. *Am J Cardiol* **101**, 26E-34E (2008).
30. Chevalier, L. *et al.* High-protein diets differentially modulate protein content and protein synthesis in visceral and peripheral tissues in rats. *Nutrition* **25**, 932-939 (2009).
31. van Norren, K. *et al.* Dietary supplementation with a specific combination of high protein, leucine, and fish oil improves muscle function and daily activity in tumour-bearing cachectic mice. *Br J Cancer* **100**, 713-722 (2009).
32. Deldicque, L. *et al.* Antagonistic effects of leucine and glutamine on the mTOR pathway in myogenic C2C12 cells. *Amino acids* **35**, 147-155 (2008).
33. Nakajo, T. *et al.* Glutamine is a key regulator for amino acid-controlled cell growth through the mTOR signaling pathway in rat intestinal epithelial cells. *Biochem Biophys Res Commun* **326**, 174-180 (2005).
34. Byfield, M.P., Murray, J.T. & Backer, J.M. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J Biol Chem* **280**, 33076-33082 (2005).
35. Findlay, G.M., Yan, L., Procter, J., Mieulet, V. & Lamb, R.F. A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem J* **403**, 13-20 (2007).

36. Suryawan, A. *et al.* Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. *Am J Physiol Endocrinol Metab* **295**, E868-875 (2008).
37. Norton, L.E. *et al.* The leucine content of a complete meal directs peak activation but not duration of skeletal muscle protein synthesis and mammalian target of rapamycin signaling in rats. *J Nutr* **139**, 1103-1109 (2009).
38. Doucet, M. *et al.* Muscle atrophy and hypertrophy signaling in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **176**, 261-269 (2007).
39. Lumeng, C.N. & Saltiel, A.R. Insulin htts on autophagy. *Autophagy* **2**, 250-253 (2006).
40. Murton, A.J., Constantin, D. & Greenhaff, P.L. The involvement of the ubiquitin proteasome system in human skeletal muscle remodelling and atrophy. *Biochim Biophys Acta* **1782**, 730-743 (2008).
41. Sacheck, J.M., Ohtsuka, A., McLary, S.C. & Goldberg, A.L. IGF-I stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin ligases, atrogin-1 and MuRF1. *Am J Physiol Endocrinol Metab* **287**, E591-601 (2004).
42. Stitt, T.N. *et al.* The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* **14**, 395-403 (2004).
43. Sandri, M. *et al.* Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**, 399-412 (2004).
44. Mammucari, C. *et al.* FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**, 458-471 (2007).
45. Sugawara, T., Ito, Y., Nishizawa, N. & Nagasawa, T. Supplementation with dietary leucine to a protein-deficient diet suppresses myofibrillar protein degradation in rats. *Journal of nutritional science and vitaminology* **53**, 552-555 (2007).
46. Sadiq, F., Hazlerigg, D.G. & Lomax, M.A. Amino acids and insulin act additively to regulate components of the ubiquitin-proteasome pathway in C2C12 myotubes. *BMC Mol Biol* **8**, 23 (2007).
47. Herningtyas, E.H. *et al.* Branched-chain amino acids and arginine suppress MaFbx/atrogin-1 mRNA expression via mTOR pathway in C2C12 cell line. *Biochim Biophys Acta* **1780**, 1115-1120 (2008).
48. Latres, E. *et al.* Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. *J Biol Chem* **280**, 2737-2744 (2005).

Chapter 4

Nuclear transcription factor κB activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia

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Abstract

Experimental models of cancer cachexia have indicated that systemic inflammation induces muscle-protein breakdown and wasting via muscular nuclear transcription factor κB (NF-κB) activation. This process may limit the efficacy of nutritional intervention. We assessed muscle NF-κB activity and protein turnover signaling in progressive stages of clinical lung cancer cachexia and assessed whether circulating factors can induce muscular NF-κB activity. Patients with lung cancer precachexia (n = 10) and cachexia (n = 16) were cross-sectionally compared with 22 healthy control subjects. mRNA transcripts of muscle proteolytic (ubiquitin proteasome system and autophagy lysosomal pathway) and myogenic markers and protein expression of PI3K/Akt, myostatin, and autophagy signaling were measured. A multiplex analysis showed the systemic inflammatory status, whereas plasma exposure to stable NF-κB-luciferase-reporter muscle cells revealed NF-κB inducibility. Compared with healthy control subjects, cachectic patients had reduced (appendicular) muscle mass (-10%), muscle fiber atrophy (-27%), and decreased quadriceps strength (-31%). Subtle alterations in the muscle morphology were also detectable in precachectic patients, without changes in body composition. Despite increased Akt phosphorylation, downstream phosphosubstrates glycogen synthase kinase 3b, mammalian target of rapamycin, and Forkhead box protein were unaltered. The expression of autophagy effectors B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3 and microtubule-associated proteins 1A/1B light chain 3B gradually increased from precachectic to cachectic patients, without differences in E3 ubiquitin ligases. Systemic and local inflammation was evident in cachexia and intermediate in precachexia, but the plasma of both patients groups caused ex vivo muscle NF-κB activation. In lung cancer, muscular NF-κB activity is induced by factors contained within the circulation. Autophagy may contribute to increased muscle proteolysis in lung cancer cachexia, whereas the absence of downstream changes in phosphosubstrates despite increased Akt phosphorylation suggests impaired anabolic signaling that may require targeted nutritional intervention.

Introduction

It has been well established that cachexia is a severely debilitating syndrome that accompanies cancer, and it was recently postulated that cancer cachexia develops in a spectrum that traverses mild to advanced stages (i.e. precachexia, cachexia, and refractory cachexia)[1]. The syndrome has received growing attention as unmet medical need because it is directly responsible for 20% of cancer-related deaths[2]. Patients who suffer from pulmonary malignancies have shown a high prevalence and rapid progression of cachexia, but currently, effective interventions to prevent or reverse cachexia in lung cancer are not available[1, 3].

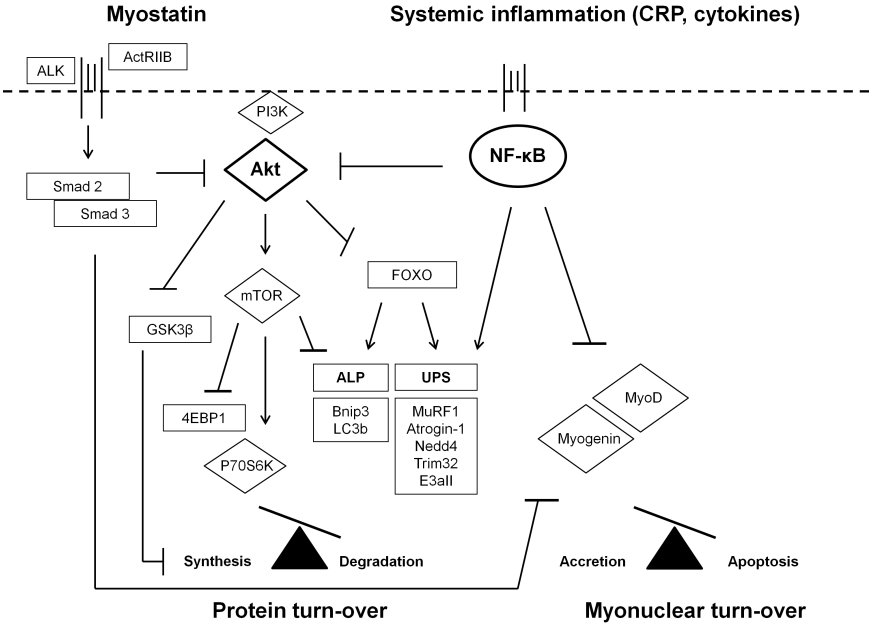
Studies in the 1990s already showed that it is particularly the loss of skeletal muscle cell mass that is accountable for the poor prognosis and declined performance status in lung cancer cachexia[4-6]. Consequently, skeletal muscle wasting can be considered an important indicator of cachexia progression as well as a potent target of nutritional or pharmacologic intervention[1]. High- quality protein diets as well as specific amino acids that target the nutrient-sensing signaling pathways have shown promising effects on muscle mass maintenance in chronic lung disease[7]. To identify the potential of such anabolic interventions in lung cancer cachexia, which may differ depending on the stage of cachexia, it is crucial to obtain more insight in molecular alterations of skeletal muscle protein turnover in patients who suffer from cancer cachexia.

In muscle-protein metabolism, protein synthesis and degradation are subject to extensive (patho)physiologic regulation, and the balance between synthesis and degradation ultimately determines the net muscle protein turnover[8, 9]. Experimental studies have especially postulated a role of ubiquitin 26S-proteasome system (UPS)–dependent proteolysis in cancer cachexia and, to a lesser extent, autophagy lysosomal pathway–dependent protein degradation[10-12]. Myonuclear turnover may constitute an additional determinant of muscle mass by the accretion (myogenesis) and loss of muscle nuclei[12]. Current insights in the mechanisms that dictate muscle-mass plasticity in cancer cachexia have revealed intricate interactions between these processes, but data have been primarily obtained from experimental research[8, 9, 12]. A schematic representation of mediators identified in experimental cancer cachexia is shown in **Figure 1**.

Systemic inflammation is considered an important host-related alteration that induces muscle atrophy in cancer cachexia[13, 14]. Systemic inflammation conveys its action via local inflammatory signaling through the nuclear transcription factor κB (NF-κB)[15-17]. However, although increased systemic inflammation profiles[18, 19] and elevated muscular NF-κB signaling have been observed in cancer patients[20], it remains to be addressed whether mediators in the circulation can be causally linked to the activation of muscle NF-κB signaling in lung cancer cachexia.

The objective of the current study was to investigate the expression of signaling molecules involved in protein metabolism during different stages of lung cancer cachexia and assess whether factors within the circulation can induce muscle inflammatory NF-κB signaling. These indexes were studied in a cross-sectional study design, in which healthy control subjects and precachectic and cachectic patients with non–small cell lung cancer (NSCLC) were compared.

Figure 1



Schematic representation of signaling molecules involved in muscle mass turnover in cancer cachexia [inhibitory signal (—|); activating signal (/)]. The predominant signaling cascade

involved in muscle protein synthesis is the PI3K/Akt pathway. The protein kinase Akt is a central mediator that induces stimulatory or inhibitory phosphorylation of downstream mediators such as GSK-3b as well as mTOR. Furthermore, Akt activity blocks catabolic signaling via inhibitory phosphorylation of FOXO 1 and 3a, which are potent inducers of proteolytic cues (ie, UPS and ALP). Experimental research revealed that the UPS and ALP are important proteolytic systems involved in muscle-protein depletion during catabolic conditions. In the UPS, individual proteins are targeted for degradation by the 26S proteasome through covalent binding of a polyubiquitin chain. In experimental cancer cachexia, E3-ubiquitin-ligase enzymes are considered rate limiting in this process of protein targeting, and the activation of NF-kB has been linked to the activation of this pathway. In the ALP, autophagosomes fuse with lysosomal structures to degrade cellular components. BNIP3 and LC3B are important markers of autophagosome formation. Myostatin is a transforming growth factor-b super family member and potent negative regulator of muscle mass. Intracellular myostatin signaling in muscle occurs after the binding of myostatin to the ActRIIB receptor and recruitment of ALK. Subsequently the intracellular phosphorylation of Smad 2 and 3 proteins occurs, which, on complex formation, transfer to the nucleus to convey their actions. Impaired myonuclear accretion (myogenesis) might also contribute to muscle atrophy. Experimental research has indicated that the expression of MRFs such as MyoD and myogenin, which are essential in myogenesis, is altered in experimental cancer-induced muscle wasting. ActRIIB; activin receptor type II B; ALK, activin receptor like kinase; ALP, autophagy lysosomal pathway; Bnip3, B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3; CRP, C-reactive protein; E3aII, ubiquitin-protein ligase E3a-II; GSK3b, glycogen synthase kinase 3b; LC3b, microtubule-associated proteins 1A/1B light chain 3B; MRF, myogenic regulatory factor; mTOR, mammalian target of rapamycin; FOXO, Forkhead box protein; MuRF1, muscle-specific RING finger 1; Nedd4, neuronal precursor cell expressed developmentally downregulated 4; NF-kB, nuclear transcription factor kB; Trim32, tripartite motif-containing protein 32; UPS, ubiquitin 26S-proteasome system; 4EBP1, 4E-binding protein 1.

Patients and Methods

Study population

Newly diagnosed patients with advanced stage NSCLC admitted to the Department of Respiratory Medicine of the Maastricht University Medical Centre^{+} between July 2007 and July 2010 were eligible for participation in the study. Participants were divided into precachectic and cachectic groups according to the definition in the

international cancer cachexia consensus[1]. Precachexia is defined as “an early stage in which clinical and metabolic signs such as anorexia and systemic inflammation can precede substantial (i.e. >5%) body weight loss”[1]. Diagnostic criteria for cancer cachexia are “body weight loss of >5% in the past 6 months or body weight loss of >2% in combination with either BMI (in kg/m^2) <20 or appendicular skeletal muscle index consistent with sarcopenia”[1]. NSCLC was confirmed by pathologic analysis, and tumor stage was determined by using the 6th Tumor-Node-Metastasis International Staging System for Lung Cancer[21, 22].

Smoking is not only an important risk factor for lung cancer but also for chronic obstructive pulmonary disease (COPD), which is another condition associated with cachexia, especially in advanced stages[23, 24]. To study a representative sample of lung cancer patients but minimize the interference of advanced comorbidities or drugs that could have potential effects on the studied variables, patients with the following characteristics were excluded: Global Initiative for Chronic Obstructive Lung Disease stage IV COPD, Congestive Heart Failure New York Heart Association stage III-IV, and active infectious disease as well as patients who were taking hormones or continual oral corticosteroids. Additional exclusion criteria were the presence of other malignant disease and the initiation of antitumor therapy.

Healthy control subjects were selected by using advertisements in newspapers. It was confirmed that healthy control subjects had no recent body weight loss or any of the previously mentioned diseases or used any of the earlier described medications.

The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre$^+$ and conducted according to local ethical guidelines. The study was not registered in an additional public trial registry because inclusion started before the moment that this became an obligation (i.e. July 2008). All participants signed informed consent forms.

All tests mentioned hereafter were performed on the same day after 8 h fasting.

Pulmonary function

Pulmonary function was tested to assess the extent of airflow obstruction in the current population because the coincident presence of COPD could influence the

tests described hereafter. Forced expiratory volume in 1 s and forced vital capacity were assessed by using spirometry.

Body composition

Dual-energy X-ray absorptiometry (DPX-L; Lunar Radiation Corp) was used to measure different body compartments (i.e. fat mass, lean mass, and bone mineral content).

Muscle strength

The isometric strength of quadriceps muscle of the contra lateral leg where the muscle biopsy was taken was measured by using a dynamometer (Biodex system version 3.3; Biodex). Subjects were seated upright on the dynamometer, and straps were attached at the level of the thigh and ankle. Isometric muscle-strength testing was performed at an angle of 60° (3 repetitions). Muscle strength was defined as the highest muscular force output (peak torque) in newton meters.

Plasma inflammatory markers

Venous blood sampling was performed by using a blood collection tube containing EDTA (Sherwood Medical). Blood was processed in 2 successive steps. First, the collected samples were centrifuged at 3000 relative centrifugal force for 10 min (48C). Plasma was collected and subjected to a second centrifugation step (5 min; 3000 relative centrifugal force; 4°C) to remove any remaining cellular constituents. Aliquots of blood samples were stored at -80°C until analyses were performed.

C-reactive protein (CRP) was measured by using a CardioPhase high-sensitivity CRP kit according to the manufacturers' protocol (Siemens Healthcare Diagnostics). The lower detection limit was 0.2 mg/L. A Human Multiplex Antibody assay was run with the Luminex System (Invitrogen; Life Technologies) to determine plasma TNF-a, soluble TNF (sTNF)–receptor 1 and 2, IL-6, -8, and -10, and interferon-g concentrations (lower detection limit ranged from 5 to 28 pg/mL). All samples were analyzed in duplicate, and these assays were performed at Invitrogen Luminex Testing Services.

Muscle biopsies

Percutaneous needle biopsies of quadriceps muscle (vastus lateralis muscle) were obtained of all subjects. The technique used for muscle biopsies was described by Bergström[25]. Muscle specimens were processed for either histochemical (immunohistochemistry) or biochemical [quantitative real-time polymerase chain reaction (Q-PCR) and Western blot analysis] analysis.

Muscle specimens for the histochemical analysis were embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek Europe BV) and subsequently frozen in melting isopentane, which was precooled in liquid nitrogen. Samples were stored at -80°C. Before histochemical analysis, serial cryostat cross-sections (5 mm) were cut on a cryostat (Leica Biosystems) at -20°C and mounted on SuperFrost microscope slides (Menzel- Gla¨ser).

Muscle specimens for biochemical assays were snap frozen in liquid nitrogen and subsequently stored at -80°C. Before analyses, muscle biopsies were crushed with a mortar and pestle in liquid nitrogen.

Cross-sectional area of muscle fibers

Immunohistochemical staining of laminin was used to determine the muscle fiber cross-sectional area. Muscle sections were air dried overnight when the sections were treated with phosphate-buffered saline (PBS) for 30 min and subsequently with 0.5% Triton X-100 (Sigma-Aldrich) solution in PBS for 5 min. Sections were incubated for 45 min with primary antilaminin antibody (L- 9393; dilution 1:50;(Sigma). Sections were rinsed once with 0.05% Tween (Sigma-Aldrich) solution in PBS and twice with regular PBS for 5 min. Subsequently, sections were incubated for 45 min with secondary antibody Alexa Fluor 350 (Invitrogen; Life Technologies; A-11069; dilution 1:100). Slides were twice rinsed with 0.05% and regular PBS for 5 min. Images for analysis were obtained by using fluorescent microscopy (objective: 310). Computer image analysis was performed with Lucia Software (version 4.81; Laboratory Imaging). Per biopsy, an average of 200 fibers were analyzed (≥100). Detached, damaged, or non–cross-sectional fibers were excluded from the analysis.

Messenger RNA abundance

For messenger RNA (mRNA) expression analysis, a ToTALLY RNA Kit (Ambion Ltd) was used according to the manufacturers' protocol. Muscle specimens (10–30 mg) were homogenized by using a Polytron PT 1600 E (Kinematica AG) sample homogenizer, and total RNA was extracted with the use of the ToTALLY RNA Kit. Subsequently, an RNeasy Mini Kit with RNase-free DNase (Qiagen) was used for the elution of contaminated genomic DNA, and RNA concentrations were measured by using spectrophotom- etry (NanoDrop ND-1000; Isogen Lifescience). A total of 400 ng total RNA was reverse transcribed to complementary DNA (cDNA) with anchored oligo(dT) primers according to the supplier's protocol (Transcriptor First Strand cDNA Synthesis kit; Roche Diagnostics). Q-PCR primers (Sigma Genosys) were designed for the detection of mRNA transcripts of nuclear factor of k light polypeptide gene enhancer in B cells inhibitor a [IkBa (inhibitory protein of NF-kB); IkBa mRNA expression was considered an indirect measure of NF-κB activity because IkBa constitutes a target gene of NF-κB, which ensures negative feedback regulation to prevent uncontrolled and sustained NF-κB activity[26]) (5#-CTACACCTTGCCTGTGAGCA-3# and 3#-TCCTG- AGCATTGACATCAGC-5), myostatin (5#-AACCTTCCCAGG ACCAGGAGAA-3# and 3#-TGTCTGTTACCTTGACCTCTA- AAAACGG-5), Muscle-specific RING finger 1 (MuRF1) (5#- GCGAGGTGGCCCCATT-3# and (3#- GATGGTCTGCACACG GTCATT-5), muscle atrophy F-box (MAFbx)/atrogin-1 (atrogin-1) (5#-GAAGAAACTCTGCCAGTACCACTTC-3# and 3# CCCTT-TGTCTGACAGAATTAATCG-5#), tripartite motif-containing protein 32 (5#-ATTTTGCTTCCTTATCTCACTGTGTTCTTT-3# and 3#-CATAATAGTGCTTTTGGCTGAATTTTGAC-5#), neuronal precursor cell expressed developmentally downregulated 4 (5#- TCACTGGCACATCTCGGGTG-3# and 3#-TCATAAGGTGGC AAGTCCAGGC-5#), ubiquitin-protein ligase E3a-II (5#-ACA GAGCACCTGTGGGAGGACTAG-3#) and 3#-CACATTAAAGC-AAGTATGAGATGTAGGTAACCT-5#), microtubule-associated proteins 1A/1B light chain 3B (LC3B) (5#- ACCATGCCG- TCGGAGAAGAC-3#and 3#-TCTCGAATAAGTCGGACATC- TTCTACTCT-5#), B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) (5#-AGCGCCCGGGA- TGCA-3# and 3#- CCCGTTCCCATTATTGCTGAA-5#), MyoD (5#-CACAGCGCGGTTTTTTCC-3# and 3#-TGAACCTAGCC- CCTCAAGGTT-5#), and

myogenin (5#-TCAGCGCCAACCC- AGG-3# and 3#-GGTGAGGGAGTGCAGGTTGT-5#). Q-PCRs contained 13 SensiMix SYBR & Fluorescein Kit (Bioline) with 300-nmol/L primers and were run in Hard-Shell 96-well Semi-skirted PCR plates (Bio-Rad) on a MyiQ thermocycler (Bio-Rad) according the following program: an initial 15-min incubation at 95°C, thermal cycling was performed by using 40 cycles of 95°C for 15 s and 60°C for 45 s. A geNorm factor (qbase+; Biogazelle) was calculated from mRNA transcript expression of cyclophilin (5#-CATCTGCACTGCCAAGACTGA-3# and 3#-TTCATGCCT TCTTTCACTTTGC-5#), b-actin (5#-AAGCCACCCCACTTCT- CTCTAA-3# and 3#-AATGCTATCACCTCCCCTGTGT-5#), and ribosomal phosphoprotein LP0 (RPLP0) (5#-TCTACAACCCT- GAAGTGCTTGATATC-3# and 3#-GCAGACAGACACTGGCA-ACATT-5#) reference genes to normalize expression of target genes[27]. Standard curves, which were prepared from pooled cDNA, and melt curves were analyzed to verify the efficiency and specificity of amplification.

Protein and DNA

For protein analysis, equal amounts of w50 mg muscle tissue were subsequently dissolved in 400 mL lysis buffer [tris pH 7.4 (50 mmol/L), NaCl (150 mmol/L), glycerol (10%), nonyl phenoxypolyethoxylethanol (0.5%), EDTA (1 mmol/L), Na_3VO_4 (1 mmol/L), NaF (5 mmol/L), b-glycerophosphate (10 mmol/L), Na-pyro-PO_4 (1 mmol/L), dithiothreitol (1 mmol/L), leupeptin (10 mg/mL), aprotenin (1%), and phenylmethylsulfonyl fluoride (1 mmol/L)] and homogenized with Polytron PT homogenizer (Polytron PT 1600 E; Kinematica AG). After 30 min incubation on ice, muscle homogenates were sonicated and centrifuged at 4°C (16000 relative centrifugal force during 30 min). Supernatant fluid (referred to as cytoplasmic fraction) was separated from the pellet (referred to as the myofibrillar fraction). Protein concentrations of cytoplasmic and myofibrillar fractions were determined by using a BCA Protein Assay Kit (Pierce, Thermo Fisher), according to the manufacturer's instructions. A Quant-iT double-stranded DNA assay kit (Promega) was used according to manufacturers' instructions to measure the DNA content in the myofibrillar fraction.

For Western blot analysis of signaling proteins, a sample buffer (43 stacking buffer: 0.250 mol/L Tris-HCL, 8% SDS, 40% glycerol, 0.4 mol/L dithiothreitol, and 0.02%

Bromphenol blue) was added in a 1:4 dilution to the cytoplasmic fraction, and samples were incubated 5 min at 95°C. Equal amounts of protein were loaded per lane of a Wells Criterion XT 4–12% bis-tris precast gel (Bio-Rad). Two standard samples were included in every blot to correct for blot-to-blot variation. Electrophoresis was performed by using an Electrophoresis Cell system (Bio-Rad). Gels were transferred to nitrocellulose membranes (Whatman; GE Healthcare). Membranes were blocked during 60 min in 2% bovine serum albumin or 5% milk in Tris-buffered saline with 0.05% Tween 20 (Sigma-Aldrich) and exposed to primary antibodies. Primary antibodies of total and phosphorylated Akt-Ser473 (total: 9272; phosphorylated: 9271; Cell Signaling Technology), Fork- head box protein 1 [FOXO 1 (total: 2880; serine 256 phosphorylated: 9461; Cell Signaling Technology)], FOXO 3a (total: 2497; threonine 32 phosphorylated: 9464; Cell Signaling Technology), mammalian target of rapamycin (mTOR) (total: 2983; serine 2448 phosphorylated: 2971; Cell Signaling Technology), glycogen synthase kinase 3b (GSK-3b) (total:27C10; serine 9 phosphorylated: 9336; Cell Signaling Technology), 4E-binding protein 1 (total: 9452 and threonine 37/46 phosphorylated: 9459: Cell Signaling Technology), P70S6 kinase (total: 9202; threonine 389 phosphorylated: 9206; Cell Signaling Technology), Smad 2 (total: 5339; serine 465/467 phosphorylated: 3108; Cell Signaling Tech- nology), Smad 3 (total: 9523; serine 423/425 phosphorylated: 9520; Cell Signaling Technology), and LC3B (2775; Cell Signaling Technology) were used. GAPDH (2118; Cell Technology) was used as a loading control. Primary antibodies were incubated overnight at 4°C. Next, membranes were incubated with secondary anti-bodies (1:5000) of anti-mouse IgG peroxidase (A85PI-1000.S1; Bio-Connect) or anti-rabbit IgG peroxidase (A85PI-2000.S1; Bio-Connect). Detection was performed by using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer's manual. Densitometric quantification was performed with the use of Quantitiy One software (version 4.6.2; Bio-Rad).

Plasma transfer experiments

C2C12 murine myoblasts stably transfected with a luciferase reporter construct that contained 3 tandem NF-κB luciferase responsive elements were cultured and differentiated into mature myotubes for 5 d as described before[28]. A differentiation medium (Dulbecco's Modified Eagle's Medium containing 0.5% fetal bovine serum, 50 U penicillin/mL, and 50 mg streptomycin/mL) was supplemented with plasma

(10% final; vol/vol) of individual subjects in the presence of 50 U heparin/mL (Leo). Myotubes were incubated for 4 h. This time point was identified in pilot experiments with the pooled plasma of study subjects. Subsequent analyses were performed by using individual samples. For analysis, myotubes were harvested in 500 mL 13 luciferase buffer on ice and stored at -80°C. Luciferase activity was measured according to manufacturer's protocol (Promega) by using a luminometer (Berthold Technologies). Luciferase activity was corrected for total protein by using the Bradford assay according to the manufacturer's protocol (Bio-Rad).

Statistics

Data were analyzed with Statistical Package for the Social Sciences software (SPSS version 15 for Windows; SPSS Inc). Except for baseline body weight loss, which represented the weight loss within individual patients in the 6 months before diagnosis, all data represent comparisons between healthy control subjects and precachectic and cachectic patient groups. When changes are described in percentages (except for baseline weight loss), the change in a specific patient group relative to healthy control subjects is represented. Continuous variables were compared by using 1-factor ANOVA followed by a least-significant difference post hoc analysis. Because post hoc least-significant difference comparisons involved only 3 groups, no additional adjustments for an experiment-wise error rate were performed[29]. Pearson's chi-square test and, in addition, Fisher's exact test (when expected counts were <5) were used for comparison of categorical variables. Both tests showed the same results for all variables. Correlations were evaluated by using Pearson's correlation test. Data in tables are represented as means 6 SDs. Error bars in figures represent the SEMs. Significance was set at $P < 0.05$.

Results

Distinct appendicular muscle depletion in cachectic but not precachectic lung cancer patients

Subject characteristics are presented in **Table 1**. There were no significant differences in sex, age, or tumor stage between study groups. As a result of the smoking history of many lung cancer patients, lung function was significantly lower in patients, but no significant correlations were shown between lung function (forced expiratory volume in 1 s) and body weight loss, lean mass index, or other indexes of cachexia in any of the groups (data not shown).

At NSCLC diagnosis, patients with precachexia had a mean within-patient body weight loss of 1.7% in the 6 months before diagnosis, whereas patients with cachexia showed a mean body weight loss of 12% in the 6 months before diagnosis ($P < 0.05$) (**Table 1**).

Comparisons of healthy control, precachectic, and cachectic groups at the moment of diagnosis revealed that lean mass atrophy was evidently present in cachectic patients compared with in healthy control subjects but not in precachectic patients (**Figure 2A**), which was in correspondence with the cancer cachexia consensus definition[1]. Specifically, the appendicular lean mass index was lower (-20%; $P < 0.05$) in cachectic patients compared with in healthy control subjects, whereas trunk lean mass was not significantly different between any of the study groups (**Figure 2A**). No differences were observed in the fat mass index and bone mineral content (**Figure 2A**).

Table 1

Basic characteristics of study population

	Healthy controls (N = 22)	Pre-cachexia (N = 10)	Cachexia (N = 16)
Gender			
Male (%)	59	80	56
Female (%)	41	20	44
Age (years)	61.4 ± 7.0[2]	62.4 ± 10.4	59.8 ± 8.2
Height (m)	1.73 ± 0.10	1.77 ± 0.06	1.72 ± 0.10
Premorbid body (weight 6 months prior to diagnosis) (kg)	72.7 ± 11.5	81.5 ± 10.3	76.8 ± 17.4
Body weight at diagnosis (kg)	72.7 ± 11.5	80.2 ± 10.4	67.7 ± 16.3***
Body weight loss (kg) within patients in 6 months prior to diagnosis	0 ± 0	1.3 ± 1.2	9.2 ± 4.3 **,***
Body weight loss (%) within patients in 6 months prior to diagnosis	0 ± 0	1.7 ± 1.4	12.0 ± 5.5 **,***
Disease stage of NSCLC[1]			
IIIB (%)	-	60	25
IV (%)	-	40	75
Histology of NSCLC			
Adenocarcinoma (%)	-	70	56
Squamous cell (%)	-	30	44
Smoking			
Current %	5[†]	20[†]	50[†]
Former %	54	80	44
Never %	22	0	6

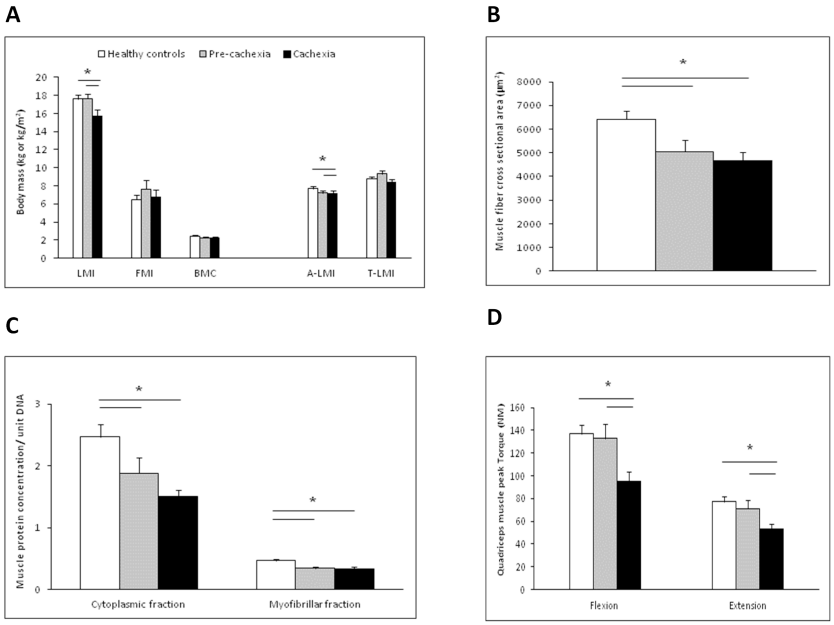
Lung function			
FEV1 (% predicted)	115.7 ± 19.3	77.0 ± 18.4*	61.9 ± 17.2**
FVC (% predicted)	125.4 ± 1.1	100.0 ± 9.9*	75.5 ± 22.0**,***
Tiffeneau-index	0.74 ± 0.08	0.60 ± 0.12*	0.65 ± 0.13**
COPD GOLD stage (on the basis of spirometry in the current study)			
No COPD	73	20	44
I (%)	23	10	13
II (%)	4	30	19
III (%)	0	10	19
IV (%)	0	0	0
No spirometry data	0	30	5

*[1] *, **, ***One-factor ANOVA and LSD post hoc testing: *P < 0.05 (precachexia compared with control subjects), **P < 0.05 (cachexia compared with control subjects), ***P < 0.05 (precachexia compared with cachexia). [†] P < 0.05 (Pearson's chi-square or Fisher's exact test). COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; LSD, least-significant difference; NSCLC, non–small cell lung cancer tumor-node-metastasis stage. [2] Mean ± SD (all such values).*

Muscle fiber atrophy and decreased muscle protein content in cachectic and precachectic lung cancer patients

The muscle fiber mean cross-sectional area (-27%) and muscle protein concentration per unit DNA (-30–40%) as well as quadriceps muscle strength (-31%) were substantially lower in the cachectic group than in healthy control subjects ($P < 0.05$) (**Figure 2, B–D**). Compared with healthy control subjects, precachectic patients showed a consistent pattern of intermediate values for these indexes of muscle mass without significant changes in muscle strength but a significantly lower muscle fiber cross-sectional area (-21%; $P < 0.05$) and muscle protein con- centration per unit DNA ratio (-24% to 27%; $P < 0.05$) (**Figure 2, B–D**).

Figure 2



*Mean (±SEM) progressive appendicular muscle (fiber) atrophy, decreased muscle-protein content, and reduced muscle strength in precachectic and cachectic lung cancer patients. A: The composition of different body compartments was evaluated by using dual-energy X-ray absorptiometry. Indexes were calculated to correct for differences in height [LMI (in kg/m^2), FMI (in kg/m^2); BMC (in kg); A-LMI (in kg/m^2); T-LMI (in kg/m^2)]. B: Cross-sectional area of individual muscle fibers was determined by using immunohistochemical staining of laminin on cryosections of quadriceps muscle biopsies. C: Protein concentrations in myofibrillar and cytoplasmic fractions obtained from crushed muscle biopsies were expressed as a ratio of the DNA concentration. D: The quadriceps muscle isometric peak torque was assessed by using a dynamometer (Biodex system version 3.3; Biodex). *P < 0.05 between indicated groups (1-factor ANOVA and least-significant difference post hoc testing). A-LMI, appendicular lean mass index; BMC, total bone mineral content; FMI, total fat mass index; LMI, total lean mass index; NM, newton meters; T-LMI, trunk lean mass index.*

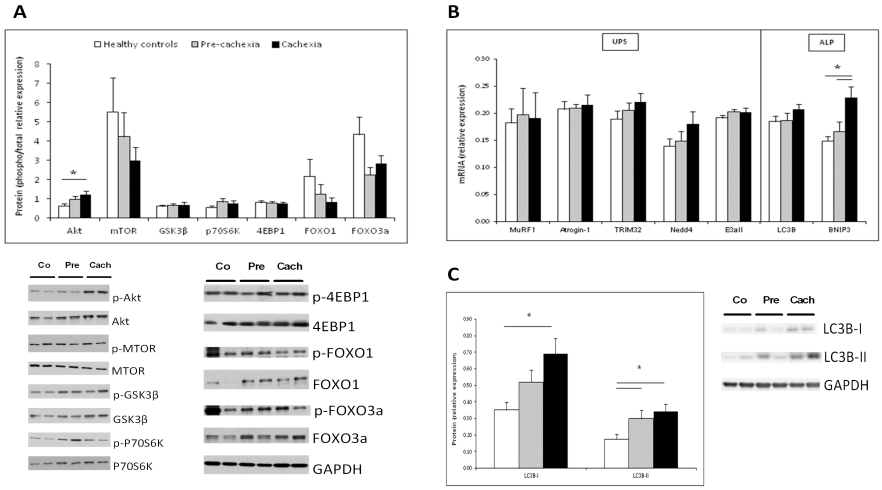
Differential activation of anabolic and catabolic signaling pathways in precachectic and cachectic patients with NSCLC

To assess the anabolic activity in skeletal muscle, the phosphorylation status of Akt and its downstream phosphosubstrates GSK-3b and FOXO as well as mTOR phosphorylation were assessed. Akt Ser473 phosphorylation to the total protein ratio was significantly increased in cachectic patients compared with in healthy control subjects ($P < 0.05$), whereas precachectic patients showed intermediate expression (**Figure 3A**). In contrast, none of the Akt phosphosubstrates (i.e. GSK-3b, FOXO, and mTOR) displayed significant alterations in phosphorylation status (**Figure 3A**).

To assess proteolytic signaling in muscle, the expression of main constituents of the UPS and autophagy lysosomal pathway was determined. No changes in mRNA expression of E3-ubiquitin- ligases MuRF1, atrogin-1, tripartite motif-containing protein 32 (TRIM32), neuronal precursor cell expressed developmentally downregulated 4 (NEDD-4), or E3a-II were observed between patient groups (**Figure 3B**). Conversely, significantly higher BNIP3 mRNA transcriptional expression and LC3B protein expression was observed in muscle biopsies of patients with cachexia than in healthy control subjects ($P < 0.05$) (**Figure 3, B and C**). mRNA transcripts of LC3B were not different between study groups (**Figure 3B**).

Figure 3

Activation of signaling pathways



*Mean (±SEM) differential activation of anabolic and catabolic signaling pathways in precachectic and cachectic patients with non-small cell lung cancer. Quadriceps muscle biopsies were processed for analysis of mRNA transcripts and protein expression. A: Protein expression of Akt and downstream phosphosubstrates. Protein expression of phosphorylated to the total protein ratio of Akt/PI3K anabolic pathway constituents were determined in skeletal muscle by using Western blot analysis. The expression of phosphorylated and total protein expression was normalized by using GAPDH as a loading control. B: mRNA transcripts of constituents of the UPS and ALP proteolytic systems. mRNA transcripts of constituents of the UPS (E3-ubiquitin-ligases MuRF1, muscle atrophy F-box/atrogin-1, TRIM32, Nedd4, and E3aII) and ALP (LC3B and BNIP3) were determined in skeletal muscle biopsies. mRNA transcripts of target genes were normalized to a geNorm factor (qbase+; Biogazelle) that was calculated from expression of cyclophilin, b-actin, and ribosomal phosphoprotein LP0 (RPLP0) reference genes. C: Protein expression of the ALP proteolytic system. LC3B-I and LC3B-II were determined as markers of the ALP in skeletal muscle biopsies using Western blot analysis. Protein expression was normalized by using GAPDH as loading control. *P < 0.05 (1-factor ANOVA and least-significant difference post hoc testing). ALP, autophagy lysosomal pathway; Atrogin-1, muscle atrophy F-box/ atrogin-1; BNIP3, B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3; Cach, cachectic patient; Co, healthy control subjects; E3aII, ubiquitin-protein ligase E3a-II; FOXO 1, Forkhead box protein 1; FOXO 3a, Forkhead box protein 3a; GSK-3b, glycogen synthase kinase 3b; LC3B, microtubule-associated proteins 1A/1B light chain 3B; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; MuRF1, muscle-specific*

RING finger 1; Nedd4, neuronal precursor cell expressed developmentally downregulated 4; p-Akt, phosphorylated Akt; p-4EBP1, phosphorylated 44E-binding protein 1; p-FOXO 1, phosphorylated Forkhead box protein 1; p-FOXO 3a, phosphorylated Forkhead box protein 3a; p-GSK-3b, phosphorylated glycogen synthase kinase 3b; p-mTOR, phosphorylated mammalian target of rapamycin; Pre, precachectic patients; P70S6K, P70S6 kinase; p-P70S6K, phosphorylated P70S6 kinase; TRIM32, tripartite motif-containing protein 32; UPS, ubiquitin 26S-proteasome system; 4EBP1, 4E-binding protein 1.

No marked alterations in regulators of myogenesis

mRNA transcript expression of the negative muscle mass regulator myostatin as well as protein phosphorylation of its downstream signaling constituents (i.e., Smad 2 and 3) was not altered in lung cancer patients compared with in healthy control subjects (see Supplemental data **Figure 1, A and B**). With respect to myogenic signaling, MyoD and myogenin transcripts were unaltered in lung cancer patients compared with in control subjects, whereas myogenin mRNA transcripts differed between precachectic and cachectic patients ($P < 0.05$) (see Supplemental data **Figure 1C** under “Supplemental data” in the online issue).

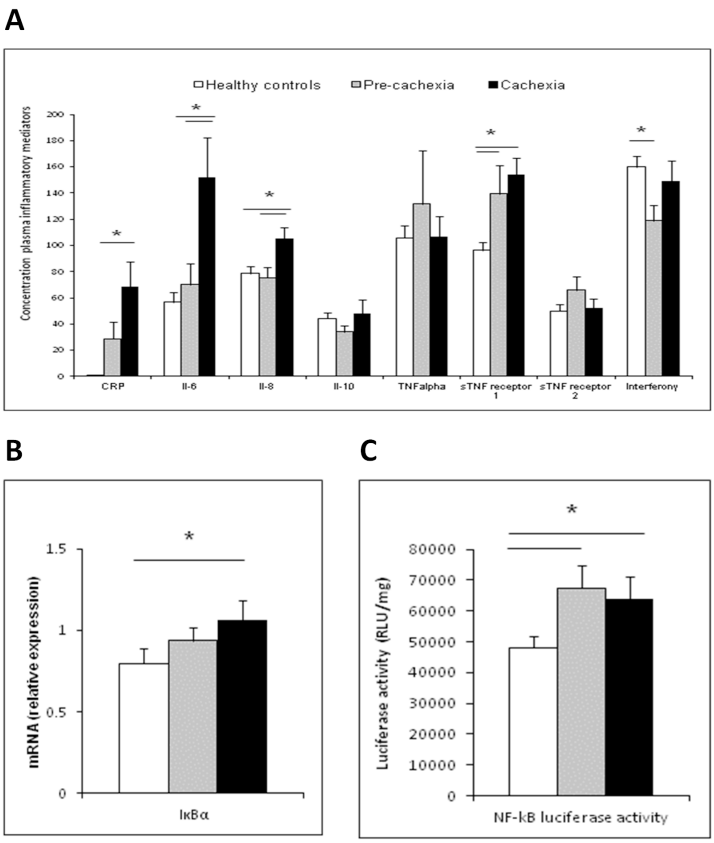
Factors in plasma of both precachectic and cachectic patients induce inflammatory signaling in skeletal muscle

To investigate the systemic and local inflammatory status in the cachexia spectrum, inflammatory markers in plasma and muscle were determined. Significantly higher concentrations of various systemic inflammatory mediators such as CRP, IL-6 and -8, and sTNF receptor 1 was observed in plasma of cachectic patients ($P < 0.05$) (**Figure 4A**). This was accompanied by activated inflammatory signaling in the skeletal muscle of cachectic patients, as shown by the increased mRNA expression of IkBa ($P < 0.05$), which is a target gene of NF-kB (**Figure 4B**). In precachectic patients, similar trends toward increased systemic and local inflammatory signaling were observed, albeit to a lesser extent, an only reached significance for sTNF receptor 1 plasma concentrations ($P < 0.05$) (**Figure 4, A and B**).

To explore the notion that inflammatory mediators present in the circulation can induce local inflammatory signaling, ex vivo plasma transfer experiments were conducted on cultured NF-κB–luciferase reporter muscle cells. These experiments

showed increased NF-κB activation when cultured muscle cells were exposed to plasma of both precachectic and cachectic patients ($P < 0.05$) (**Figure 4C**).

Figure 4 Plasma factors



Mean (±SEM) factors in plasma of both precachectic and cachectic patients induce inflammatory signaling in skeletal muscle. A: Systemic inflammatory status. A Luminex assay (Invitrogen; Life Technologies) was run on plasma of study subjects to assess concentrations of, TNFa (pg/mL), sTNF receptor 1 and 2 (pg/mL; values divided by 25), IL-6, -8, and -10 (pg/mL), and interferonγ (lower detection limit ranged from 5 to 28 pg/mL). CRP was measured using a CardioPhase high-sensitivity CRP (Siemens Healthcare Diagnostics). B: Muscle inflammatory signaling. mRNA transcripts of IkBa in quadriceps biopsies were assessed as an indirect measure of muscular NF-kB activity. Expression of IkBa were normalized to a geNorm

*factor (qbase+; Biogazelle) that was calculated from cyclophilin, b-actin, and ribosomal phosphoprotein LP0 reference genes. C: Inducibility of muscle NF-kB activity by factors contained within the circulation. Murine muscle cells were stably transfected with a luciferase reporter construct containing a promoter with NF-kB responsive elements. NF-kB activity was assessed after exposing muscle cells to plasma of study participants for 4 h by determining luciferase activity. *P < 0.05 (1-factor ANOVA and least-significant difference post hoc testing). CRP, C-reactive protein; IkBa, nuclear factor of k light polypeptide gene enhancer in B cells inhibitor a; NF-kB, nuclear transcription factor kB; sTNF, soluble TNF.*

Discussion

This study was conducted to identify whether mechanisms in the control of cachexia in experimental models are evident in patients with progressive stages of lung cancer cachexia. To the best of our knowledge, this was the first study comprehensively assessing regulation of muscle protein metabolism and transition of inflammatory signaling in precachectic and cachectic patients stratified according to the recent international cancer cachexia consensus[1]. Insight in the molecular mechanisms responsible for alterations of nutritional status in cancer cachexia is essential for the design and timing of tailored (nutritional) intervention strategies that alleviate the negative consequences of this destructive syndrome.

As shown by the anthropometric data, cachectic patients predominantly exhibited lean mass depletion, especially in the appendicular body compartment. Because the appendicular lean body compartment primarily consists of muscle mass[30, 31], this result indicated a specific loss of skeletal muscle. The wasting of muscle mass despite a similar fat mass index is indicative of an active catabolic state[1]. As a result of appendicular muscle mass depletion, quadriceps muscle strength was significantly declined in cachectic patients (**Figure 2D**). In line with and to a similar extent as the impaired muscle strength, muscle morphologic analyses revealed a substantial lower muscle fiber cross-sectional area and muscle protein per unit DNA ratio in the muscle of cachectic patients (**Figure 2, B and C**). Subtle alterations in muscle morphology were already detectable in the precachectic group, which were not identified by the body composition and muscle-function analysis (**Figure 2, A–D**). Future research is indicated to study if other noninvasive imaging techniques (i.e.,

MRI and computed tomography) may be more sensitive to show these subtle but clinically relevant changes in muscle morphology.

With respect to signaling pathways of muscle protein turnover, the PI3K/Akt pathway is considered essential for muscle protein synthesis and, more importantly, sensitive to nutritional modulation[8, 9]. The current data showed that Akt phosphorylation gradually increased in precachexia and was significantly elevated in cachexia (**Figure 3A**), which might have implied that there was increased anabolic activity as a compensatory response to muscle mass loss. However, Akt activation was not accompanied by phosphorylation of its downstream substrates (GSK-3b and FOXO), and correspondingly, no increases in mTOR phosphorylation were observed (**Figure 3A**)[8, 9]. This discrepancy between Akt activation and downstream signals suggested im- paired Akt activity, which might have implied a resistance to anabolic stimuli at the level of Akt. This resistance can have important consequences on muscle mass regulation because downstream molecules such as mTOR and FOXO are important regulators of muscle protein turnover[8, 9]. Because nutritional interventions with branched chain amino acids have been shown to stimulate the anabolic activity by directly affecting these mediators downstream of Akt, these findings implied a potential for nutritional interventions to circumvent putative anabolic resistance at the level of Akt in cancer cachexia[32]. To our knowledge, only one other report comprehensively studied PI3K/Akt signaling in cancer cachexia and showed a general decrease of the PI3K/Akt signaling cascade in patients with pancreatic cancer[33]. Previous studies in lung cancer patients only assessed isolated mediators (GSK-3b) of the PI3K/Akt pathway or myofibrillar protein fractional synthetic rate, which both were unaltered[34, 35].

Akt functions as a nodal point between anabolic and proteolytic pathways, and its control of protein degradation relies on the regulation of members of the FOXO transcription factors family. Different FOXO subtypes can activate proteolytic pathways when not phosphorylated by Akt[9, 12] by inducing an increased expression of regulatory constituents of the UPS and autophagy lysosomal pathway (ALP) proteolytic systems[36, 37]. However, the current study showed no differences in mRNA expression of a comprehensive set of UPS E3 ligases in any of the cachexia stages (**Figure 3B**). This seems discrepant because E3 ligases are involved and even considered rate limiting for UPS- mediated proteolysis in experimental cancer

cachexia, especially MuRF1 and atrogin-1[38, 39]. It is probable that the rapid development and disproportionate high tumor-to-total mass ratio resulting in more acute and more unrestrained host responses contributed to the significant UPS activation in experimental models[40]. As concerns clinical cachexia, a general problem of studies that investigated UPS activity was that they were conducted in small and heterogeneous populations[41-43]. With respect to E3 ligases, in line with the current study, expression is almost consistently unaffected in clinical cancer cachexia[35, 44-48]. Therefore, we conclude that, when lung cancer-related muscle atrophy involves increased proteolysis, it is unlikely to rely on sustained elevations in mRNA transcripts of E3 ubiquitin ligases.

In contrast to E3 ubiquitin ligases, the expression of ALP-associated effectors was increased in cachectic patients (i.e. mRNA transcripts encoding BNIP3 and protein expression of LC3B were upregulated in cachectic patients) (**Figure 3, B and C**). The activation of ALP may imply that, in contrast to highly regulated degradation of individual proteins in UPS-mediated proteolysis, cancer cachexia is characterized by bulk degradation of cytoplasmic components via ALP-dependent proteolysis. The observation that expression of LC3B mRNA transcripts were not increased (**Figure 3B**) may be explained because its activation is not necessarily accompanied by increased mRNA expression but is rather regulated on the protein level[49]. Autophagic flux measurement would be required to ultimately determine ALP activity because the increased expression of BNIP3 and LC3B markers might also reflect accumulation as a result of an obstruction of the ALP at some point, which would not result in increased autophagic activity[49]. However, because the patient population exhibited distinct muscle atrophy that was not accompanied by increased UPS activity, it is plausible that the current findings reflect a role for the ALP in cancer-induced muscle catabolism.

An evaluation of important myogenic regulators revealed no marked alterations at the level of myogenesis. Our data suggested a limited role for myostatin in the catabolic process in lung cancer because neither the expressions of myostatin mRNA transcripts nor Smad 2 and 3 protein signaling were altered in different stages of cachexia (see Supplemental data **Figure 1, A and B**, under “Supplemental data” in the online issue). This result was consistent with findings in non–weight-losing lung cancer patients[34] and cachectic colorectal cancer patients[35]. Contrarily, increased

myostatin expression was shown in the skeletal muscle of patients with gastrointestinal cancer, which may have indicated tumor-specific effects[34].

No consistent alterations in myogenic regulatory factor expression in the muscle of lung cancer patients were observed compared with in control subjects (see Supplemental data **Figure 1C** under "Supplemental data" in the online issue) despite affected MyoD and myogenin expression in experimental models[50, 51] and decreased muscular MyoD expression in a set of colorectal patients[52].

Because systemic factors can induce local muscular inflammatory signaling and could be a suitable target for intervention strategies, another aim of this study was to verify whether systemic to local transition of inflammatory signaling also occurs in clinical cachexia. In analogy with many studies, the current data showed a prominent systemic inflammatory response in cachectic patients with lung cancer[18, 20, 53], whereas intermediate increases were apparent in precachectic patients (**Figure** 4A). In addition, our findings extended these observations because the target gene expression reflective of muscle NF-κB activation was elevated (**Figure 4B**). This local inflammatory response and subsequent proteolytic signaling appear to be derived from the circulation because the current study showed, for the first time to our knowledge, that factors contained within the plasma of patients with lung cancer can induce local muscular inflammation (**Figure 4C**).

In conclusion, this study reveals that muscle atrophy in cachectic patients with lung cancer is accompanied by increased systemic and local muscle inflammation, whereas precachectic patients show intermediate expression. Moreover, factors contained within the circulation of both precachectic and cachectic patients with lung cancer can induce inflammatory signaling in skeletal muscle. As concerns muscle protein turnover, increased ALP signaling and Akt phosphorylation without alterations in downstream Akt phosphosubstrates are observed in cachectic patients. This finding implies impaired anabolic signaling that could, in combination with increased proteolytic activity, contribute to the net loss of muscle protein in cancer cachexia and provides further support for a more-targeted nutritional modulation beyond merely macronutrient supplementation.

Chapter 4

Supplemental Data

Nuclear transcription factor κB activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia

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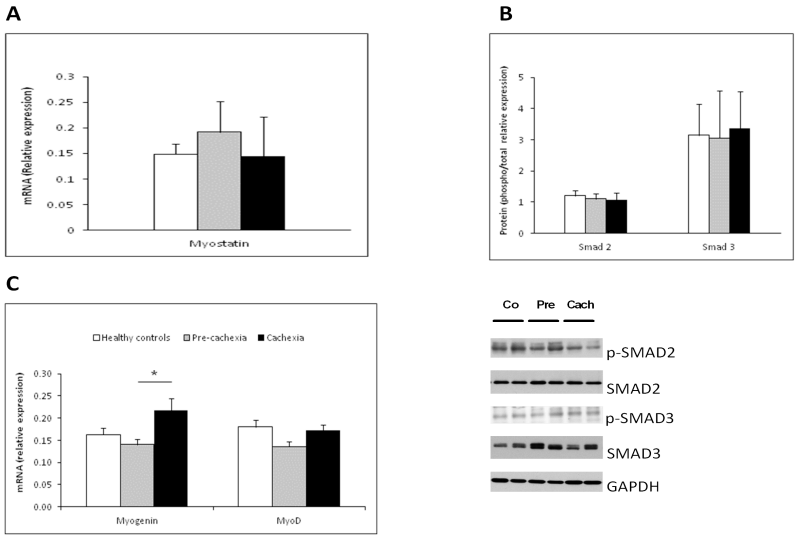
Supplemental data

Supplemental data figure 1



Muscle MyoD, Myogenin and Myostatin expression levels are unaltered in lung cancer cachexia.

A. Myostatin mRNA expression. Transcript levels of myostatin were determined in quadriceps muscle biopsies and normalized to a geNorm factor that was calculated from cyclophilin, beta-actin and RPLPO reference genes.

B. Myostatin signaling constituents. Phosphorylated and total levels of Smad 2 and Smad 3 were determined in homogenates of skeletal muscle biopsies and expressed as a ratio. Expression of phosphorylated and total protein levels were normalized using GAPDH as loading control.

C. Myogenic signaling. Transcript levels of muscular regulatory factors Myogenin and MyoD were determined in quadriceps muscle biopsies and normalized to a geNorm factor that was calculated from cyclophilin, beta-actin and RPLPO reference genes.

Co, Healthy controls; Pre, Pre-cachectic patients, Cach, Cachectic patients.

** P < 0.05 in One way ANOVA and LSD post-hoc testing. Data is represent as mean ± SEM.*

References

1. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489-495 (2011).
2. von Haehling, S. Cachexia as major underestimated and unmet medical need: facts and numbers. *J Cachex Sarcopenia Muscle* **1**, 1-5 (2010).
3. Tan, B.H. & Fearon, K.C. Cachexia: prevalence and impact in medicine. *Curr Opin Clin Nutr Metab Care* **11**, 400-407 (2008).
4. Fredrix, E.W., Staal-van den Brekel, A.J. & Wouters, E.F. Energy balance in nonsmall cell lung carcinoma patients before and after surgical resection of their tumors. *Cancer* **79**, 717-723 (1997).
5. Staal-van den Brekel, A.J. *et al.* Metabolism in patients with small cell lung carcinoma compared with patients with non-small cell lung carcinoma and healthy controls. *Thorax* **52**, 338-341 (1997).
6. Staal-van den Brekel, A.J., Schols, A.M., ten Velde, G.P., Buurman, W.A. & Wouters, E.F. Analysis of the energy balance in lung cancer patients. *Cancer Res* **54**, 6430-6433 (1994).
7. Collins, P.F., Stratton, R.J. & Elia, M. Nutritional support in chronic obstructive pulmonary disease: a systematic review and meta-analysis. *Am J Clin Nutr* **95**, 1385-1395 (2012).
8. Banerjee, A. & Guttridge, D.C. Mechanisms for maintaining muscle. *Curr Opin Support Palliat Care* **6**, 451-456 (2012).
9. Glass, D.J. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* **37**, 1974-1984 (2005).
10. Acharyya, S. & Guttridge, D.C. Cancer cachexia signaling pathways continue to emerge yet much still points to the proteasome. *Clin Cancer Res* **13**, 1356-1361 (2007).
11. Attaix, D., Combaret, L., Tilignac, T. & Taillandier, D. Adaptation of the ubiquitin-proteasome proteolytic pathway in cancer cachexia. *Mol Biol Rep* **26**, 77-82 (1999).
12. Glass, D.J. Signaling pathways perturbing muscle mass. *Curr Opin Clin Nutr Metab Care* **13**, 225-229 (2010).
13. Deans, C. & Wigmore, S.J. Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr Opin Clin Nutr Metab Care* **8**, 265-269 (2005).
14. Richards, C.H. *et al.* The relationships between body composition and the systemic inflammatory response in patients with primary operable colorectal cancer. *PLoS One* **7**, e41883 (2012).
15. Cai, D. *et al.* IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* **119**, 285-298 (2004).
16. Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**, 6853-6866 (1999).
17. Zhou, W. *et al.* Role of NF-kappaB and cytokine in experimental cancer cachexia. *World J Gastroenterol* **9**, 1567-1570 (2003).
18. Carson, J.A. & Baltgalvis, K.A. Interleukin 6 as a key regulator of muscle mass during cachexia. *Exerc Sport Sci Rev* **38**, 168-176 (2010).
19. Op den Kamp, C.M. *et al.* 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. *Lung Cancer* **76**, 112-117 (2012).

20. Rhoads, M.G., Kandarian, S.C., Pacelli, F., Doglietto, G.B. & Bossola, M. Expression of NF-kappaB and IkappaB proteins in skeletal muscle of gastric cancer patients. *Eur J Cancer* **46**, 191-197 (2010).
21. Greene FL, P.D., Fleming ID *AJCC Cancer Staging Manual, 6th ed*. (Springer-Verlag, 2002).
22. Sobin L, W.C. *UICC TNM Classification of Malignant Tumors, 6th Ed*. (Wiley-Liss, New York 2002).
23. Decramer, M., Janssens, W. & Miravitlles, M. Chronic obstructive pulmonary disease. *Lancet* **379**, 1341-1351 (2012).
24. Schols, A.M. & Gosker, H.R. The pathophysiology of cachexia in chronic obstructive pulmonary disease. *Curr Opin Support Palliat Care* **3**, 282-287 (2009).
25. Bergstrom, J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* **35**, 609-616 (1975).
26. Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D. & Miyamoto, S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **9**, 2723-2735 (1995).
27. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034 (2002).
28. Verhees, K.J. *et al.* Glycogen synthase kinase-3beta is required for the induction of skeletal muscle atrophy. *Am J Physiol Cell Physiol* **301**, C995-C1007 (2011).
29. Meier, U. A note on the power of Fisher's least significant difference procedure. *Pharm Stat* **5**, 253-263 (2006).
30. Fuller, N.J., Laskey, M.A. & Elia, M. Assessment of the composition of major body regions by dual-energy X-ray absorptiometry (DEXA), with special reference to limb muscle mass. *Clin Physiol* **12**, 253-266 (1992).
31. Heymsfield, S.B. *et al.* Appendicular skeletal muscle mass: measurement by dual-photon absorptiometry. *Am J Clin Nutr* **52**, 214-218 (1990).
32. Vary, T.C. & Lynch, C.J. Nutrient signaling components controlling protein synthesis in striated muscle. *J Nutr* **137**, 1835-1843 (2007).
33. Schmitt, T.L. *et al.* Activity of the Akt-dependent anabolic and catabolic pathways in muscle and liver samples in cancer-related cachexia. *J Mol Med (Berl)* **85**, 647-654 (2007).
34. Aversa, Z. *et al.* Changes in myostatin signaling in non-weight-losing cancer patients. *Ann Surg Oncol* **19**, 1350-1356 (2012).
35. Williams, J.P. *et al.* Effect of tumor burden and subsequent surgical resection on skeletal muscle mass and protein turnover in colorectal cancer patients. *Am J Clin Nutr* **96**, 1064-1070 (2012).
36. Mammucari, C. *et al.* FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**, 458-471 (2007).
37. Mammucari, C., Schiaffino, S. & Sandri, M. Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. *Autophagy* **4**, 524-526 (2008).
38. Bodine, S.C. *et al.* Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-1708 (2001).
39. Cao, P.R., Kim, H.J. & Lecker, S.H. Ubiquitin-protein ligases in muscle wasting. *Int J Biochem Cell Biol* **37**, 2088-2097 (2005).
40. Bennani-Baiti, N. & Walsh, D. Animal models of the cancer anorexia-cachexia syndrome. *Support Care Cancer* **19**, 1451-1463 (2011).

41. Khal, J., Hine, A.V., Fearon, K.C., Dejong, C.H. & Tisdale, M.J. Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss. *Int J Biochem Cell Biol* **37**, 2196-2206 (2005).
42. Smith, I.J. *et al.* Calpain activity is increased in skeletal muscle from gastric cancer patients with no or minimal weight loss. *Muscle Nerve* **43**, 410-414 (2011).
43. Williams, A., Sun, X., Fischer, J.E. & Hasselgren, P.O. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery* **126**, 744-749; discussion 749-750 (1999).
44. Bossola, M. *et al.* Increased muscle ubiquitin mRNA levels in gastric cancer patients. *Am J Physiol Regul Integr Comp Physiol* **280**, R1518-1523 (2001).
45. Bossola, M. *et al.* Increased muscle proteasome activity correlates with disease severity in gastric cancer patients. *Ann Surg* **237**, 384-389 (2003).
46. Gallagher, I.J. *et al.* Suppression of skeletal muscle turnover in cancer cachexia: evidence from the transcriptome in sequential human muscle biopsies. *Clin Cancer Res* **18**, 2817-2827 (2012).
47. Polge, C. *et al.* Muscle actin is polyubiquitinylated in vitro and in vivo and targeted for breakdown by the E3 ligase MuRF1. *FASEB J* **25**, 3790-3802 (2011).
48. Sun, Y.S., Ye, Z.Y., Qian, Z.Y., Xu, X.D. & Hu, J.F. Expression of TRAF6 and ubiquitin mRNA in skeletal muscle of gastric cancer patients. *J Exp Clin Cancer Res* **31**, 81 (2012).
49. Klionsky, D.J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4**, 151-175 (2008).
50. Costelli, P. *et al.* Skeletal muscle wasting in tumor-bearing rats is associated with MyoD down-regulation. *Int J Oncol* **26**, 1663-1668 (2005).
51. Penna, F. *et al.* Muscle wasting and impaired myogenesis in tumor bearing mice are prevented by ERK inhibition. *PLoS One* **5**, e13604 (2010).
52. Busquets, S. *et al.* Myostatin blockage using actRIIB antagonism in mice bearing the Lewis lung carcinoma results in the improvement of muscle wasting and physical performance. *J Cachexia Sarcopenia Muscle* **3**, 37-43 (2012).
53. Gioulbasanis, I. *et al.* Baseline plasma levels of interleukin-8 in stage IV non-small-cell lung cancer patients: relationship with nutritional status and prognosis. *Nutr Cancer* **64**, 41-47 (2012).

Chapter 5

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 ($\dot{V}O_2peak$) 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.

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, 3]. 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 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.

Patients and Methods

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.

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.

Excercise 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.

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).

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).

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).

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.

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 $P < 0.05$.

Results

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 ($P < 0.001$) (**Figure 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**).

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) [1]	65.9 ± 7.5	63.7 ± 5.6	0.427
Weight loss (%) [2]	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) [3]	78 ± 18	115 ± 22	< 0.001*
FEV_1/FVC [4] **(%)**	62 ± 17	78 ± 4	< 0.001*
COPD [5] **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/minute)	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)		

[1] *Data are represented as means ± SD, all such values*

[2] *Body weight loss in 6 months prior to diagnosis*

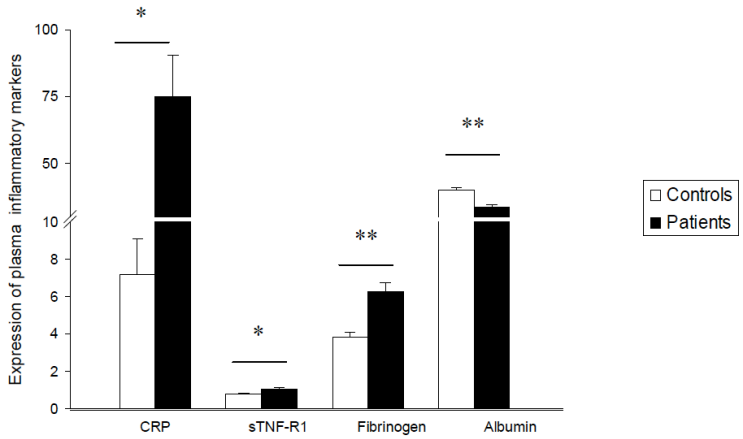
[3] *Forced expiratory volume in 1 second*

[4] *Forced vital capacity*

[5] *Chronic Obstructive Pulmonary Disease*

* *P < 0.05*

Figure 1

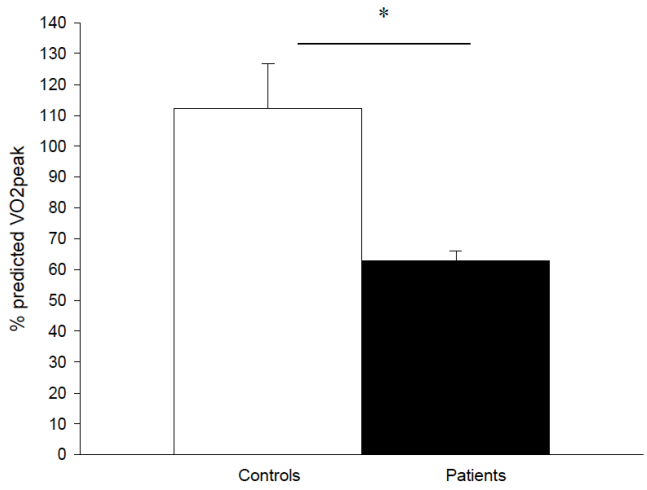


Plasma levels of inflammatory mediators of healthy control subjects and NSCLC patients. Patient with pre-cachexia have an increased inflammatory profile compared with healthy controls. CRP: C-reactive protein (µg/ml); sTNF-R1: soluble Tumor Necrosis Factor receptor 1 (sTNF-R1) (µg/ml); fibrinogen (g/l)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

Excercise 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) (**Figure 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) (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 correlations were observed between $\dot{V}O_2$ peak and fat free mass (*R*: −0.28, P = 0.909).

Figure 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. $^{*}P < 0.05$.

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**).

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[1] (AU)[2]	0.24 ± 0.16[2]	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

[1] *Data are represented as means ± SD*

[2] *AU, arbitrary units*

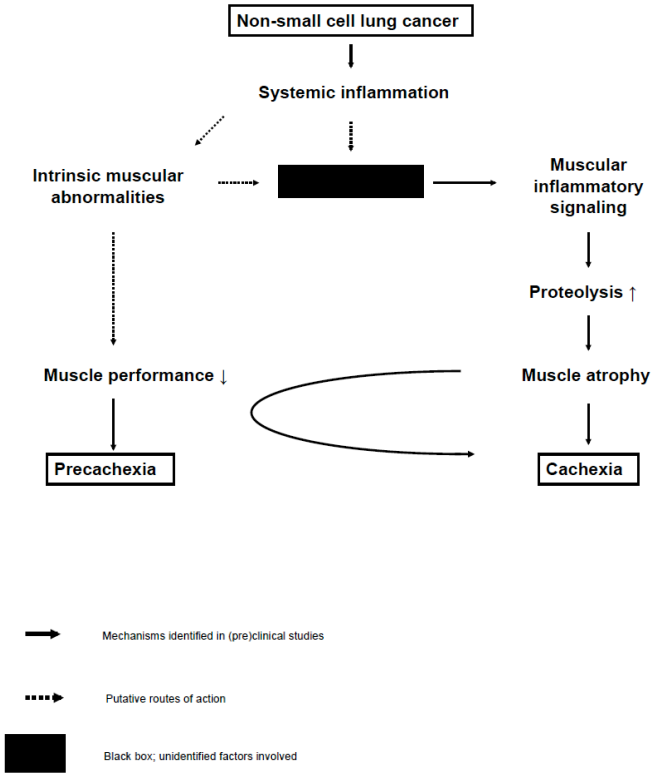
Discussion

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 **Figure 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

Figure 3

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



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[25]. 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 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.

References

1. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489-495 (2011).
2. Ross, P.J. *et al.* Do patients with weight loss have a worse outcome when undergoing chemotherapy for lung cancers? *Br J Cancer* **90**, 1905-1911 (2004).
3. Tan, B.H. & Fearon, K.C. Cachexia: prevalence and impact in medicine. *Curr Opin Clin Nutr Metab Care* **11**, 400-407 (2008).
4. Tisdale, M.J. Cachexia in cancer patients. *Nat Rev Cancer* **2**, 862-871 (2002).
5. von Haehling, S. & Anker, S.D. Cachexia as a major underestimated and unmet medical need: facts and numbers. *J Cachexia Sarcopenia Muscle* **1**, 1-5 (2010).
6. Bossola, M., Pacelli, F., Tortorelli, A. & Doglietto, G.B. Cancer cachexia: it's time for more clinical trials. *Ann Surg Oncol* **14**, 276-285 (2007).
7. Argiles, J.M., Busquets, S. & Lopez-Soriano, F.J. The pivotal role of cytokines in muscle wasting during cancer. *Int J Biochem Cell Biol* **37**, 2036-2046 (2005).
8. Fong, Y. *et al.* Cachectin/TNF or IL-1 alpha induces cachexia with redistribution of body proteins. *Am J Physiol* **256**, R659-665 (1989).
9. Cai, D. *et al.* IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* **119**, 285-298 (2004).
10. Camps, C., Iranzo, V., Bremnes, R.M. & Sirera, R. Anorexia-Cachexia syndrome in cancer: implications of the ubiquitin-proteasome pathway. *Support Care Cancer* **14**, 1173-1183 (2006).
11. Ciechanover, A. & Iwai, K. The ubiquitin system: from basic mechanisms to the patient bed. *IUBMB Life* **56**, 193-201 (2004).
12. Bodine, S.C. *et al.* Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-1708 (2001).
13. Tanoue, L.T. & Detterbeck, F.C. New TNM classification for non-small-cell lung cancer. *Expert Rev Anticancer Ther* **9**, 413-423 (2009).
14. Mazess, R.B., Barden, H.S., Bisek, J.P. & Hanson, J. Dual-energy x-ray absorptiometry for total-body and regional bone-mineral and soft-tissue composition. *Am J Clin Nutr* **51**, 1106-1112 (1990).
15. Jones, N.L., Makrides, L., Hitchcock, C., Chypchar, T. & McCartney, N. Normal standards for an incremental progressive cycle ergometer test. *Am Rev Respir Dis* **131**, 700-708 (1985).
16. Gosker, H.R. *et al.* Striking similarities in systemic factors contributing to decreased exercise capacity in patients with severe chronic heart failure or COPD. *Chest* **123**, 1416-1424 (2003).
17. Plasqui, G., Joosen, A.M., Kester, A.D., Goris, A.H. & Westerterp, K.R. Measuring free-living energy expenditure and physical activity with triaxial accelerometry. *Obes Res* **13**, 1363-1369 (2005).
18. Leeuwenberg, J.F., Dentener, M.A. & Buurman, W.A. Lipopolysaccharide LPS-mediated soluble TNF receptor release and TNF receptor expression by monocytes. Role of CD14, LPS binding protein, and bactericidal/permeability-increasing protein. *J Immunol* **152**, 5070-5076 (1994).

19. Bergstrom, J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* **35**, 609-616 (1975).
20. Hobler, S.C. *et al.* Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis. *Am J Physiol* **277**, R434-440 (1999).
21. Minnaard, R. *et al.* Ubiquitin-proteasome-dependent proteolytic activity remains elevated after zymosan-induced sepsis in rats while muscle mass recovers. *Int J Biochem Cell Biol* **37**, 2217-2225 (2005).
22. Khal, J., Hine, A.V., Fearon, K.C., Dejong, C.H. & Tisdale, M.J. Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss. *Int J Biochem Cell Biol* **37**, 2196-2206 (2005).
23. Williams, A., Sun, X., Fischer, J.E. & Hasselgren, P.O. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery* **126**, 744-749; discussion 749-750 (1999).
24. Jagoe, R.T., Redfern, C.P., Roberts, R.G., Gibson, G.J. & Goodship, T.H. Skeletal muscle mRNA levels for cathepsin B, but not components of the ubiquitin-proteasome pathway, are increased in patients with lung cancer referred for thoracotomy. *Clin Sci (Lond)* **102**, 353-361 (2002).
25. Smith, I.J. *et al.* Calpain activity is increased in skeletal muscle from gastric cancer patients with no or minimal weight loss. *Muscle Nerve* **43**, 410-414 (2011).
26. Bobbio, A. *et al.* Exercise capacity assessment in patients undergoing lung resection. *Eur J Cardiothorac Surg* **35**, 419-422 (2009).
27. Jones, L.W. *et al.* Peak oxygen consumption and long-term all-cause mortality in nonsmall cell lung cancer. *Cancer* **116**, 4825-4832 (2010).
28. Broekhuizen, R., Wouters, E.F., Creutzberg, E.C. & Schols, A.M. Raised CRP levels mark metabolic and functional impairment in advanced COPD. *Thorax* **61**, 17-22 (2006).
29. Yende, S. *et al.* Inflammatory markers are associated with ventilatory limitation and muscle dysfunction in obstructive lung disease in well functioning elderly subjects. *Thorax* **61**, 10-16 (2006).

Chapter 6

Preserved muscle oxidative metabolic phenotype in clinical cancer cachexia

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Submitted

Abstract

Cachexia augments cancer-related mortality and has devastating effects on quality of life. Preclinical studies indicate that systemic inflammation-induced loss of muscle oxidative phenotype (Oxphen) could stimulate cancer-induced muscle wasting. The aim of the current study was to test the hypothesis that muscle Oxphen loss is observed in patients with lung cancer cachexia and associated with enhanced systemic inflammation. Quadriceps muscle biopsies of comprehensively phenotyped pre-cachectic (n=10) and cachectic (n=16) patients with non-small cell lung cancer were compared with healthy age matched controls (n=22). Oxphen was determined by assessing muscle fiber type distribution (immunohistochemistry), enzyme activity (spectrometry), and protein expression levels of mitochondrial complexes (Western blot) as well as transcript levels of (regulatory) oxidative genes (QPCR). Additionally, muscle fiber cross sectional area (CSA) (immunohistochemistry) and systemic inflammation (multiplex analysis) were assessed. Plasma levels of inflammatory markers IL-6 (268%) and soluble TNF-receptor-1 (160%) were significantly increased in the cancer cachexia subgroup and to a lesser degree in the pre-cachectic group. Patients with cachexia however showed no alterations in muscle fiber distribution or enzyme activities as compared to pre-cachexia and healthy controls. Furthermore, mitochondrial protein expression and transcript levels of regulatory oxidative genes were not altered. Moreover, muscle CSA of all fiber types (oxidative and glycolytic) was decreased in cachectic patients ($P<0.05$). In conclusion, despite evident systemic inflammation, muscle Oxphen is preserved and therefore not an important trigger of muscle wasting in patients with lung cancer cachexia.

Introduction

Cachexia has been well recognised as an adverse effect of cancer. In successive stages of severity, i.e. pre-cachexia, cachexia, and refractory cachexia, the syndrome is reflected by progressive body weight loss, wasting of peripheral muscle and impairment of muscle function[1]. Weight loss and muscle weakness have major negative implications on mortality and quality of life in cancer.

Loss of muscle oxidative phenotype (Oxphen) includes a decreased proportion of oxidative slow-twitch type I fibers as well as loss of mitochondrial function and capacity. Mitochondrial pathways recently have emerged as central players in muscle mass maintenance[2-4]. Indeed, in numerous experimental models of cancer cachexia, mitochondrial impairments have clearly been related to muscle atrophy and activation of muscle proteolytic pathways[5-12]. Moreover, mice suffering from cancer cachexia also demonstrated a decreased proportion of oxidative type I muscle fibers and an increased proportion of glycolytic type II fibers in soleus muscle[13, 14]. Since type II fibers are more susceptible to catabolic stimuli[13, 15], this fiber type shift may further enhance muscle wasting in cancer cachexia. However, such a loss of muscle Oxphen and moreover, its putative associations with the pathophysiology of cachexia, have never been validated in patients with cancer cachexia. Previously, a clinical study conducted by our own group provided indirect evidence by demonstrating a decreased cycle exercise capacity in lung cancer patients even in those with pre-cachectic[16]. This could be contributed to a loss of Oxphen, as this is a well-established determinant of exercise capacity[17, 18]. Therefore, our first hypothesis was that cancer pre-cachexia is accompanied by a subtle decreased muscle Oxphen, which is deteriorated in advanced cancer cachexia.

Increased systemic pro-inflammatory signaling has been causally related to the loss of Oxphen observed in skeletal muscle in an experimental model of intestinal cancer cachexia[7, 19]. Further experimental *in vitro* research indeed confirms that, the pro-inflammatory mediators Interleukin 6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α) can induce alterations in oxidative metabolism by decreasing muscular activation of the master regulator of oxidative metabolism, peroxisome proliferator-activated

receptor gamma co-activator 1-alpha (PGC-1α)[20]. Because elevated levels of pro-inflammatory cytokines is a common finding in patients with cancer cachexia[1, 16], this might also apply to clinical cancer cachexia. Moreover, pro-inflammatory cytokines are also considered responsible for the activation of muscular proteolytic pathways in cancer cachexia[21-23], which further strengthens the notion that energy metabolism and protein turnover are intertwined in cancer cachexia[24]. Hence, our second hypothesis was that the anticipated gradual loss of muscle Oxphen is associated with increasing systemic inflammation in cancer cachexia.

The aim of this study is therefore to determine if muscle Oxphen loss indeed occurs in patients with cancer cachexia and is associated with systemic inflammation. To this end we analyzed muscle biopsies obtained from pre-cachectic and cachectic non-small cell lung cancer (NSCLC) patients - as clinical cancer cachexia model - and compared them with healthy controls.

Patients and Methods

Study population

Sixteen newly diagnosed pre-cachectic patients with locally 26 patients with newly diagnosed stage III or IV NSCLC and 22 matched healthy control subjects were enrolled in the study. Patients were originally subdivided in a group with less or more than 5% weight loss but these stages were re-evaluated when the international cachexia consensus was published. According to the international expert consensus[1] pre-cachexia is a stage in which early clinical and metabolic signs precede substantial involuntary weight loss (i.e. ≤5%) and patients with more than 5% weight loss, or a body mass index (BMI) less than 20 kg/m^2 or sarcopenia combined with ongoing weight loss of more than 2% are classified as having cachexia. As 5% weight loss is still the main criteria for cachexia diagnosis, this only minimally changed the stratification and resulted in a group of 16 patients with cachexia. Of the patients that did not meet the cachexia definition (N=10), i.e. non-cachectic patients, the vast majority exhibited weight loss to some extent and intermediate levels of inflammatory mediators could be observed in this patient group (**Table 1**). Based on

these characteristics, the non-cachectic patients are further referred to as pre-cachectic patients. Additional inclusion and exclusion criteria are described in the Supplement.

Patients were recruited at the Department of Respiratory Medicine, Maastricht University Medical Centre+ between July 2007 and July 2010. Healthy controls were recruited via local newspaper advertisements and were matched to the NSCLC patients with respect to age and sex. Written informed consent was obtained from all subjects and the ethical review board of the Maastricht University Medical Centre+ approved the study (reference number 08-2-059). Because public trial registration was not implemented in clinical practice (WHO guideline indicates January 2009) at start of enrollment (July 2007), the study was not registered in a public trial registry.

Assessment of the parameters described below was performed in the morning after 8 hours fasting.

Body composition

Dual energy X-ray absorptiometry (DEXA; DPX-L, Lunar Radiation Corp., Madison, WI) was used to determine whole-body composition, including fat free mass index (FFMI). DEXA measurements were performed in the fasted state.

Spirometry

Forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were assessed by spirometry to assess airflow obstruction as a potential result of smoking history of patients. As metabolic derangements in skeletal muscle occur in COPD and could therefore influence outcome parameters, relations between lung function and muscular metabolic characteristics were assessed.

Physical activity and quadriceps muscle function

The Medical Studies Study Short Form-20 (SF-20) questionnaire was used to assess physical activity and the European Organisation for Research and Treatment of Cancer (EORTC) QLQ-C30 questionnaire was used to assess quality of life, which includes physical performance. Isometric and isokinetic strength of quadriceps and hamstrings muscles was measured by Biodex dynamometer (Biodex system version 3.3). Details of the functional measurements are included in the Supplement.

Plasma inflammatory markers

Plasma inflammatory mediators were assessed using a Human Multiplex Antibody assay (Luminex® System, Invitrogen, Life Technologies) to determine plasma TNF-α, soluble TNF (sTNF)-receptor 1 and Interleukin 6 (IL-6) (lower detection limit 5-28 pg/mL). All samples were analyzed at Invitrogen Luminex Testing Services (Paisley, United Kingdom).

Muscle biopsies collection and processing

Muscle biopsies of *vastus lateralis* muscle (part of quadriceps muscle) were obtained by needle biopsies using a technique described by Bergström[25]. For further muscle biopsy processing, please see the Supplement section.

Muscle fiber type distribution

Immunohistochemical staining of laminin was used to determine muscle fiber cross sectional area (CSA). Subsequently, a combination of immunohistochemical staining and myosin adenosine 5'-triphosphatase (mATPase) staining was used (as described before [26]) to identify fibers expressing different Myosin Heavy Chain (MyHC) subtypes, i.e. oxidative type I, hybrid I/II and glycolytic II fibers. Details of this procedure are described in the Supplement section.

Muscle protein expression analysis

Western blot analysis was used to detect protein expression levels of Oxphos ATP synthase and complexes I-IV. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. For a more detailed description of this analysis, please see the online Supplement section.

Muscle enzyme activity analysis

Analysis was performed as described earlier[26]. In summary, crushed muscle tissue was dissolved in 5% (w/v) SET buffer solution containing Sucrose (250 mM), EDTA (2.5 mM) and Tris (10 mM) and subsequently homogenized using a Polytron PT (Polytron PT 1600 E, Kinematica AG). Enzyme assays for CS, HAD, and PFK

analyzed spectrophotometrically (Multiskan Spectrum; Thermo Labsystems, Breda, The Netherlands).

Muscle mRNA expression analysis

Quantitative real time polymerase chain reaction was used to assess transcript levels of PGC-1α, mitochondrial transcription factor A (TFAM), citrate synthase (CS), b-hydroxyacyl-CoA dehydrogenase (HAD), hexokinase II (HKII), phosphofructokinase (PFK), mitochondrially encoded cytochrome c oxidase III (COX III), cytochrome c oxidase subunit IV isoform 1 (COX IV), MyHC I. For a more detailed description of this analysis and primer sequences, please see the Supplement section.

Statistics

For the sample size calculation, please see the online Supplement section. Data was analyzed using Statistical Package for the Social Sciences (SPSS version 15 for Windows, SPSS Inc.). Except for baseline body weight loss, which represents weight loss *within* individual patients in the 6 months prior to diagnosis, *all* data represent comparisons between healthy controls, pre-cachectic and cachectic patient groups. Continuous variables were compared using one way ANOVA. Pearson Chi-Square test was used for comparison of categorical variables. Correlations were evaluated using Pearson correlations. Data in tables is represented as mean ± standard deviation (SD). Error bars in figures represent standard error of mean (SEM). Significance was set at $p < 0.05$.

Results

Subject characteristics

Baseline characteristics of the study population are shown in **Table 1**. Basic characteristics of healthy control, pre-cachectic and cachectic study participants are shown in **Table 1**. Pre-cachectic and cachectic patients with NSCLC showed no significant differences in tumor stage or histological subtype. Pre-cachectic patients showed mild within patient body weight loss (1.7%) in the 6 months prior to

diagnosis, whereas patients with cachexia showed significant weight loss in this period (12%, p<0.05). Moreover, FFMI was significantly lower in patients with cachexia, while no differences were observed in total body mass index or fat mass index between any of the study groups (**Table 1**). All except one patient had a history of cigarette smoking and as a result, lung function was decreased in patients with lung cancer. Muscle strength in cachectic patients was declined by 30-55% as compared to controls (*P*<0.05, **Table 1**) as well as reported physical functioning in quality of life and performance status questionnaires, which was declined to a similar degree (32-38%, *P*<0.05) (data not shown). Pre-cachectic patients showed less pronounced but significant decreases in quadriceps muscle strength (3-42%, *P*<0.05) and physical performance indices reported in questionnaires (16-28%, *P*<0.05) (**Table 1**).

Increased IL-6 and soluble TNF receptor-1 levels in patients with cancer cachexia

Expression of the pro-inflammatory cytokine considered most potent in inducing loss of Oxphen in experimental cancer cachexia, i.e. IL-6, was increased in cachectic patients compared to healthy controls and pre-cachectic patients (*P*<0.05) (**Table 1**). TNF-α, another putative mediator of Oxphen regulation was not differentially expressed in pre-cachectic or cachectic patients (**Table 1**). However, circulating levels of its receptor, soluble TNF receptor-1, were significantly increased in plasma of patients with cachexia when compared with healthy controls (*P*<0.05) (**Table 1**). A gradual increase was observed in plasma levels of both IL-6 and TNF-R1 from pre-cachexia to cachexia (**Table 1**).

Preserved muscle Oxphen in patients with lung cancer pre-cachexia and cachexia

As can be observed in **Figure 1**, patients with lung cancer cachexia showed no differences from healthy controls concerning the proportion of type I, hybrid I/II or II muscle fibers, although trends towards decreased Type I (P=0.091) and increased type II fibers (P=0.096) could be appreciated. Also, no indications for a fiber type shift during early development of cancer cachexia were found, i.e. no changes were observed between the cachectic and the pre-cachectic group or pre-cachectic patients and healthy controls (**Figure 1A**). Similarly, ratios of oxidative (HAD and CS)

Table 1

Subject characteristics

	Healthy controls	Pre-cachexia	Cachexia
N (m/f)	22 (13/9)	10 (8/2)	16 (9/7)
Age (years)	61.4 ± 7.0	62.4 ± 10.4	59.8 ± 8.2
Disease stage[1]: IIIB (%) / IV (%)	- / -	60 / 40	25 / 75
Histology: Adenocarcinoma (%) / Squamous cell (%)	- / -	70 / 30	56 /44
Smoking: (Current % / Former % / Never %)	5[†] / 54 / 22	20[†] / 80 / 0	50[†] / 44 / 6
FEV1[6] (% predicted)	115.7 ± 19.3	77.0 ± 18.4*	61.9 ± 17.2**
FVC[7] (% predicted)	125.4 ± 1.1	100.0 ± 9.9*	75.5 ± 22.0**,***
Tiffeneau-index	0.74 ± 0.08	0.60 ± 0.12*	0.65 ± 0.13**
Mean weight loss in 6 months prior to diagnosis (%)[2]	0 ± 0	1.7 ± 1.4	12.0 ± 5.5 **,***
Body mass index (BMI) (kg/m^2)	24.1 ± 3.3	25.7 ± 3.4	23.0 ± 4.8
Fat Mass Index (FMI) (kg/m^2)	6.5 ± 2.5	7.7 ± 3.0	6.8 ± 3.0
Fat Free Mass Index (FFMI) (kg/m^2)	18.4 ± 2.2	18.5 ± 1.6	16.5 ± 2.7 **
IL-6 (pg/ml)[3]	56.7 ± 33.2	70.1 ± 50.8	151.8 ± 122.6**,***
TNF-α (pg/ml)[4]	105.7 ± 43.8	131.6 ± 129.9	106.6 ± 58.1
Soluble TNF receptor 1 (pg/ml)[5]	2404 ± 728	3482 ± 1747*	3855 ± 1270**
Peak torque flexion 180°/sec (Nm)	64.3 ± 25.5	37.6 ± 14.2*	32.9 ± 16.3**
Peak torque extension 180°/sec (Nm)	75.8 ± 27.6	51.2 ± 17.5*	34.2 ± 15.0**
Peak torque flexion 60° (Nm)	77.4 ± 19.8	71.0 ± 25.3	53.4 ± 18.1**,***
Peak torque extension 60° (Nm)	137.1 ± 35.5	133.0 ± 40.2	95.3 ± 32.8**,***

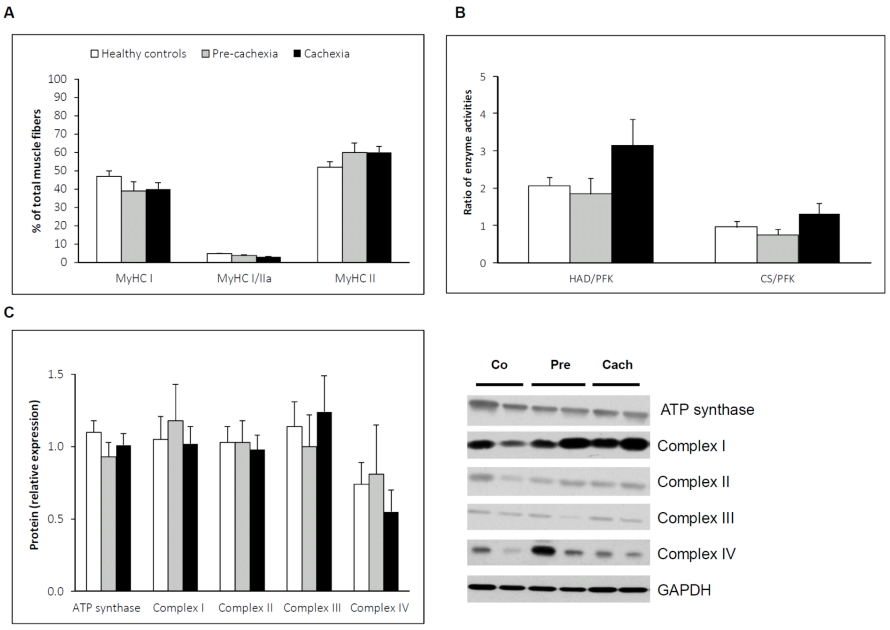
[1] Stage of non-small cell lung cancer according to the 6th Tumor-Node-Metastasis (TNM) classification system, [2] Mean percentage of within patient weight loss in the 6 months prior to diagnosis, [3] Interleukin-6, [4] Tumor Necrosis Factor alpha, [5] Soluble Tumor Necrosis Factor alpha receptor 1, [6] Quality of Life Questionnaire C30, [7] Medical Studies Study Short Form-20 (Physical performance questionnaire), [8] Forced expiratory volume in one second, [9] Forced vital capacity

, **, * One way ANOVA and LSD post-hoc testing: * P<0.05 pre-cachexia vs. controls, ** P < 0.05, cachexia vs. controls, *** P < 0.05, pre-cachexia vs. Cachexia. [†] Pearson Chi-Square or Fisher's Exact Tests: P < 0.05*

Data represent mean ± SD.

to glycolytic (PFK) enzyme activity and protein expression levels of ATP synthase and I-IV Oxphos protein complexes were unaltered in pre-cachectic and cachectic patients compared with healthy controls (**Figure 1B-C**).

Figure 1



Normal Oxphen in patients with lung cancer pre-cachexia and cachexia

Quadriceps muscle biopsies were processed for analysis of muscle fiber subtypes, enzyme activity and protein expression.

A. Distribution of oxidative type I and glycolytic type II muscle fiber types in quadriceps muscle. Assessment of fibers expressing different MyHC isoforms was performed using immunohistochemistry and mATPase staining.

B. Muscle oxidative and glycolytic enzyme activity. Activity of oxidative (HAD and CS) and glycolytic (PFK) enzymes was assessed. Ratios of oxidative to glycolytic were calculated for the different enzymes.

C. Protein expression of Oxphos proteins. Expression of ATP synthase and I-IV Oxphos protein complexes was assessed using western blot analysis. GAPDH was used as a loading control

Co, Healthy controls; Pre, Pre-cachectic patients, Cach, Cachectic patients.

** Significant difference between indicated groups, $P < 0.05$*

Preserved muscular expression of mediators regulating and representing oxidative metabolism in lung cancer cachexia

Muscle gene expression levels are shown in **Table 2**. PGC-1a, the master regulator of oxidative metabolism, was not differentially expressed in pre-cachexia or cachexia patients with lung cancer. Likewise, TFAM, another regulator of oxidative metabolism, and downstream oxidative markers (HAD, CS) and glycolytic markers (PFK, HKII) were not differentially expressed in any of the study groups (**Table 2**). mRNA transcripts of COX III and COX IV were significantly decreased in patients with pre-cachexia and cachexia compared with healthy controls.

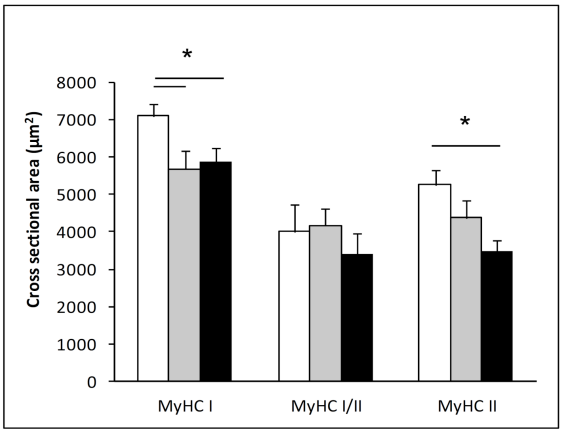
Corresponding to the absence of histological alterations in the proportion of type I fibers, no changes in gene expression levels of oxidative MyHC I were observed during the subsequent stages of cachexia (**Table 2**).

Muscle fiber atrophy is independent of fiber type in lung cancer cachexia

Muscle fiber CSA was decreased independent of the fiber type in the cachectic group ($P<0.05$), i.e. both Type I and Type II muscle fiber CSA was decreased, albeit that loss of muscle fiber cross sectional area of Type I (17%) was less extensive than in Type II (34%) fibers (**Figure 2**). In accordance, fiber type-independent muscle atrophy was observed in patients with pre-cachexia, although only the decrease in type I fibers (20%) reached significance in comparison with healthy controls (**Figure 2**). No significant alterations were observed in the cross sectional area of hybrid Type I/II fibers in any of the study groups (**Figure 2**).

There were no significant associations between FFMI as marker of muscle mass and the following metabolic markers: fiber type distribution, oxidative-glycolytic enzyme activity ratios or transcript levels of (regulatory) oxidative markers in any of the patient groups (data not shown). Conversely, FFMI was significantly and negatively associated with expression of several of the Oxphos proteins in pre-cachectic patients (complex II and complex III, $P<0.05$) and cachectic patients (ATP synthase and complex III, $P<0.05$), whereas weight loss was positively associated with expression of Oxphos proteins in cachectic patients (ATP synthase, complex II and complex III, $P<0.05$) (**Figure 3**).

Figure 2



Muscle fiber atrophy is independent of fiber type in lung cancer cachexia
Cross sectional of individual muscle fibers was assessed using immunohistochemical staining of laminin. Assessment of fibers expressing different MyHC isoforms was performed using immunohistochemistry and mATPase staining.

Table 2

Gene expression profiles

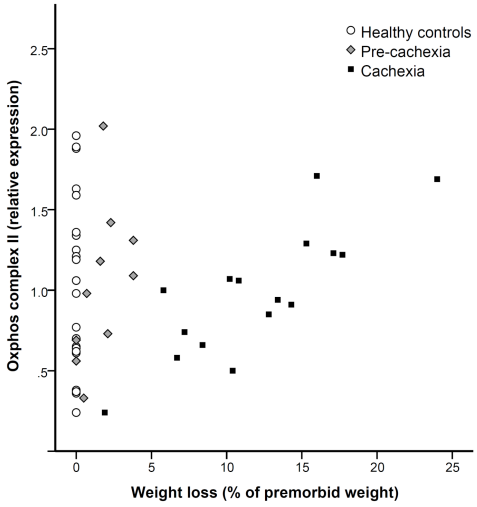
	Healthy controls	Pre-cachexia	Cachexia
PGC-1α (AU)	0.20 ± 0.19	0.13 ± 0.05	0.13 ± 0.06
TFAM (AU)	0.16 ± 0.04	0.14 ± 0.04	0.16 ± 0.04
CS (AU)	0.21 ± 0.10	0.15 ± 0.06	0.17 ± 0.05
HAD (AU)	0.19 ± 0.04	0.19 ± 0.04	0.21 ± 0.04
HKII (AU)	0.18 ± 0.12	0.12 ± 0.13	0.21 ± 0.27
PFK (AU)	0.19 ± 0.10	0.17 ± 0.09	0.18 ± 0.05
COX III (AU)	0.25 ± 0.08	0.19 ± 0.06*	0.15 ± 0.05**
COX IV (AU)	0.22 ± 0.07	0.18 ± 0.06*	0.17 ± 0.05**
MyHC I (AU)	0.20 ± 0.09	0.18 ± 0.09	0.17 ± 0.09

AU: Arbitrary Units, PGC-1α; peroxisome proliferator-activated receptor gamma co-activator 1-alpha, TFAM; mitochondrial transcription factor A, CS; citrate synthase, HAD; b-hydroxyacyl-

CoA dehydrogenase, HKII; hexokinase II, PFK; phosphofructokinase, COX III; mitochondrially encoded cytochrome c oxidase III, COX IV; cytochrome c oxidase subunit IV isoform 1, MyHC; Myosin heavy chain.

, **, * One way ANOVA and LSD post-hoc testing: * P<0.05 pre-cachexia vs. controls, ** P < 0.05, cachexia vs. controls, *** P < 0.05, pre-cachexia vs. cachexia*

Figure 3



Correlation between weight loss and protein expression of Oxphos complex II in healthy controls, pre-cachectic and cachectic patients. A significant correlation between weight loss and Oxphos complex II protein expression was found in cachectic patients (R 0.826, P <0.05) but not in healthy control subjects or pre-cachectic patients.

Discussion

In contrast to findings in experimental cancer cachexia, this clinical study demonstrates that skeletal muscle Oxphen is mainly preserved in patients with lung cancer cachexia, despite significant increases in systemic pro-inflammatory mediators as putative triggers. This is illustrated by the absence of alterations in fiber type distribution, mRNA transcript levels of regulators of oxidative signaling, protein expression of mitochondrial complexes and oxidative enzyme activity. Accordingly, it is concluded that Oxphen is not changed in patients with lung cancer cachexia.

To the best of our knowledge, this is the first study that addresses the question

whether loss of muscle Oxphen is involved in patients with cancer cachexia. It must be noted that an isolated decrease in COX III and COX IV mRNA transcripts is observed in pre-cachectic and cachectic patients but as this is not accompanied by changes in protein levels of complex IV or any other of the studied markers of oxidative metabolism, this finding seems not related to alterations in muscle Oxphen in patients with lung NSCLC (pre-) cachexia. In a previous clinical study in patients with gastro-intestinal cancer no alterations in microcirculation, capillary density or muscular energy metabolites were found[27] and although these measures do not reflect muscle Oxphen *per se*, these data are in line with the current findings. Thus, it seems that clinical findings are in large contrast with most experimental models of cancer cachexia. In contrast to the previously mentioned animal studies, increased transcript levels of the Oxphen regulator PGC-1a and its downstream mediators were observed in a rat model of cancer cachexia (hepatoma)[28]. These findings are consistent with the positive and negative correlations between Oxphos complexes and respectively weight and FFMI in the current population of patients with lung cancer. Together, this implicates that the loss of Oxphen observed in the majority of experimental cancer cachexia models is not merely a result of the presence of malignant disease but is likely dependent on (a combination of) yet to be determined additional host metabolic alterations that could be related to a more aggressive and active nature of tumor development. However, it must be noted that none of the above mentioned experimental models were real lung cancer models (i.e. a tumor in the lungs) and loss of Oxphen associated with cachexia in such a real model of lung cancer could help to interpreter the current clinical findings.

The absence of changes in Oxphen in the current patient population with lung cancer cachexia are dissimilar to observations in COPD, another smoking-related lung disorder in which cachexia and loss of skeletal muscle Oxphen are frequently observed[4, 29, 30]. An important difference between cachexia in lung cancer and COPD is the time frame in which cachexia progresses. In COPD, cachexia probably develops gradually over a long time period, i.e. months or years. The loss of Oxphen might indeed play a role as accelerator of muscle wasting in this gradually developing type of cachexia [4]. However, in lung cancer, cachexia develops relatively rapid, i.e. in weeks to months, due to the aggressive nature of the disease, and the current findings indicate that loss of Oxphen does not precede or accompany this

rapid developing cachexia, which suggests that the molecular mechanisms of muscle wasting are different in both conditions. An additional indication that different wasting mechanisms indeed occur in these respective diseases is the generalized muscle fiber atrophy of type I as well as type II fibers in the current population with lung cancer cachexia, whereas patients with COPD typically exhibit selective type II fiber atrophy[29]. A potential reason for the differences in muscle Oxphen in lung cancer cachexia and COPD could be that specific triggers, such as hypoxia or a slow adaptation to sedentary life style, which might not be as predominant in malignant disease[16], contribute significantly to intrinsic metabolic alterations in skeletal muscle of COPD patients[29]. Independent of the cause of the differences, the currently observed phenotype seems specific for lung cancer and not influenced by the presence of decreased lung function, as the proportion and diameter of glycolytic muscle fibers did not correlate with the lung function parameters in any of the current patient groups (data not shown).

Although muscle Oxphen is not altered in lung cancer patients, the question remains whether stimulation of oxidative metabolism through interventions like exercise training or pharmaceutical stimulation could still have beneficial effects on muscle mass preservation and functional performance during cancer cachexia via the potential increase in contractile efficiency, reduced muscle proteolysis and increased muscle performance. Moreover, patients in the current study were included at diagnosis, prior to anti-tumor treatment; it cannot be excluded that muscle Oxphen could be affected by therapy-induced inflammation[31, 32]. Studies performed in humans and rodents show that endurance exercise increases mRNA and protein expression of the master Oxphen regulator PGC-1α[33, 34] and muscle-specific over-expression of PGC-1α resulted in improved muscle performance in mice[35, 36]. However, pharmacological stimulation or genetic overexpression of PGC-1α could not rescue muscle mass consistently in all experimental models of cachexia[37, 38] and extreme caution should be taken into account when testing strategies like systemic stimulation of PGC-1α expression or activity, as overexpression of PGC-1α has been associated with increased tumor growth in a model of cancer cachexia[38]. Therefore, exercise programs and/or (the less demanding) neuromuscular electrical stimulation might be a safer alternative at this point[39, 40].
In summary, findings demonstrate that despite evident pro-inflammatory signaling, muscle Oxphen is preserved in (pre-) cachexia associated with NSCLC, which

implies that Oxphen loss is not involved in progression of cancer cachexia-related muscle wasting prior to tumor treatment.

Chapter 6

Supplemental Data

Preserved muscle oxidative metabolic phenotype in clinical cancer cachexia

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Submitted

Supplemental data

Study population characteristics

NSCLC was confirmed by pathology and was classified according to the 6th Tumor Node Metastasis (TNM) classification for lung cancer[41]. Only patients with TNM stages III and IV were included to minimize confounding on the studied markers by malignant disease characteristics. Patients suffering from COPD GOLD III-IV, cardiac failure NYHA IV, severe endocrine, hepatic or renal disorders, other malignancies in the last 3 years and chronic inflammatory diseases or acute infection were excluded because of potential interference with muscle energy metabolism in these conditions. For this reason, also patients who underwent recent surgery (<3 months) or received treatment with corticosteroids or hormonal therapy were excluded.

Quadriceps muscle function

During the test, subjects were seated on the dynamometer chair with belts attached at the level the thigh and ankle for stability. Isometric muscle strength was assessed by 3 maximal voluntary contractions (MVCs) at an angle of 60°. Isokinetic muscle strength was assessed at angular velocities of 60°/s (set of 5 MVCs) and 180°/s (set of 10 MVCs). Muscle strength was defined as the highest muscular force output (peak torque) in Newton meters (Nm).

Muscle tissue processing following muscle biopsy

For (immuno)histochemical analysis, a part of each muscle biopsy was covered with Tissue-Tek® OCT™ (Sakura Finetek Europe B.V.) and frozen in melting isopentane which was pre-cooled in liquid nitrogen. Serial cross sections (5 µm) were cut on a cryostat microtome at -20˚C and mounted on SuperFrost microscope slides (Menzel-Gläser) which were kept at -80˚C until analysis. The remaining muscle tissue was snap-frozen and crushed to powder in liquid nitrogen and stored at -80˚C until further biochemical analyses.

Immunohistochemical analysis

Slides were incubated with primary: anti-laminin (dilution 1:50; #L-9393, Sigma, St. Louis, Missouri, USA) anti-type I MyHC (dilution 1:40; #A4840, DSHB, Iowa City, Iowa, USA) and anti-type II MyHC (dilution 1:40; #N2261, Santa Cruz, California, USA) and secondary antibodies: Alexa Fluor 350 (dilution 1:100; #A-11069, Invitrogen, Madison, Wisconsin, USA), Alexa Fluor 488 (dilution 1:1000; #A-21121, Invitrogen, Madison, Wisconsin, USA) and Alexa Fluor 555 (dilution 1:1000; #A-21426, Invitrogen, Madison, Wisconsin, USA). Images for analysis were obtained with fluorescent microscopy. Computer image analysis was performed using Lucia Software version 4.81 (Laboratory Imaging, Czech Republic).
For mATPase staining, sections were processed using acidic pre-incubation (NaAc 7.8 g/L, KCl 7.56 g/L) at pH 4.40, followed by acidic incubation (glycine 3.75 g/L, CaCl2•2H2O 4.26 g/L, NaCl 38g/L, ATP 1.7g/L) at pH 9.4. Sections were dehydrated in ethanol (50%-70%-96%-100%-ultraclear (Fisher Scientific Emergo). Images for analysis were obtained by light microscopy.
Per biopsy, 200 fibers were analyzed on average (minimum of 100 fibers). Damaged and detached fibers were excluded from analysis.

Western blot analysis

Approximately 50 mg of muscle biopsies was dissolved in lysis buffer (400 µl) consisting of Tris pH7.4 (50 mM), NaCl (150 mM), Glycerol (10%), NP-40 (0.5%), EDTA (1 mM), Na_3VO_4 (1 mM), NaF (5 mM), β-glycerophosphate (10 mM), Na-pyro-PO_4 (1 mM), DTT (1 mM), Leupeptin (10 µg/ml), Aprotenin (1 %), PMSF (1 mM) and homogenized using a Polytron PT (Polytron PT 1600 E, Kinematica AG). Following an incubation step of 30 minutes, muscle samples were sonicated and centrifuged at 4 degrees Celsius (16000 rcf) during 30 minutes. Protein concentration was assessed using BCA Protein Assay Kit (Pierce, Thermo Fisher). Next, sample buffer (dilution 1:4; 4x Stacking buffer: 0.250 M Tris-HCL, 8% SDS, 40% glycerol, 0.4M DTT, 0.02% Bromphenol Blue) was added and samples were incubated for 5 minutes at 95°C. Electrophoresis was performed on an Electrophoresis Cell system (Bio-Rad), where equal amounts of protein were loaded per lane of a 26 Wells Criterion XT 4-12% Bis-Tris precast gel (Bio-Rad). Two standard samples were included in every blot in order to correct for blot-to-blot variation. The gels were

transferred to nitrocellulose membranes (Whatman, GE Healthcare), followed by incubation during 60 minutes in 2% BSA or 5% milk in TBS Tween 20 (v/v 0.05%) before incubation with primary antibodies. Primary antibodies for Oxphos ATP synthase and I-IV complexes were used (1:1000; #MS604, Mito Sciences) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000; #2118, Cell Signalling) was used as loading control. The membranes were incubated with primary antibodies overnight at 4 degrees Celsius. Then, a successive incubation step with secondary antibodies (dilution 1:5000) of anti-mouse IgG peroxidase (#A85PI-1000.S1, Bio-Connect) and anti-rabbit IgG peroxidase (#A85PI-2000.S1, Bio-Connect) was performed. Detection of protein signals was performed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). Densitometry was used to quantify signals using Quantitiy One (version 4.6.2, Bio-Rad).

Quantitative real time polymerase chain reaction

To extract RNA, ToTALLY RNA™ Kit (Ambion Ltd.) was used according to guidelines of the manufacturer. In short, muscle biopsies (10–30 mg) were homogenized by a Polytron PT 1600 E (Kinematica AG) and total RNA was extracted. Then, contaminating genomic DNA was removed using RNeasy Mini Kit with RNase-free DNase (Qiagen). Concentration of total RNA in the respective samples was assessed using spectrophotometry (NanoDrop ND-1000, Isogen Lifescience). Total RNA (400ng) was reverse transcribed to cDNA with anchored oligo(dT) primers following manufacturer's guidelines (Transcriptor First Strand cDNA Synthesis kit, Roche Diagnostics). Primers (Sigma Genosys) were designed for: peroxisome proliferator-activated receptor gamma co-activator 1-alpha (*PGC-1α*), mitochondrial transcription factor A (*TFAM*), citrate synthase (*CS*), β-hydroxyacyl-CoA dehydrogenase (*HAD*), hexokinase II (*HKII*), phosphofructokinase (*PFK*), mitochondrially encoded cytochrome c oxidase III (*COX III*), cytochrome c oxidase subunit IV isoform 1 (*COX IV*), *MyHC I* (Supplemental data **Table 1**). Primers were used in quantitative real time polymerase chain reaction (Q-PCR) analysis (reaction contained 1x SensiMix SYBR & Fluorescein Kit (Bioline) with 300nM primers) and Hard-Shell 96-well Semi-skirted (HSS) PCR plates (Bio-Rad) were used on a MyiQ thermocycler (Bio-Rad). The assay program consisted of an initial 15 min incubation step at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds

of thermal cycling. To correct for variance within each reaction, gene expression was corrected for a sample specific geNorm factor calculated from *CYCLOPHILIN*, *BETA-ACTIN* (*β-ACTIN*) and *RPLPO* reference gene expression. Standard curves from pooled cDNA and melt curves were analyzed to verify efficiency and specificity of amplification.

Supplemental data table 1

Primer sequences

Gene name	Sense sequence	Antisense sequence
PGC-1α	ATGCCTTTAGATGTGAGCTAACAGTAGGTAATG	CGTACAGCCATCAAAAGGGACAC
TFAM	ATAACGTTTATGTAGCTGAAAGATTCCA	TCAGAGTCAGACAGATTTTTCCAGTTT
CS	GATGTGTCAGATGAGAAGTTACGAGACT	TGGCCATAGCCTGGAACAA
HAD	TGGCTTCCCGCCTTGTC	TGGAGCCGGTCCACTATCTTC
HKII	GTAAATACAGTGGATCTCAATCTTCGGG	CAAGGATTTGAGATGATTCGCTATTCA
PFK	CCTGCCCCTCATGGAATGT	GGGCTTCGTCAAATTTCTTCTC
COX III	CGGCATCTACGGCTCAACAT	TGAGGAAAGTTGAGCCAATAATGAC
COX IV	CCATGGATGAGAAAGTCGAGT	CGTTCGAGCCCCTGTTCA
MyHC I	CCTGGAACATCTGGAGACCT	AGTCTGATGACCAACTTGCGC
CYCLOPHILIN	CATCTGCACTGCCAAGACTGA	TTCATGCCTTCTTTCACTTTGC
β-ACTIN	AAGCCACCCCACTTCTCTCTAA	AATGCTATCACCTCCCCTGTGT
RPLPO	TCTACAACCCTGAAGTGCTTGATATC	GCAGACAGACACTGGCAACATT

Sample size calculation and interpretation

Sample size calculation was based on observations of Oxphen loss in patients with COPD. A meta-analysis demonstrated a proportion of 51% (± 12%) oxidative type I fibers in healthy control *versus* 33% in COPD patients, which implies a 35% loss[42]. Additional Oxphen markers like PGC-1α gene and Oxphos protein expression showed reductions in the same order of magnitude[26]. For the current study we aimed at detecting a change of a least 20% (being intermediate of a conventional 10% change for clinical relevance and the observed 35% decrease in COPD). An estimated loss (d) of 20% type I fibers, a standard deviation (SD) of 12% based on the abovementioned healthy control group, power of 80% and significance level van 5%, will calculate: $n = 15.7 \times (SD)^2 / (d)^2 = 22$ patients. At the end of inclusion, 22 healthy controls and 26 patients with lung cancer were included.

Based on the power calculation, a decrease of 20% in type I fibers could be observed with 80% power in patients with lung cancer. However, no significant

alterations were observed, both when lung cancer patients were divided in a pre-cachectic and cachectic group and when all patients with lung cancer patients were analyzed as one group (data not shown). And despite trends towards decreased type I fibers and increased type II fibers in the cachectic group, the difference was only ~15% and none of the other oxidative parameters showed indications of loss of Oxphen.

References

1. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489-495 (2011).
2. Calvani, R. *et al.* Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. *Biological chemistry* **394**, 393-414 (2013).
3. Kang, C. & Li Ji, L. Role of PGC-1alpha signaling in skeletal muscle health and disease. *Annals of the New York Academy of Sciences* **1271**, 110-117 (2012).
4. Remels, A.H., Gosker, H.R., Langen, R.C. & Schols, A.M. The mechanisms of cachexia underlying muscle dysfunction in COPD. *J Appl Physiol* (2012).
5. Fontes-Oliveira, C.C. *et al.* Mitochondrial and sarcoplasmic reticulum abnormalities in cancer cachexia: Altered energetic efficiency? *Biochim Biophys Acta* (2012).
6. Julienne, C.M. *et al.* Cancer cachexia is associated with a decrease in skeletal muscle mitochondrial oxidative capacities without alteration of ATP production efficiency. *J Cachexia Sarcopenia Muscle* **3**, 265-275 (2012).
7. White, J.P. *et al.* Muscle oxidative capacity during IL-6-dependent cancer cachexia. *Am J Physiol Regul Integr Comp Physiol* **300**, R201-211 (2011).
8. Padrao, A.I. *et al.* Bladder cancer-induced skeletal muscle wasting: disclosing the role of mitochondria plasticity. *Int J Biochem Cell Biol* **45**, 1399-1409 (2013).
9. Fermoselle, C. *et al.* Mitochondrial dysfunction and therapeutic approaches in respiratory and limb muscles of cancer cachectic mice. *Exp Physiol* **98**, 1349-1365 (2013).
10. Tzika, A.A. *et al.* Skeletal muscle mitochondrial uncoupling in a murine cancer cachexia model. *International journal of oncology* **43**, 886-894 (2013).
11. Constantinou, C. *et al.* Nuclear magnetic resonance in conjunction with functional genomics suggests mitochondrial dysfunction in a murine model of cancer cachexia. *Int J Mol Med* **27**, 15-24 (2011).
12. Khamoui, A.V. & Kim, J.S. Candidate mechanisms underlying effects of contractile activity on muscle morphology and energetics in cancer cachexia. *Eur J Cancer Care (Engl)* **21**, 143-157 (2012).
13. Acharyya, S. *et al.* Cancer cachexia is regulated by selective targeting of skeletal muscle gene products. *J Clin Invest* **114**, 370-378 (2004).
14. Diffee, G.M., Kalfas, K., Al-Majid, S. & McCarthy, D.O. Altered expression of skeletal muscle myosin isoforms in cancer cachexia. *Am J Physiol Cell Physiol* **283**, C1376-1382 (2002).
15. Baracos, V.E., DeVivo, C., Hoyle, D.H. & Goldberg, A.L. Activation of the ATP-ubiquitin-proteasome pathway in skeletal muscle of cachectic rats bearing a hepatoma. *Am J Physiol* **268**, E996-1006 (1995).
16. Op den Kamp, C.M. *et al.* 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. *Lung Cancer* **76**, 112-117 (2012).
17. Hoppeler, H. The different relationship of VO2max to muscle mitochondria in humans and quadrupedal animals. *Respir Physiol* **80**, 137-145 (1990).
18. Ivy, J.L., Costill, D.L. & Maxwell, B.D. Skeletal muscle determinants of maximum aerobic power in man. *Eur J Appl Physiol Occup Physiol* **44**, 1-8 (1980).

19. White, J.P. *et al.* IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse. *Skelet Muscle* **2**, 14 (2012).
20. Remels, A.H. *et al.* TNF-alpha impairs regulation of muscle oxidative phenotype: implications for cachexia? *FASEB J* **24**, 5052-5062 (2010).
21. Argiles, J.M., Busquets, S. & Lopez-Soriano, F.J. The pivotal role of cytokines in muscle wasting during cancer. *Int J Biochem Cell Biol* **37**, 2036-2046 (2005).
22. Carson, J.A. & Baltgalvis, K.A. Interleukin 6 as a key regulator of muscle mass during cachexia. *Exerc Sport Sci Rev* **38**, 168-176 (2010).
23. Op den Kamp, C.M. *et al.* Nuclear transcription factor kappa B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. *Am J Clin Nutr* **98**, 738-748 (2013).
24. Puigserver, P. *et al.* Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. *Mol Cell* **8**, 971-982 (2001).
25. Bergstrom, J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* **35**, 609-616 (1975).
26. van den Borst, B. *et al.* Loss of quadriceps muscle oxidative phenotype and decreased endurance in patients with mild-to-moderate COPD. *J Appl Physiol* (2012).
27. Weber, M.A. *et al.* Morphology, metabolism, microcirculation, and strength of skeletal muscles in cancer-related cachexia. *Acta Oncol* **48**, 116-124 (2009).
28. Fuster, G. *et al.* Are peroxisome proliferator-activated receptors involved in skeletal muscle wasting during experimental cancer cachexia? Role of beta2-adrenergic agonists. *Cancer Res* **67**, 6512-6519 (2007).
29. Gosker, H.R., Wouters, E.F., van der Vusse, G.J. & Schols, A.M. Skeletal muscle dysfunction in chronic obstructive pulmonary disease and chronic heart failure: underlying mechanisms and therapy perspectives. *Am J Clin Nutr* **71**, 1033-1047 (2000).
30. Puente-Maestu, L., Lazaro, A. & Humanes, B. Metabolic derangements in COPD muscle dysfunction. *J Appl Physiol* (2013).
31. De Ruysscher, D. *et al.* Maximal neutropenia during chemotherapy and radiotherapy is significantly associated with the development of acute radiation-induced dysphagia in lung cancer patients. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **18**, 909-916 (2007).
32. Ramnath, N. *et al.* Treatment of stage III non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e314S-340S (2013).
33. Pilegaard, H., Saltin, B. & Neufer, P.D. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* **546**, 851-858 (2003).
34. Puppa, M.J. *et al.* The effect of exercise on IL-6-induced cachexia in the Apc (Min/+) mouse. *J Cachexia Sarcopenia Muscle* **3**, 117-137 (2012).
35. Calvo, J.A. *et al.* Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *J Appl Physiol* **104**, 1304-1312 (2008).

36. Tadaishi, M. *et al.* Skeletal muscle-specific expression of PGC-1alpha-b, an exercise-responsive isoform, increases exercise capacity and peak oxygen uptake. *PLoS One* **6**, e28290 (2011).
37. Brault, J.J., Jespersen, J.G. & Goldberg, A.L. Peroxisome proliferator-activated receptor gamma coactivator 1alpha or 1beta overexpression inhibits muscle protein degradation, induction of ubiquitin ligases, and disuse atrophy. *J Biol Chem* **285**, 19460-19471 (2010).
38. Wang, X., Pickrell, A.M., Zimmers, T.A. & Moraes, C.T. Increase in muscle mitochondrial biogenesis does not prevent muscle loss but increased tumor size in a mouse model of acute cancer-induced cachexia. *PLoS One* **7**, e33426 (2012).
39. Maddocks, M., Gao, W., Higginson, I.J. & Wilcock, A. Neuromuscular electrical stimulation for muscle weakness in adults with advanced disease. *Cochrane Database Syst Rev* **1**, CD009419 (2013).
40. Phillips, S.M. Resistance exercise: good for more than just Grandma and Grandpa's muscles. *Appl Physiol Nutr Metab* **32**, 1198-1205 (2007).
41. Greene, F.L. *AJCC Cancer Staging Manual, 6th ed.* (Springer-Verlag, 2002).
42. Gosker, H.R., Zeegers, M.P., Wouters, E.F. & Schols, A.M. Muscle fibre type shifting in the vastus lateralis of patients with COPD is associated with disease severity: a systematic review and meta-analysis. *Thorax* **62**, 944-949 (2007).

Chapter 7

General discussion

Introduction

The cachexia syndrome is a commonly observed paraneoplastic feature of non-small cell lung cancer (NSCLC) that culminates in adverse outcome, including reduced response to anti-tumor therapy, functional decline, decreased quality of life and a significant increase in cancer-related mortality[1]. These devastating consequences predominantly result from wasting of skeletal muscle as distinct characteristic of cancer cachexia[1]. Because management of the primary malignant disease is challenging on its own, attention to secondary symptoms, including weight loss and muscle wasting, is currently not prioritized in clinical care. Consequently, cancer cachexia is rarely assessed or managed in a systematic manner, despite its large disease burden[1]. An additional obstacle impeding adequate clinical management of cancer cachexia is the absence of evidence-based interventions due to incomplete understanding of the pathophysiology[1].

The current dissertation presents a comprehensive characterization of phenotypic aspects and molecular signatures involved in progressive stages of cachexia in patients with non-small cell lung cancer (NSCLC). The overall aim was to assess and validate insights from experimental research, with the ultimate goal to contribute to improved and more personalized clinical management of NSCLC cachexia.

The following section will summarize the highlights of the current thesis in perspective of the currently available cancer cachexia literature. As elucidation of NSCLC cachexia pathophysiology is still in an early stage, the current findings are additionally compared to research in chronic obstructive pulmonary disease (COPD), a chronic disease also affecting the lung and with a high prevalence of cachexia. Management of cachexia in COPD is more established and insights in similarities or differences of underlying pathophysiology could aid in guiding future cancer cachexia therapy.

Main findings

Radiaton-esophagitis is not the primary trigger for early weigth loss in patients treated with concurrent chemoradiotherapy for non-small cell lung cancer

During the disease course of NSCLC, patients are constantly exposed to the risk of catabolism and weight loss, which can negatively influence response and tolerance to therapy, and decrease overall survival[2-4]. Tumor-dependent factors as well as therapy-related toxicities can contribute to weight loss and cachexia in NSCLC by decreasing dietary intake and increasing energy requirements[1-3]. In **chapter 2**, body weight alterations were studied during concurrent chemoradiotherapy (CT-RT) for locally advanced NSCLC. Body weight loss is commonly observed during concurrent CT-RT and though it is intuitively attributed to diminished dietary intake resulting from therapy-induced esophagitis, a combined retrospective and prospective research approach demonstrated that weight loss starts early after initiation of concurrent CT-RT and precedes the presence of significant esophagitis symptoms or decreased dietary intake. As further illustration, body weight loss was already observed in ~54% of patients in week 1 of concurrent CT-RT and this number increased to ~70-90% of patients in weeks 2 and 3 of treatment. Furthermore, a linear mixed model statistical approach indicated that the observed weight loss is not associated with severe esophagitis (grade ≥2) in the first weeks of concurrent CT-RT.

In addition to the alterations in body weight, muscle upper and lower extremity strength also rapidly declined by 20-30% during the first weeks of concurrent CT-RT. Since muscle strength is related to muscle mass[1, 5, 6], the significant loss of muscle strength could be determined by a net loss of muscle mass. In line with this notion, the data in **chapter 4** demonstrated that a decline of ~30% in muscle strength is indeed accompanied by a ~30% decrease in muscle fiber cross-sectional area. Interestingly, total and appendicular lean mass, as measured by dual-energy X-ray absorptiometry (DEXA), revealed only a ~10-20% decrease in this patient population, which could indicate that the detection of mild alterations in muscle mass by DEXA is limited, resulting in underestimation of muscle atrophy. Future research should determine if alternative non-invasive measures such as CT-scan and MRI imaging

techniques, or biomarkers in blood and urine might be more sensitive to detect (mild) alterations in muscle mass[7-9].

The etiology of the esophagitis-independent body weight loss remains to be determined but possibly, therapy-related elevation of energy expenditure associated with the intense concurrent CT-RT treatment regimen could be involved. This is illustrated by the work of Garcia-Peris et al., which demonstrated that after an initial decline at the start of concurrent CT-RT, resting energy expenditure as measured by indirect calorimetry was indeed unexpectedly elevated towards the end and following concurrent CT-RT in patients with head and neck cancer[10]. Whether changes in energy expenditure occur during concurrent CT-RT for NSCLC remains to be determined. Obtaining adequate data on energy requirements would require frequent assessment of energy expenditure. Using indirect calorimetric techniques that assess oxygen and carbon dioxide to estimate energy expenditure would be unfavorable due to the (time) demanding character of these measurements. Doubly labeled water is an accurate alternative that would be less demanding in patients undergoing the intense concurrent CT-RT treatment regimen[11].

The underlying mechanism for elevated (resting) energy requirements during early stage concurrent CT-RT could depend on inflammatory reactions associated with high-dose radiotherapy in combination with chemotherapy, and might be (partly) induced by acute cellular toxicity, e.g. inflammatory responses due to mucosal cell damage[12]. This can increase energy needs required for formation of inflammatory mediators and the acute phase response. Furthermore, amino acids could be redistributed from the muscle to the liver for acute phase protein synthesis[13]. When not adequately compensated by the diet, increased energy and amino acid requirements result in deficits that could subsequently lead to or enhance (muscle) catabolism[1]. Furthermore, (spill over of local inflammatory responses to) a systemic inflammatory reaction may alter neuroendocrine regulation of dietary intake[13-15], and prevent spontaneous dietary compensation of the elevated energy and nutrient requirements. To validate these hypotheses, it would be imperative to assess whether the balance between muscle protein synthesis and degradation is indeed disturbed in concurrent CT-RT, and whether this corresponds with the presence, intensity and kinetics of local and/or inflammatory responses.

Because weight loss in cancer patients can have detrimental effects on prognosis and quality of life, optimal supportive care to prevent or reverse the observed weight loss during chemo-radiation therapy is needed[1, 16]. Patients should therefore be

adequately monitored, which could minimally include regular assessment of body weight, scoring of dietary intake, measurement of catabolic drivers, such as inflammatory markers, and muscle function during concurrent CT-RT[1]. Assessment of these parameters, and conceivably also therapeutic measures, should be started early in the treatment regimen and prolonged for a substantial amount of time following concurrent CT-RT, at least as long as body weight is not recovered. The type of therapeutic intervention that is most effective during concurrent CT-RT remains to be determined but it seems conceivable that a multimodal approach is needed targeting both impaired energy balance as well as the triggers and signaling cues of skeletal muscle weakness[1]. Increasing insight in molecular alterations of muscle wasting in clinical cachexia could identify selective targets and help to guide development of effective therapies.

Altered protein turn-over in skeletal muscle of patients with NSCLC cachexia

Chapter 4 represents the first study that investigated the molecular basis of lung cancer cachexia in patient groups stratified according to cachexia stage using the recent international cancer cachexia consensus guideline[1]. Stratification criteria included weight loss and objective assessment of body composition. This systematic approach is novel, as previous clinical data was predominantly obtained from studies investigating heterogeneous populations, i.e. patients with different cancer types and cachexia stages were studied in single study designs and different definition criteria for cancer cachexia were used[17-20]. Often a single cut-off point for weight loss determined stratification, which resulted in inclusion of patients with wide-ranging weight loss and therefore, different cancer cachexia severity stages[1]. As the heterogeneity of patient populations included in previous studies likely introduced heterogeneity in subsequent analyses as well, this may have complicated identification of (relatively small) changes, resulting in inconclusive information on potentially involved underlying pathophysiologic mechanisms. Though recent studies are starting to implement the new cancer cachexia definition guidelines, all domains necessary for cancer cachexia diagnosis, i.e. weight loss, metabolic alterations and body composition, are not consistently used for stratification yet, as patients remain often stratified based on weight loss only[21]. This was the first approach to decrease heterogeneity introduced by the stage of cachexia. Moreover, studying pre-cachectic

alongside cachectic patients in the same analysis allows evaluation of the molecular alterations in the course of cancer cachexia.

With respect to molecular signaling in skeletal muscle, previous research has shown that muscle mass can be influenced by accretion or apoptosis of myonuclei[22]. The data in **chapter 4** demonstrated no consistent alterations in mRNA expression of myogenic markers MyoD and Myogenin in muscle of cachectic NSCLC patients compared to healthy controls, which is in line with stable MyoD mRNA transcript levels in patients with advanced stage gastric cancer (in stage IA and B gastric cancer an increase in MyoD mRNA transcripts was observed)[23]. In contrast, protein expression of MyoD was decreased in a set of weight-losing gastro-intestinal patients[24]. The discrepancy between transcriptional activity and protein expression could implicate regulation based on post-translational modification rather than on transcription level. This notion is supported by findings in COPD, i.e. a study in muscle-atrophied COPD patients showed that Myogenin mRNA expression was not different from controls[25], whereas other studies showed decreased protein expression of Myogenin and MyoD in cachectic patients compared with healthy controls or non-cachectic patients, respectively[26, 27]. However, as MyoD protein levels were not different between muscle-atrophied COPD patients and healthy controls[25], it needs to be elucidated whether post-translational alterations of myogenic factors are definitively important in cancer-imposed muscle wasting.
Although myonuclear apoptosis was not assessed in the studies that comprise the current dissertation, one study in gastro-intestinal cancer patients exhibiting >5% weight loss showed increased apoptotic activity (DNA fragmentation and poly-ADP-ribosyl polimerase (PARP) cleavage)[24]. In contrast, weight-losing gastric cancer patients showed no differences in apoptotic myonuclei[28]. Discrepancy concerning apoptotic activity has also been observed in patients with COPD, i.e. increased DNA fragmentation has been observed in underweight COPD patients[29], whereas DNA fragmentation and caspase-3 expression was not different in muscle-atrophied COPD compared with healthy controls[30]. The role of apoptosis in muscle wasting clearly requires further investigation.

A general concept in cachexia-related muscle wasting is that the balance between muscle protein synthesis and degradation, which normally preserves muscle mass, is disturbed[1, 31-33]. A remarkable finding described in **chapter 4** concerns the increased

proteolytic signaling associated with autophagy lysosomal pathway (ALP) activation in patients with NSCLC cachexia, rather than increased signaling of the anticipated ubiquitin 26S-proteasome system (UPS). Expression of ALP markers BNIP-3 and LC3B was ~2 fold increased in patients with NSCLC cachexia compared with healthy controls. While experimental studies indicate that autophagy might be involved in muscle wasting in cancer cachexia[34, 35], these findings are novel in the clinical setting. Conversely, the discrepancy between the current observations and findings in experimental research with respect to the role for E3-ligase expression in cancer cachexia-associated muscle wasting[36-38] is possibly a result of the characteristics of experimental cachexia models, in which excessive tumor load could lead to more acute and more unrestrained host responses[39]. This may be required for UPS activation. Data on UPS activity in clinical studies is contradicting but E3-ligase expression is generally unaffected in cancer patients suffering from cancer cachexia[40-42]. Therefore, it seems unlikely that muscle wasting in patients with NSCLC cachexia relies on sustained increases in expression of E3-ligases, although transient elevations in E3-ligase activity cannot be excluded. This result appears to be in contrast with increased expression of muscle UPS-related markers in patients suffering from COPD cachexia, as increased expression of muscle E3-ligases is observed in several studies[27, 43]. Potentially ALP signaling is also activated in COPD but this remains inconclusive yet. While one study in patients with COPD-induced muscle atrophy found no differences in ALP activation compared with healthy controls[25], Hussain and colleagues speculated that increased ALP signaling might be present in muscle of COPD patients based on their preliminary findings of increased LC3B, BECLIN1 and SQSTM1 expression[44]. The exact contribution of these alternative degradation systems and their regulation is an exciting research topic that warrants further exploration.

While catabolic signaling may play an important role in cancer cachexia-related muscle wasting, it is unlikely that muscle proteolysis is the only affected domain, as anabolic and catabolic pathways are coordinated at multiple levels[45, 46]. Protein synthesis in skeletal muscle is sensitive to activity of the PI3K/Akt pathway, in which the kinase Akt constitutes an important and potent stimulator of downstream mediators involved in protein synthesis[47]. Phosphorylation of the anabolic integrator Akt (P-Akt) was ~2 fold increased in patients suffering from NSCLC cachexia when compared to healthy control subjects. Considering the muscle atrophy observed in

these patients with NSCLC, it unlikely that this increased anabolic signaling effectively increases protein synthetic rate in NSCLC cachexia. Accordingly, activation of Akt was not accompanied by significant alterations in phosphorylation status of downstream molecules in the PI3K/Akt pathway, i.e. GSK-3β and mTOR. This indicates that despite increased P-Akt, no stimulation of protein synthesis signaling via the GSK-3β/eIF-2B and mTOR/eIF4/S6 axes is activated[46, 47]. Activation of Akt is also associated with attenuation of protein degradation via inhibitory phosphorylation of FOXO transcription factors, which orchestrate proteolytic UPS and ALP activity[47-49]. However, also FOXO phosphorylation was not altered in patients suffering from NSCLC cachexia, which is in line with the increased proteolytic signaling. The current findings concerning increased Akt activity are in contrast to the absence of alterations in Akt signaling in muscle-atrophied colorectal cancer patients compared with healthy controls[41], and decreased expression of Akt and alteration in down-stream mediators reported in patients with pancreatic cancer cachexia compared with non-cachectic patients[50]. The fact that the increased Akt signaling in the latter study was only compared to signaling in non-cachectic pancreatic (cancer) patients and not to signaling in healthy control subjects makes a direct comparison impossible and therefore, additional research is required to identify whether differential Akt signaling is actually present in different types of cancer. Increased P-Akt is also observed in cachectic patients with COPD[26, 43], and while some reports show activation of downstream molecules (4-EBP1 and P70S6K) in response to the increased Akt activation, activity of proteolytic E3-ligases Atrogin-1 and MuRF1 is not consistently inhibited in the presence of increased Akt phosphorylation[26, 43, 51]. The activation of Akt in cachexia in both lung cancer and COPD without consistent downstream signaling could be explained as a failed attempt to launch a compensatory mechanism to prevent the loss of muscle mass, i.e. increased catabolic signaling without adequate counterbalancing anabolic responses results in a net loss of muscle protein. A further indication for this notion is that the absence of P-Akt downstream signaling allows FOXO to convey its effects on proteolytic ALP signaling as was found in the current dissertation[48, 52]. However, ALP signaling might also depend on alternative regulators, like the important inflammatory integrator Nuclear Factor kappa B (NF-κB), of which muscle activation has also been associated with increased ALP signaling and which could be activated by disease-induced inflammatory signaling[53, 54].

Systemic and muscle inflammatory signals as triggers of cancer cachexia

Systemic and local inflammatory responses are considered potential triggers for muscle wasting in cancer cachexia[1, 55]. The results in **chapter 4** showed that increased concentrations of the inflammatory mediators C-reactive protein (CRP), Interleukin-6 (IL-6), Interleukin-8 (IL-8) and soluble TNF receptor 1 (sTNF receptor 1) as well as increased muscle NF-κB signaling (measured by transcript levels of NF-κB target gene IκBα) were observed in patients with NSCLC cachexia. While increased systemic inflammatory responses have repeatedly been observed in patients with cancer cachexia and some reports additionally suggests that NF-κB activity is increased in patients with gastro-intestinal cancer[56, 57], it had never been studied whether there is a causal link between systemic factors present in plasma and the induction of local inflammatory signaling in muscle. Using *ex vivo* plasma transfer experiments, in which cultured muscle cells were exposed to plasma of NSCLC patients, it was shown that the plasma of lung cancer patients contains mediators that induce muscle NF-κB activity. It would be extremely informative to identify whether systemic inflammatory mediators are actually the triggers responsible for the activation of NF-κB in skeletal muscle, as these could be important targets for selective treatment. Antibody-mediated inhibition of alleged inflammatory mediators could elucidate whether alleged inflammatory mediators, like IL-6 or TNF-α, can activate NF-κB signaling in muscle of patients with lung cancer cachexia.

A relation between increased systemic inflammatory responses and weight loss is also observed in patients with COPD, and some of the affected inflammatory mediators tend to overlap between cancer and COPD patients, i.e. increased concentrations of acute phase proteins and cytokines like CRP, TNF-α and IL-6 have been reported[58-60]. However, an important difference in the inflammatory responses between lung cancer and COPD is that the inflammatory response in stable COPD disease tends to be low grade, while more profound expression of the acute phase proteins and cytokines are reported in cancer. The exact origin of the systemic inflammatory responses remains to be determined as production of mediators could derive from multiple tissues and cell types. In (lung) cancer, the tumor has been implicated as significant source of cytokine production[61, 62] while in COPD, inflammatory processes in the respiratory system could spill over to the circulation[51]. However, as no correlation was found between concentrations of pulmonary and

systemic inflammatory mediators[63], production of inflammatory responses might also originate from other cells and tissues, such as leukocytes[64] or fat cells[65]. Whether the low-grade systemic inflammation in stable COPD disease is able to activate muscle NF-κB needs to be elucidated because contradicting findings on NF-κB activity in COPD patients have been reported[25, 66, 67]. Putatively, a certain intensity or prolonged duration might influence the potential of systemic inflammation to induce NF-κB signaling and subsequently, proteolytic activity in skeletal muscle. When considering that expression of inflammatory mediators and activity of proteolytic systems is more pronounced during exacerbations episodes in COPD than in stable disease[51, 64, 68, 69], the activity of NF-κB might also be increased during exacerbations and could contribute to accelerated muscle wasting during acute exacerbation episodes. When further elaborating on this notion, it might be hypothesized that muscle wasting in chronic diseases and cancer is as a dynamic process in which the relative activity of triggers of cachexia ultimately determines the progression of cachexia. The relative contributions of these triggers can fluctuate over time as a result of specific disease episodes, like exacerbations. Obviously, this hypothesis needs to be studied in clinical studies that include patients in 'stable' or 'acute' disease episodes.

A more recently identified regulatory molecule implicated in cachexia pathophysiology is myostatin, an extremely potent negative regulator of muscle mass that influences protein and myonuclear turn-over[70-72]. A recent report in non-weight losing lung and gastric cancer patients demonstrated that myostatin protein expression was increased in patients with gastric cancer but not in patients with lung cancer[73]. The latter is confirmed by the data in the current dissertation, i.e. no changes in myostatin signaling were observed in patients with NSCLC pre-cachexia and cachexia (**chapter 5**). These first results do not suggest an important role for alterations in myostatin signaling in lung cancer cachexia, whereas a study of muscle-atrophied patients with COPD showed increased myostatin mRNA expression[25]. However, another study found no differences in myostatin protein expression between non-cachectic and cachectic COPD patients[26]. Though the available data does not suggest differential myostatin signaling in lung cancer cachexia, inhibition of the (basal) activity of this pathway might still have beneficial

effects of muscle mass regulation when considering the extremely potent influence on muscle mass of the myostatin signaling pathway[71].

Decreased muscle function in cancer cachexia is not associated with altered muscle oxidative phenotype

Because fatigue and declined performance status can contribute to deterioration of quality of life and negatively influence prognosis in cancer cachexia[1], another aim of this thesis was to assess alterations in physical performance and characterize potential adaptations in muscle energy metabolism during NSCLC cachexia. In **chapter 5** it was demonstrated that NSCLC patients without signs of muscle atrophy show a decline of ~50% in peak oxygen consumption in incremental cycle ergometry without depletion of cardiac or respiratory reserves. Recent experimental studies in cancer cachexia models indicate that systemic inflammation might be involved in muscle exercise incapacity by altering dynamics in muscle energy metabolism[74-76]. Inflammatory signals can lead to decreased oxidative metabolism and increased glycolytic metabolism, i.e. a shift from slow-oxidative type I fibers towards fast-glycolytic type II fibers, loss of oxidative capacity and mitochondrial dysfunction. The data in **chapter 6** demonstrated that muscle oxidative and glycolytic metabolic profiles were not different in patients with NSCLC (pre-) cachexia compared with healthy controls, despite the significantly pro-inflammatory profiles. Though the exact role for differences between experimental findings and the current clinical findings need to be elucidated, a clinical study in patients with gastro-intestinal cancer cachexia confirmed the absence of alterations in muscle energy metabolism during cancer cachexia, i.e. no alterations were observed in microcirculation, capillary density en energy metabolites[77]. Contrarily, a pilot study in 8 colon cancer patients without lean mass depletion undergoing surgery shows decreased activity and protein expression of oxidative mitochondrial enzymes[57]. A comprehensive analysis of Oxphen in patients suffering from colon cancer is needed to reveal whether oxidative metabolism is indeed differently affected in alternative types of cancer. Another notion is that muscle oxidative metabolism was studied at rest in the current thesis. The absence of alterations in oxidative metabolism at rest does not exclude mechanical inefficiency or impaired mitochondrial function during physical exercise. A putative approach for further exploration of the observed muscle dysfunction would be to obtain muscle samples of patients during or shortly following physical exercise

and assess mechanical efficiency, mitochondrial function or expression of signaling molecules involved in energy metabolism[78, 79].

In COPD, patients show distinct features of decreased muscle oxidative metabolism as illustrated by an oxidative-glycolytic fiber type shift, mitochondrial oxidative enzymes and impaired mitochondrial function, especially in cachectic patients[80-84]. Also mRNA and protein expression of regulators of oxidative metabolism, such as PGC-1α and TFAM, are decreased in COPD patients[80-84]. These findings suggest a distinct difference in muscle oxidative metabolism between patients with lung cancer and COPD. Inflammatory signaling is considered an important trigger for alterations in muscle oxidative metabolism in COPD[80]. Possibly, the contrast between prolonged exposure to low-grade systemic inflammation in the relatively slow developing COPD cachexia and the more profound systemic inflammation in the relatively rapid developing NSCLC might contribute to the observed difference. Alternatively, (co-) factors that are more dominant in COPD compared to NSCLC, such as hypoxemia could determine the transition to a glycolytic phenotype in COPD. Hypoxemia might be more profound in COPD because decreased gas diffusion is widespread due to generalized remodeling of lung tissue[82], whereas in lung cancer mostly only a limited part of the lung is affected.

Pre-cachexia constitutes an early stage cachexia-phenotype and is associated with intermediate changes of molecular mediators identified in cachexia

Cancer cachexia presents itself in heterogeneous forms, varying from mild weight loss and fat depletion to extremely emaciated phenotypes in terminal disease[32]. In the cancer cachexia consensus, special attention is acknowledged for the early stage cachexia, i.e. pre-cachexia, as this stage could be optimal in terms of responsiveness to interventions that aim at delaying or even preventing progression into cancer cachexia[1]. Insights in systemic and molecular alterations in pre-cachexia are important for identification of potential targets for effective intervention strategies. Therefore, investigation of systemic and molecular alterations in pre-cachectic patients with NSCLC was another focus of the current thesis. The data in **chapters 4, 5 and 6** showed that according to the definition, patients with pre-cachexia demonstrated subtle changes in body weight, i.e. ~2-3% body weight loss, without significant alterations in anthropometric measurements or profound muscle atrophy.

However, intermediate changes were observed in morphological determinants, i.e. a ~20% decrease in muscle fiber cross-sectional area and protein concentration per unit DNA. This indicates that alterations in muscle morphology that cannot be detected by current DEXA measurements are already present in pre-cachexia. Despite the mild weight loss and subtle changes in DEXA-measured body composition, a significant decrease in muscle strength (up to ~40%) and endurance (~50%) indicates that functional parameters can already be compromised in pre-cachexia when compared to healthy controls.

Although patients with pre-cachexia do not exhibit significant alterations in muscle catabolic and anabolic signals (e.g. UPS and ALP vs. PI3/Akt activity), subtle alterations of several of these systems could be observed compared with healthy controls. Akt phosphorylation and expression of ALP markers was intermediate between expression in healthy controls and cachectic patients (**chapter 4**). These alterations in expression were in line with the intermediate alterations in muscle morphology and suggest that these subtle alterations are signs that precede significant changes in body composition and lean mass.

In line with the observations in lung cancer cachexia, systemic inflammatory signals might also be involved as trigger for the intermediate alterations observed in pre-cachexia, i.e. concentrations of several pro-inflammatory mediators like CRP, sTNF-receptor 1, fibrinogen and negative acute phase reactant albumin were intermediately altered in pre-cachectic patients when compared to healthy controls and cachectic patients (**chapter 4, 5 and 6**). Interestingly, the data presented in this dissertation reveal that despite the presence of mediators in plasma capable of inducing NF-κB activity in cultured muscle cells, NF-κB target gene expression was not significantly increased in muscle of patients with NSCLC pre-cachexia (**chapter 4**). This may suggest the activation of a muscle intrinsic, NF-kB suppressive response in pre-cachectic muscle, preventing profound catabolic responses at this stage. Identification of such a protective signaling mechanisms could have important implications for the treatment of cancer (pre-) cachexia.

In conclusion, pre-cachexia in NSCLC can be regarded as an early stage cancer cachexia, both in phenotypic and molecular perspective, although it has to be noted that it was not assessed whether the pre-cachectic patients studied in the current dissertation will actually develop cancer cachexia. The identification of subtle alterations in line with mechanisms of muscle wasting in cancer cachexia indicates

that intervention strategies, such as anti-inflammatory agents, stimulation of anabolism or prevention of catabolism proposed in cachexia could be beneficial in pre-cachexia as well. Possibly, however, the subtle changes in body composition associated with pre-cachexia might already be attenuated by a single interventions, like nutritional support, while the management of cancer cachexia presumably requires more potent multimodal support.

Treatment options for cancer cachexia: lessons learned from COPD?

Cancer cachexia is a multifactorial syndrome that involves many facets, e.g. reduced dietary intake, inflammatory responses and molecular alterations in muscle mass regulation[1]. Currently, NSCLC cachexia is considered irreversible[1]. Previous studies in which efficacy of mono-therapies were tested have shown only subtle beneficial effects on cancer cachexia. Therefore, it is believed that most potential is to be expected from a multimodal therapeutic approach[1]. The exact design of such multimodal interventions however, needs to be investigated. Based on results from the current thesis, a combination of the following approaches could be effective.

Nutritional interventions targeting muscle protein turn-over

With respect to alterations protein turn-over, the data in this dissertation indicate that muscle protein degradation is increased and anabolic signaling is impaired in NSCLC cachexia. Therefore, it seems conceivable that nutritional interventions stimulating anabolism and/or inhibiting catabolism could have beneficial effects on muscle mass regulation. Because the regulation of muscle protein turnover is pre-dominantly determined by the availability of amino acids in the circulation, the efficacy of protein supplementation in attenuation of cachexia-related muscle atrophy was reviewed in **chapter 3**. Data concerning this topic appears pre-dominantly derived from *in vitro* and experimental models of muscle wasting, and especially the paucity of studies in clinical cancer cachexia is remarkable. Based on the experimental data, it seems conceivable that there is a beneficial role for protein supplementation in the stimulation of muscle protein synthesis and attenuation of muscle protein degradation. Specifically, branched chain amino acids (BCAAs) showed promising result in *in vitro* studies. It was shown that BCAAs, and especially leucine, can directly activate protein synthesis and inhibit protein degradation via the mammalian

target of rapamycin (mTOR), a mediator downstream of Akt. Thereby, BCAAs would circumvent the anabolic resistance at the level of Akt as found in the current dissertation in patients with NSCLC cachexia. However, the potential of these *in vitro* findings requires further study, as the increase in anabolic signaling was not always accompanied by actual muscle maintenance in animal models of (cancer) cachexia. Clinical trials investigating the role of protein supplementation or administration of BCAAs in cancer patients are scarce but some studies were conducted in previous decades. In these studies supplementation with BCAAs in parental nutrition had beneficial effects on tolerance to treatment[85] and showed subtle increases in total body leucine balances and protein utilization[86, 87]_ENREF_84. However, difficulties in implementation of parental nutrition in medical care and the associated complications in absence of convincing beneficial effects on cancer cachexia outcomes have prevented the implementation of these strategies[88]. To explore the actual potential of protein supplementation on muscle mass regulation in NSCLC cachexia, further experimental and clinical studies are needed. A specific point of attention with respect to nutritional supplementation in patients with cancer is that it has recently been suggested that temporarily starvation sensitizes malignant cells to chemotherapy, while normal cells are relatively protected[89-91]. As this temporarily starvation might improve therapy outcome and reduce toxicity, it needs to be taken into consideration when designing trials using nutritional supplementation in cancer cachexia.

Anti-inflammatory interventions

Inhibiting inflammatory responses could be an alternative approach to attenuate cancer cachexia as the data in this thesis clearly showed that systemic and local muscle inflammatory signaling is increased in patients suffering from NSCLC cachexia. Some studies in cancer patients indicate that non-steroidal anti-inflammatory drugs (NSAID's) might improve body weight, physical performance and quality of life. However, the studies were performed in patients groups with different types of cancer, different stages of cachexia, a wide-range of treatment duration (2-125 weeks) and non-consistent study outcome parameters. Furthermore, patient populations tended to be small[92]. In a systematic literature review it is therefore concluded that there might be beneficial effects of NSAID's on weight course and other outcomes, but that evidence level of these studies is insufficient to recommend

NSAID's for attenuation of cancer cachexia[92]. The same accounts for the beneficial effects of omega-3 fatty acids, which are present in high concentrations in fish oil and are known for their anti-inflammatory effects by reducing synthesis of eicosanoids and influence of inflammatory signaling and gene expression[93]. Although some reports suggest improvement of muscle mass and anti-tumor treatment tolerance, the study designs are widely heterogeneous and methodological quality is not optimal. Therefore, current evidence is not sufficient to draw definite conclusions[94, 95]. Additionally, while early phase I/II clinical trials have indicated that ghrelin might increase food intake and lean mass by reducing cytokine production, additional research is ongoing[96].

Another anti-inflammatory approach is to directly ameliorate the activity of cytokines like IL-6 and TNF-alpha by monoclonal anti-bodies. Though definite efficacy needs to be determined, phase I and II studies using IL-6 antibody ALD518 showed less weight loss and improved fatigue and was tolerated well in patients with NSCLC[97]. On the other hand, the monoclonal anti-TNF-alpha antibody infliximab did not improve cachexia symptoms in pancreatic cancer patients receiving gemcitabine[98].

As the current data in **chapter 4** indicated, plasma mediators are able to activate local inflammatory signaling in skeletal muscle. Plasma transfer experiments similar as described in **chapter 4** could be used to identify the plasma factor(s) responsible for activating inflammatory signaling at the muscle level by systemically assessing NF-κB activity in presence of inhibiting antibodies against specific inflammatory mediators. Further study on the putative factor(s) identified in these experiments could aid development of effective targeted therapies.

Exercise training

The data in the current dissertation indicated that patients with NSCLC show a significant decline in muscle strength and muscle exercise capacity, which can significantly impair physical performance and quality of life[1]. Whereas decreases in muscle strength probably depend, at least partly, on the presence of muscle atrophy, the background of muscle endurance impairment is currently unknown. The current thesis indicated that alterations in oxidative or glycolytic metabolism do not play an important role in patients with NSCLC cachexia. Based on these findings, the role for interventions using aerobic training to improve muscle oxidative phenotype might be limited in NSCLC cachexia, whereas resistance training might have more potential by

improving muscle strength[99, 100]. Small (feasibility) studies indicate that progressive resistance exercise training at 75% of workload is sufficient to increase muscle strength in gastro-intestinal cancer patients[101] and similar results have been reported for resistance training in a small group of lung cancer patients[102]. However, implementation of exercise training is limited due to practical issues of tolerance and motivation, i.e. only 44-80% of patient completed offered exercise programs, which seems to be related to disease advancement and physical function of patients[102-104]. An alternative for intensive exercise rehabilitation programs might be neuromuscular electrical stimulation (NMES). Early studies, including a pilot study in NSCLC patients, however, show that this strategy might be less effective in increasing muscle mass and function than physical exercise programs[101, 105].

Multimodal approaches

Since, as pointed out above, a multimodal approach for cancer cachexia is likely required, the optimal combination of intervention strategies needs to be evaluated. Though some early phase clinical trials have found positive effects on cancer cachexia symptoms of alternative combinations of anti-oxidants, pharmaconutritional support and anti-inflammatory drugs[106, 107], there are no well-structured programs that are consistently implemented in clinical care yet. Well-structured multimodal nutritional rehabilitation programs are increasingly used in COPD care and show positive effects on cachexia symptoms[108]. Generally, integrated rehabilitation programs indicate that rehabilitation using (endurance) exercise training combined with NMES, nutritional supplementation, anabolic hormones, anti-inflammatory drugs and anti-oxidant drugs can have beneficial effects on body weight, muscle mass and muscle function[109, 110]. Possibly all these strategies might be beneficial in patients with lung cancer cachexia as well because multimodality strategies in COPD target pro-cachectic aspects that seem similarly affected in cancer cachexia, i.e. nutritional support for decreased dietary intake and alterations in muscle protein turn-over, anti-inflammatory drugs to inhibit inflammatory responses, exercise therapy to increase muscle mass and physical function and pharmacological anabolic agents to further increase muscle anabolism[1]. The optimal combination probably depends on the stage of cachexia, presence of pro-catabolic triggers and tolerance and preference of patients[1]. There is a need for well designed randomized clinical trials and

implementation of multimodality treatment intervention in clinical cancer cachexia care.

Future directions of cancer cachexia research

Based on the findings presented in the current thesis, as well as recently produced preliminary data described below, and literature on cancer cachexia, suggestions for future directions of research and clinical implementation to optimize management of cancer cachexia will be addressed in this paragraph.

Design of clinical studies investigating molecular mechanisms of cancer cachexia

An important point that has been addressed several times in this dissertation is that clinical studies that aim to identify underlying mechanisms often have included very heterogeneous patient populations, i.e. simultaneous inclusion of patients with early stages (pre-cachexia) and advanced stages (cachexia, or even refractory cachexia) of cachexia[1]. This thesis was a first attempt to systematically stratify patients according to criteria defined in a uniform and operational international consensus[1]. The results demonstrate that patients with progressive stages of NSCLC cachexia are characterized by different metabolic profiles and emphasize the need for clinical trials evaluating optimal and tailored interventions based on uniform metabolic phenotypes.

Another limitation of clinical studies addressing mechanisms of cancer cachexia involves the fact that most studies have a cross-sectional design, including studies in the current thesis. Additional data on the course of cachexia progression could identify underlying mechanisms of transition through the cachexia stages, e.g. following pre-cachectic patients would provide insight in the molecular mechanisms in different phases within one patient and could provide clues for the etiology of transition within the cachexia stages.

Furthermore, current research is mainly performed in standardized fasting conditions, when patients are at rest. However, certain pathological alterations that can influence metabolic regulation might not be detectable in these circumstances, e.g.

disturbances in anabolic responses to nutritional intake or exercise might be of importance muscle protein metabolism in cancer cachexia. Experimental conditions altering nutritional status or exercise evoking a robust and reproducible molecular signaling response could reveal potential impairments in anabolic or catabolic regulatory responses.

In addition, heterogeneity in studied outcome parameters contributes to the difficulty of drawing conclusions from clinical studies in cancer cachexia. UPS activity is a clear example, i.e. several studies assessed various outcome parameters like transcriptional activity and protein expression of ubiquitin, E3-ubiquitin ligases or proteasome subunits as well as assessment of ubiquitin conjugation and proteasome activity has been performed[18, 111, 112]. Consensus on key outcome parameters is therefore imperative.

Newly identified regulatory molecules in cancer cachexia: micro-RNAs

A main focus of this thesis was the verification of putative mechanisms of muscle atrophy postulated in studies of experimental models in muscle biopsies of patients with lung cancer cachexia. This also included addressing the potential involvement of transcriptional regulators of muscle atrophy signaling, including NF-kB, FOXO and Smad2/3 (as part of myostatin signaling) (**chapter 4 and 5**). However, culminating evidence points at a role of post-transcriptional regulation of tissue homeostasis by microRNAs (miRs), which are involved in coordination and fine-tuning of many cellular processes. These ~22nt short, single-stranded RNA molecules constitute a layer of post-transcriptional regulation of gene expression, as their binding to complementary sequences in the (primarily) 3'UTR of 'target' mRNAs predominantly results in negative impact on mRNA stability and translation[113].

miRs have been implicated in skeletal muscle plasticity. In fact, miRs specific to, or highly expressed in, skeletal muscle (myomiRs) have been identified as essential determinants in regulatory networks of myogenesis[114, 115], and muscle fiber type composition[116].

miRs subject to myogenic regulation by MyoD and MEF2 include miR1, -133, -206, and -486[113, 117]. Conversely, miRs target regulators of myogenesis involved in satellite cell activation, proliferation and myogenic differentiation. Although these also include signaling pathways like IGF-I/Akt as identified for miR29 and -128a, their

potential relevance to muscle hypertrophy and atrophy remains to be established[118, 119].

In experimental models of inflammation- and glucocorticoid (GC)-induced muscle atrophy, down-regulation of specific miRs has been implicated in the loss of muscle mass[120, 121]. Specifically, miR23a suppresses Atrogin-1 and MuRF1 expression, and miR23a over-expressing mice displayed resistance to GC-induced muscle atrophy. However, thus far no information on miR regulation in skeletal muscle during cancer cachexia is available.

As a first step in investigating the potential contribution of miRs to skeletal muscle atrophy in cancer cachexia, RNA prepared from the muscle biopsies collected as part of the studies described in **chapters 4 and 6** was subjected miR expression profiling. Two different platforms were deployed: miRNome (microRNA Profilers, Systems Biosciences), in collaboration with Dr. R. de Cabo (National Institute on Aging, Baltimore, MD, USA), and a TaqMan Array (Human MicroRNA A+B cards set v3.0) in collaboration with Prof. A. Harell-Belan (Laboratoire Epigenitique et Cancer, Saclay, Paris, France). The expression of a total of 759 miRNAs was compared between 7 biopsies of NSCLC patients with cachexia and 7 gender and age-matched, healthy control subjects. Between these groups the abundance of 32 miRs differed (>2-fold, $p<0.05$). Currently, differential expression of 17 miRs has been validated with RT Q-PCR, and interestingly, the expression of only 3 miRs was increased, whereas the transcript levels of 14 miRs were decreased. These findings reveal that lung cancer cachexia is accompanied by aberrant regulation of miRs in skeletal muscle. Further characterization of the regulation and function of these miRs, which putatively may turn out to be atrophy-specific microRNA's or 'AtromiRs', will yield insights that may serve as basis for therapeutic interventions aimed at reversing the intramuscular processes that drive cancer cachexia. However, the availability of representative experimental models is essential for the generation of robust pre-clinical data that merit the transition of mechanistic understanding to clinical application.

Optimization of experimental models that mimic cancer cachexia

Experimental models represent an important tool to explore molecular mechanisms of pathological states that enhance understanding of pathophysiology and prevents unnecessary clinical assessment that contributes to patient burden[39]. However, a frequent observation in the studies in this dissertation and other studies is that the findings in experimental cancer cachexia do not align with findings in patients with cancer. The reason for the discrepancies found between experimental findings and clinical findings may be explained by the status of current lung cancer experimental models. First, while current experimental cancer cachexia models exhibit differential aspects of cachexia, no single model seems to mimic the full repertoire of alterations in cancer cachexia, i.e. some models predominantly have increased inflammatory responses while others show cachectic effects without significant systemic inflammation[39]. Secondly, experimental cancer cachexia models might not be optimal in simulating clinical lung cancer in patients due to disproportional high tumor load and rapidity of cachexia development in the models, which results in a very aggressive and rapid phenotype that might have other characteristics than clinical lung cancer cachexia[39]. Additionally, a pitfall of many preclinical studies testing pharmacological interventions or genetic loss or gain of function studies is that the experimental design is aimed at prevention instead of reversing muscle wasting. An elegant exception concerns one study in which muscle wasting was completely reversed by pharmacological inhibition of ActRIIB signaling, which significantly increased survival, even despite the sustained presence of tumor growth and inflammatory signaling[122]. Therefore, more focus should be provided for the selection of one or a combination of experimental models that adequately exhibits aspects tailored to the relevant research questions.

Improvement of study design, timing and outcome parameters of clinical trials investigating intervention strategies in cachexia

As was discussed before, it seems conceivable that a multimodal therapeutic approach could have potential in counteracting cancer cachexia as the origin of this syndrome is considered multifactorial and addressing a single domain by mono-therapy has shown to be only minimally effective. For design of such intervention

regimens, successful rehabilitation programs that have been tested in COPD can provide important clues. However, it should be taken into account that the window of opportunity for different interventions in lung cancer is often limited in this regard. For instance, in COPD, long-term exercise programs have proven to be beneficial for better performance and maintenance of muscle mass, while the long-term training might not be possible in rapidly developing lung cancer.

Furthermore, performance status of patients with lung cancer is often declining to such an extent, partly because of intense treatment regimens, that it is not feasible to subject patients to intense and time-consuming training programs. In addition, benefits might not outweigh the effort of patients to overcome these obstacles with respect to prognosis and quality of life. Less invasive and less demanding alternatives of regular interventions such as neuroelectrical muscle stimulation (NMES) in combination with pharmacological (anti-inflammatory, hormonal therapy or appetite stimulants) or nutritional interventions (branched chain amino acid diets) should therefore be considered in clinical lung cancer cachexia[1, 99]. Preferably, the increasing insights in molecular mechanisms of cancer cachexia will shortly result in the development of novel, targeted and tailored therapies that can be used as an additive to these interventions.

Furthermore, when studying intervention strategies in cancer cachexia adequate outcome parameters should be determined. While currently, weight gain is often assessed, the benefit of weight gain without information on the body compartment that increased, i.e. adipose tissue versus muscle, might not be adequate in determining positive effects in cachectic patients. As especially the loss of muscle mass has been associated with increased mortality[1], decreased performance status and declined quality of life, it seems most conceivable to assess the response of muscle mass to a specific treatment and evaluate the effects on the domains mortality, performance status and quality of life. However, as it is difficult to assess subtle alterations in muscle mass with conventional techniques such as arm or leg circumference, bioelectrical impedance (BIA) and DEXA, new techniques that are sensitive to such alterations should be identified, e.g. CT-scan or MRI[7, 123].

Conclusions from thesis

In the current dissertation it was revealed that systemic and local (muscle) inflammation can be appreciated in NSCLC cachexia and that factors present in the circulation are able to induce the transition to local inflammatory signaling.

With respect to muscle protein turnover, NSCLC cachexia proteolytic signaling was characterized by increased autophagy-related signaling, while no indications for sustained elevations in activity of the UPS were found. Increased Akt phosphorylation without activation of downstream targets such as mTOR or FOXO points at a futile compensatory response to a catabolic milieu. Combined, the increased proteolytic signaling and impairment of anabolic signaling at the level of Akt can be the cause for the net loss of muscle protein in NSCLC cachexia.

With respect to muscle function, muscle strength and exercise endurance was significantly declined in patients suffering from cancer cachexia. Muscle strength was declined to the same extent as muscle fiber cross sectional area but not total body lean mass atrophy as measured by DEXA. The decrease in muscle function does to rely on structural muscular adaptations with respect to muscle oxidative or glycolytic metabolism.

Pre-cachexia can be considered an early stage of NSCLC cachexia with respect to phenotype and molecular signatures, including lean mass atrophy, muscle function, systemic and local inflammatory signaling, and muscle protein turnover.

Optimization of experimental cachexia models and design of human studies is required to provide further insights in etiology of (pre-)cachexia in NSCLC cachexia. Uniformity of study populations and outcome parameters could also contribute to development of adequate intervention strategies for cachexia, presumably in a multimodal structure.

References

1. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol* **12**, 489-495 (2011).
2. Fearon, K.C., Voss, A.C., Hustead, D.S. & Cancer Cachexia Study, G. Definition of cancer cachexia: effect of weight loss, reduced food intake, and systemic inflammation on functional status and prognosis. *Am J Clin Nutr* **83**, 1345-1350 (2006).
3. Ramnath, N. *et al.* Treatment of stage III non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **143**, e314S-340S (2013).
4. Thiel, H.J., Fietkau, R. & Sauer, R. Malnutrition and the role of nutritional support for radiation therapy patients. *Recent Results Cancer Res* **108**, 205-226 (1988).
5. Jubrias, S.A., Odderson, I.R., Esselman, P.C. & Conley, K.E. Decline in isokinetic force with age: muscle cross-sectional area and specific force. *Pflugers Arch* **434**, 246-253 (1997).
6. Larsson, L., Grimby, G. & Karlsson, J. Muscle strength and speed of movement in relation to age and muscle morphology. *J Appl Physiol* **46**, 451-456 (1979).
7. Prado, C.M., Birdsell, L.A. & Baracos, V.E. The emerging role of computerized tomography in assessing cancer cachexia. *Curr Opin Support Palliat Care* **3**, 269-275 (2009).
8. Gray, C. *et al.* Magnetic resonance imaging with k-means clustering objectively measures whole muscle volume compartments in sarcopenia/cancer cachexia. *Clin Nutr* **30**, 106-111 (2011).
9. Prado, C.M. Body composition in chemotherapy: the promising role of CT scans. *Curr Opin Clin Nutr Metab Care* (2013).
10. Garcia-Peris, P. *et al.* Prospective study of resting energy expenditure changes in head and neck cancer patients treated with chemoradiotherapy measured by indirect calorimetry. *Nutrition* **21**, 1107-1112 (2005).
11. Pinheiro Volp, A.C., Esteves de Oliveira, F.C., Duarte Moreira Alves, R., Esteves, E.A. & Bressan, J. Energy expenditure: components and evaluation methods. *Nutr Hosp* **26**, 430-440 (2011).
12. Hildebrandt, M.A. *et al.* Genetic variants in inflammation-related genes are associated with radiation-induced toxicity following treatment for non-small cell lung cancer. *PLoS One* **5**, e12402 (2010).
13. Donohoe, C.L., Ryan, A.M. & Reynolds, J.V. Cancer cachexia: mechanisms and clinical implications. *Gastroenterol Res Pract* **2011**, 601434 (2011).
14. Plata-Salaman, C.R. Central nervous system mechanisms contributing to the cachexia-anorexia syndrome. *Nutrition* **16**, 1009-1012 (2000).
15. Plata-Salaman, C.R. Anorexia during acute and chronic disease. *Nutrition* **12**, 69-78 (1996).
16. von Haehling, S. Cachexia as major underestimated and unmet medical need: facts and numbers. *J Cachex Sarcopenia Muscle* **1**, 1-5 (2010).
17. Bossola, M. *et al.* Increased muscle ubiquitin mRNA levels in gastric cancer patients. *Am J Physiol Regul Integr Comp Physiol* **280**, R1518-1523 (2001).
18. Bossola, M. *et al.* Increased muscle proteasome activity correlates with disease severity in gastric cancer patients. *Ann Surg* **237**, 384-389 (2003).

19. Khal, J., Hine, A.V., Fearon, K.C., Dejong, C.H. & Tisdale, M.J. Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss. *Int J Biochem Cell Biol* **37**, 2196-2206 (2005).
20. Williams, A., Sun, X., Fischer, J.E. & Hasselgren, P.O. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery* **126**, 744-749; discussion 749-750 (1999).
21. D'Orlando, C. *et al.* Gastric cancer does not affect the expression of atrophy-related genes in human skeletal muscle. *Muscle Nerve* (2013).
22. Allen, D.L., Roy, R.R. & Edgerton, V.R. Myonuclear domains in muscle adaptation and disease. *Muscle Nerve* **22**, 1350-1360 (1999).
23. Pessina, P. *et al.* Skeletal muscle of gastric cancer patients expresses genes involved in muscle regeneration. *Oncol Rep* **24**, 741-745 (2010).
24. Busquets, S. *et al.* Apoptosis is present in skeletal muscle of cachectic gastro-intestinal cancer patients. *Clin Nutr* **26**, 614-618 (2007).
25. Plant, P.J. *et al.* Cellular markers of muscle atrophy in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **42**, 461-471 (2010).
26. Vogiatzis, I. *et al.* Effect of pulmonary rehabilitation on muscle remodelling in cachectic patients with COPD. *Eur Respir J* **36**, 301-310 (2010).
27. Fermoselle, C. *et al.* Does oxidative stress modulate limb muscle atrophy in severe COPD patients? *Eur Respir J* **40**, 851-862 (2012).
28. Bossola, M. *et al.* Skeletal muscle apoptosis is not increased in gastric cancer patients with mild-moderate weight loss. *Int J Biochem Cell Biol* **38**, 1561-1570 (2006).
29. Agusti, A.G. *et al.* Skeletal muscle apoptosis and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **166**, 485-489 (2002).
30. Gosker, H.R. *et al.* Myopathological features in skeletal muscle of patients with chronic obstructive pulmonary disease. *Eur Respir J* **22**, 280-285 (2003).
31. Acharyya, S. & Guttridge, D.C. Cancer cachexia signaling pathways continue to emerge yet much still points to the proteasome. *Clin Cancer Res* **13**, 1356-1361 (2007).
32. Fearon, K.C., Glass, D.J. & Guttridge, D.C. Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell Metab* **16**, 153-166 (2012).
33. Tisdale, M.J. Cachexia in cancer patients. *Nat Rev Cancer* **2**, 862-871 (2002).
34. Ham, D.J., Murphy, K.T., Chee, A., Lynch, G.S. & Koopman, R. Glycine administration attenuates skeletal muscle wasting in a mouse model of cancer cachexia. *Clin Nutr* (2013).
35. Penna, F. *et al.* Autophagic degradation contributes to muscle wasting in cancer cachexia. *Am J Pathol* **182**, 1367-1378 (2013).
36. Bodine, S.C. *et al.* Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-1708 (2001).
37. Cao, P.R., Kim, H.J. & Lecker, S.H. Ubiquitin-protein ligases in muscle wasting. *Int J Biochem Cell Biol* **37**, 2088-2097 (2005).
38. Lecker, S.H. *et al.* Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J* **18**, 39-51 (2004).
39. Bennani-Baiti, N. & Walsh, D. Animal models of the cancer anorexia-cachexia syndrome. *Support Care Cancer* **19**, 1451-1463 (2011).

40. Gallagher, I.J. *et al.* Suppression of skeletal muscle turnover in cancer cachexia: evidence from the transcriptome in sequential human muscle biopsies. *Clin Cancer Res* **18**, 2817-2827 (2012).
41. Williams, J.P. *et al.* Effect of tumor burden and subsequent surgical resection on skeletal muscle mass and protein turnover in colorectal cancer patients. *Am J Clin Nutr* **96**, 1064-1070 (2012).
42. Smith, I.J. *et al.* Calpain activity is increased in skeletal muscle from gastric cancer patients with no or minimal weight loss. *Muscle Nerve* **43**, 410-414 (2011).
43. Doucet, M. *et al.* Muscle atrophy and hypertrophy signaling in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **176**, 261-269 (2007).
44. Hussain, S.N. & Sandri, M. Role of autophagy in COPD skeletal muscle dysfunction. *J Appl Physiol* **114**, 1273-1281 (2013).
45. Argiles, J.M., Lopez-Soriano, F.J. & Busquets, S. Mechanisms to explain wasting of muscle and fat in cancer cachexia. *Curr Opin Support Palliat Care* **1**, 293-298 (2007).
46. Glass, D.J. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* **37**, 1974-1984 (2005).
47. Glass, D.J. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. *Curr Top Microbiol Immunol* **346**, 267-278 (2010).
48. Mammucari, C., Schiaffino, S. & Sandri, M. Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. *Autophagy* **4**, 524-526 (2008).
49. Stitt, T.N. *et al.* The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* **14**, 395-403 (2004).
50. Schmitt, T.L. *et al.* Activity of the Akt-dependent anabolic and catabolic pathways in muscle and liver samples in cancer-related cachexia. *J Mol Med (Berl)* **85**, 647-654 (2007).
51. Langen, R.C., Gosker, H.R., Remels, A.H. & Schols, A.M. Triggers and mechanisms of skeletal muscle wasting in chronic obstructive pulmonary disease. *Int J Biochem Cell Biol* (2013).
52. Mammucari, C. *et al.* FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**, 458-471 (2007).
53. Pietrocola, F. *et al.* Regulation of autophagy by stress-responsive transcription factors. *Semin Cancer Biol* (2013).
54. Zeng, M. *et al.* NF-kappaB-mediated induction of autophagy in cardiac ischemia/reperfusion injury. *Biochem Biophys Res Commun* **436**, 180-185 (2013).
55. Deans, C. & Wigmore, S.J. Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr Opin Clin Nutr Metab Care* **8**, 265-269 (2005).
56. Rhoads, M.G., Kandarian, S.C., Pacelli, F., Doglietto, G.B. & Bossola, M. Expression of NF-kappaB and IkappaB proteins in skeletal muscle of gastric cancer patients. *Eur J Cancer* **46**, 191-197 (2010).
57. Phillips, B.E. *et al.* Effect of colon cancer and surgical resection on skeletal muscle mitochondrial enzyme activity in colon cancer patients: a pilot study. *J Cachexia Sarcopenia Muscle* **4**, 71-77 (2013).
58. Nussbaumer-Ochsner, Y. & Rabe, K.F. Systemic manifestations of COPD. *Chest* **139**, 165-173 (2011).

59. Di Francia, M., Barbier, D., Mege, J.L. & Orehek, J. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **150**, 1453-1455 (1994).
60. Eid, A.A. *et al.* Inflammatory response and body composition in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **164**, 1414-1418 (2001).
61. Zhang, Y. *et al.* Potential mechanism of interleukin-8 production from lung cancer cells: an involvement of EGF-EGFR-PI3K-Akt-Erk pathway. *J Cell Physiol* **227**, 35-43 (2012).
62. Argiles, J.M., Busquets, S. & Lopez-Soriano, F.J. Anti-inflammatory therapies in cancer cachexia. *Eur J Pharmacol* **668 Suppl 1**, S81-86 (2011).
63. Vernooy, J.H. *et al.* Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am J Respir Crit Care Med* **166**, 1218-1224 (2002).
64. Oudijk, E.J. *et al.* Systemic inflammation in COPD visualised by gene profiling in peripheral blood neutrophils. *Thorax* **60**, 538-544 (2005).
65. van den Borst, B. *et al.* Low-grade adipose tissue inflammation in patients with mild-to-moderate chronic obstructive pulmonary disease. *Am J Clin Nutr* **94**, 1504-1512 (2011).
66. Mercken, E.M., Hageman, G.J., Langen, R.C., Wouters, E.F. & Schols, A.M. Decreased exercise-induced expression of nuclear factor-kappaB-regulated genes in muscle of patients with COPD. *Chest* **139**, 337-346 (2011).
67. Agusti, A., Morla, M., Sauleda, J., Saus, C. & Busquets, X. NF-kappaB activation and iNOS upregulation in skeletal muscle of patients with COPD and low body weight. *Thorax* **59**, 483-487 (2004).
68. Wouters, E.F., Groenewegen, K.H., Dentener, M.A. & Vernooy, J.H. Systemic inflammation in chronic obstructive pulmonary disease: the role of exacerbations. *Proc Am Thorac Soc* **4**, 626-634 (2007).
69. Crul, T. *et al.* Gene expression profiling in vastus lateralis muscle during an acute exacerbation of COPD. *Cell Physiol Biochem* **25**, 491-500 (2010).
70. Glass, D.J. Signaling pathways perturbing muscle mass. *Curr Opin Clin Nutr Metab Care* **13**, 225-229 (2010).
71. McPherron, A.C., Lawler, A.M. & Lee, S.J. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90 (1997).
72. Morley, J.E., Thomas, D.R. & Wilson, M.M. Cachexia: pathophysiology and clinical relevance. *Am J Clin Nutr* **83**, 735-743 (2006).
73. Aversa, Z. *et al.* Changes in myostatin signaling in non-weight-losing cancer patients. *Ann Surg Oncol* **19**, 1350-1356 (2012).
74. Tzika, A.A. *et al.* Skeletal muscle mitochondrial uncoupling in a murine cancer cachexia model. *Int J Oncol* **43**, 886-894 (2013).
75. Fontes-Oliveira, C.C. *et al.* Mitochondrial and sarcoplasmic reticulum abnormalities in cancer cachexia: altered energetic efficiency? *Biochim Biophys Acta* **1830**, 2770-2778 (2013).
76. Julienne, C.M. *et al.* Cancer cachexia is associated with a decrease in skeletal muscle mitochondrial oxidative capacities without alteration of ATP production efficiency. *J Cachexia Sarcopenia Muscle* **3**, 265-275 (2012).
77. Weber, M.A. *et al.* Morphology, metabolism, microcirculation, and strength of skeletal muscles in cancer-related cachexia. *Acta Oncol* **48**, 116-124 (2009).

78. Baarends, E.M., Schols, A.M., Akkermans, M.A. & Wouters, E.F. Decreased mechanical efficiency in clinically stable patients with COPD. *Thorax* **52**, 981-986 (1997).
79. Saey, D. *et al.* Quadriceps metabolism during constant workrate cycling exercise in chronic obstructive pulmonary disease. *J Appl Physiol* **110**, 116-124 (2011).
80. Remels, A.H., Gosker, H.R., Langen, R.C. & Schols, A.M. The mechanisms of cachexia underlying muscle dysfunction in COPD. *J Appl Physiol* **114**, 1253-1262 (2013).
81. Allaire, J. *et al.* Peripheral muscle endurance and the oxidative profile of the quadriceps in patients with COPD. *Thorax* **59**, 673-678 (2004).
82. Gosker, H.R. *et al.* Striking similarities in systemic factors contributing to decreased exercise capacity in patients with severe chronic heart failure or COPD. *Chest* **123**, 1416-1424 (2003).
83. Gosker, H.R., Wouters, E.F., van der Vusse, G.J. & Schols, A.M. Skeletal muscle dysfunction in chronic obstructive pulmonary disease and chronic heart failure: underlying mechanisms and therapy perspectives. *Am J Clin Nutr* **71**, 1033-1047 (2000).
84. van den Borst, B. *et al.* Loss of quadriceps muscle oxidative phenotype and decreased endurance in patients with mild-to-moderate COPD. *J Appl Physiol* **114**, 1319-1328 (2013).
85. Paccagnella, A., Morassutti, I. & Rosti, G. Nutritional intervention for improving treatment tolerance in cancer patients. *Curr Opin Oncol* **23**, 322-330 (2011).
86. Hunter, D.C., Weintraub, M., Blackburn, G.L. & Bistrian, B.R. Branched chain amino acids as the protein component of parenteral nutrition in cancer cachexia. *Br J Surg* **76**, 149-153 (1989).
87. Tayek, J.A. *et al.* Improved protein kinetics and albumin synthesis by branched chain amino acid-enriched total parenteral nutrition in cancer cachexia. A prospective randomized crossover trial. *Cancer* **58**, 147-157 (1986).
88. Bosaeus, I. Nutritional support in multimodal therapy for cancer cachexia. *Support Care Cancer* **16**, 447-451 (2008).
89. Raffaghello, L. *et al.* Fasting and differential chemotherapy protection in patients. *Cell Cycle* **9**, 4474-4476 (2010).
90. Safdie, F.M. *et al.* Fasting and cancer treatment in humans: A case series report. *Aging (Albany NY)* **1**, 988-1007 (2009).
91. Raffaghello, L. *et al.* Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc Natl Acad Sci U S A* **105**, 8215-8220 (2008).
92. Solheim, T.S., Fearon, K.C., Blum, D. & Kaasa, S. Non-steroidal anti-inflammatory treatment in cancer cachexia: a systematic literature review. *Acta Oncol* **52**, 6-17 (2013).
93. Simopoulos, A.P. Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* **21**, 495-505 (2002).
94. Murphy, R.A., Mourtzakis, M. & Mazurak, V.C. n-3 polyunsaturated fatty acids: the potential role for supplementation in cancer. *Curr Opin Clin Nutr Metab Care* **15**, 246-251 (2012).
95. Ries, A. *et al.* A systematic review on the role of fish oil for the treatment of cachexia in advanced cancer: an EPCRC cachexia guidelines project. *Palliat Med* **26**, 294-304 (2012).

96. Argiles, J.M. & Stemmler, B. The potential of ghrelin in the treatment of cancer cachexia. *Expert Opin Biol Ther* **13**, 67-76 (2013).
97. Bayliss, T.J., Smith, J.T., Schuster, M., Dragnev, K.H. & Rigas, J.R. A humanized anti-IL-6 antibody (ALD518) in non-small cell lung cancer. *Expert Opin Biol Ther* **11**, 1663-1668 (2011).
98. Wiedenmann, B. *et al.* A multicenter, phase II study of infliximab plus gemcitabine in pancreatic cancer cachexia. *J Support Oncol* **6**, 18-25 (2008).
99. Maddocks, M., Gao, W., Higginson, I.J. & Wilcock, A. Neuromuscular electrical stimulation for muscle weakness in adults with advanced disease. *Cochrane Database Syst Rev* **1**, CD009419 (2013).
100. Maddocks, M., Murton, A.J. & Wilcock, A. Therapeutic exercise in cancer cachexia. *Crit Rev Oncog* **17**, 285-292 (2012).
101. Maddocks, M., Murton, A.J. & Wilcock, A. Improving muscle mass and function in cachexia: non-drug approaches. *Curr Opin Support Palliat Care* **5**, 361-364 (2011).
102. Peddle-McIntyre, C.J., Bell, G., Fenton, D., McCargar, L. & Courneya, K.S. Feasibility and preliminary efficacy of progressive resistance exercise training in lung cancer survivors. *Lung Cancer* **75**, 126-132 (2012).
103. Glare, P., Jongs, W. & Zafiropoulos, B. Establishing a cancer nutrition rehabilitation program (CNRP) for ambulatory patients attending an Australian cancer center. *Support Care Cancer* **19**, 445-454 (2011).
104. Temel, J.S. *et al.* A structured exercise program for patients with advanced non-small cell lung cancer. *J Thorac Oncol* **4**, 595-601 (2009).
105. Maddocks, M. *et al.* Randomized controlled pilot study of neuromuscular electrical stimulation of the quadriceps in patients with non-small cell lung cancer. *J Pain Symptom Manage* **38**, 950-956 (2009).
106. Maccio, A., Madeddu, C. & Mantovani, G. Current pharmacotherapy options for cancer anorexia and cachexia. *Expert Opin Pharmacother* **13**, 2453-2472 (2012).
107. Mantovani, G. *et al.* A phase II study with antioxidants, both in the diet and supplemented, pharmaconutritional support, progestagen, and anti-cyclooxygenase-2 showing efficacy and safety in patients with cancer-related anorexia/cachexia and oxidative stress. *Cancer Epidemiol Biomarkers Prev* **15**, 1030-1034 (2006).
108. Solheim, T.S. & Laird, B.J. Evidence base for multimodal therapy in cachexia. *Curr Opin Support Palliat Care* **6**, 424-431 (2012).
109. Schols, A.M., Soeters, P.B., Mostert, R., Pluymers, R.J. & Wouters, E.F. Physiologic effects of nutritional support and anabolic steroids in patients with chronic obstructive pulmonary disease. A placebo-controlled randomized trial. *Am J Respir Crit Care Med* **152**, 1268-1274 (1995).
110. Man, W.D., Kemp, P., Moxham, J. & Polkey, M.I. Exercise and muscle dysfunction in COPD: implications for pulmonary rehabilitation. *Clin Sci (Lond)* **117**, 281-291 (2009).
111. Op den Kamp, C.M. *et al.* 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. *Lung Cancer* **76**, 112-117 (2012).
112. Sun, Y.S., Ye, Z.Y., Qian, Z.Y., Xu, X.D. & Hu, J.F. Expression of TRAF6 and ubiquitin mRNA in skeletal muscle of gastric cancer patients. *J Exp Clin Cancer Res* **31**, 81 (2012).

113. Wang, X.H. MicroRNA in myogenesis and muscle atrophy. *Curr Opin Clin Nutr Metab Care* **16**, 258-266 (2013).
114. Naguibneva, I. *et al.* The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. *Nat Cell Biol* **8**, 278-284 (2006).
115. Wang, H., Sun, H. & Guttridge, D.C. microRNAs: novel components in a muscle gene regulatory network. *Cell Cycle* **8**, 1833-1837 (2009).
116. van Rooij, E. *et al.* A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. *Dev Cell* **17**, 662-673 (2009).
117. Ge, Y. & Chen, J. MicroRNAs in skeletal myogenesis. *Cell Cycle* **10**, 441-448 (2011).
118. Wei, W. *et al.* miR-29 targets Akt3 to reduce proliferation and facilitate differentiation of myoblasts in skeletal muscle development. *Cell Death Dis* **4**, e668 (2013).
119. Motohashi, N. *et al.* Regulation of IRS1/Akt insulin signaling by microRNA-128a during myogenesis. *J Cell Sci* **126**, 2678-2691 (2013).
120. Panguluri, S.K. *et al.* Genomic profiling of messenger RNAs and microRNAs reveals potential mechanisms of TWEAK-induced skeletal muscle wasting in mice. *PLoS One* **5**, e8760 (2010).
121. Wada, S. *et al.* Translational suppression of atrophic regulators by microRNA-23a integrates resistance to skeletal muscle atrophy. *J Biol Chem* **286**, 38456-38465 (2011).
122. Zhou, X. *et al.* Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. *Cell* **142**, 531-543 (2010).
123. Di Sebastiano, K.M. & Mourtzakis, M. A critical evaluation of body composition modalities used to assess adipose and skeletal muscle tissue in cancer. *Appl Physiol Nutr Metab* **37**, 811-821 (2012).

Summary

Summary

Cancer cachexia is a paraneoplastic feature that frequently occurs in patients with non-small cell lung cancer (NSCLC). Progressive body weight loss and disproportionate wasting of skeletal muscle are the most distinct characteristics of the syndrome. The presence of cancer cachexia is associated with major negative consequences, i.e. low tolerance and responsiveness to anti-tumor therapy, decreased muscle performance, declined quality of life and a significant increase in cancer-related mortality. Currently, no effective therapeutic intervention can prevent or reverse these negative consequences of cancer cachexia. Although studies in experimental cancer cachexia have increased our understanding on putative molecular mechanisms that lie at the basis of cancer cachexia, most of these remain to be validated in the different stages of cancer cachexia in the clinical setting. The current dissertation provides a comprehensive characterization of phenotypic aspects and molecular signatures of muscle atrophy, involved in progressive stages of cachexia in patients with non-small cell lung cancer (NSCLC).

Radiation-esophagitis is not the primary trigger for early weight loss in patients treated with concurrent chemoradiotherapy for non-small cell lung cancer

Concurrent administration of chemotherapy and radiotherapy (CT-RT) is the treatment of choice for many patients with locally advanced non-small cell lung cancer (NSCLC). It has been demonstrated that this intensive multimodal treatment regimen results in significantly longer disease free and overall survival. Concurrent administration of CT-RT is associated with a high incidence of severe esophagitis. Intuitively, the frequently observed body weight loss during concurrent CT-RT may be a result of impaired dietary intake due to esophagitis-related dysphagia. However, clinical observations indicated that body weight loss occurs prior to treatment-induced esophagitis and might therefore not solely depend on a dysphagia-related decline in nutritional intake. In a retrospective and prospective study, it was investigated whether body weight loss during concurrent CT-RT was associated with significant esophagitis, i.e. grade ≥2 esophagitis. In **chapter 2**, it was revealed that

loss of body weight is a frequent consequence of concurrent CT-RT, starts early following initiation of treatment and is independent of treatment-induced esophagitis in the first weeks of CT-RT. In addition, total caloric intake was not decreased, while muscle function rapidly declined, which may implicate active catabolism. These findings indicate that other treatment-dependent metabolic alterations may contribute to 'early' weight loss and advocate for more supportive and early initiated nutritional intervention, possibly in a multimodal approach to optimize concurrent CT-RT management.

Muscle atrophy in cachexia: can dietary protein tip the balance?

As the identification of therapeutic strategies to reverse or postpone cancer cachexia remains challenging in clinical care, the efficacy of commonly used interventions needs evaluation. In **chapter 3** the literature on (clinical) data regarding the efficacy of dietary protein supplementation in compensation of muscle atrophy during cachexia was reviewed. Based on the currently available data, it can be hypothesized that supplementation of dietary protein (>1.5 g/kg per day) alone or in combination with other anabolic stimuli such as exercise training maintains or even improves muscle mass. Furthermore, the putative molecular mechanisms by which proteins or specific amino acids may attenuate muscle mass loss were evaluated. The literature study presented in **chapter 3** showed that in-vitro studies provide evidence for pro-anabolic and anti-catabolic effects of amino acids but that limited data are available in experimental animal models and in patients suffering from cachexia. In addition, results on muscle function are controversial and no clinical studies have yet directly linked alterations in cellular signaling or metabolic signatures of protein intake-induced muscle anabolism to muscle weight gain. Further randomized clinical trials are needed in adequately phenotyped patients using sensitive measures of muscle mass and function to provide more insight in the efficacy of protein supplementation in (cancer) cachexia.

Skeletal muscle alterations during the progression of lung cancer cachexia

From **chapter 4** on, alterations in skeletal muscle protein and myonuclear turn-over as well as measures of (oxidative) energy metabolism were investigated in muscle biopsies of patients with progressive stages of NSCLC cachexia. The patients in **chapter 4 and 6** were stratified according to the recent international cancer cachexia consensus. Consequently, this dissertation contributes to the validation of this definition as an instrument for standardizing cancer cachexia classification and management.

Nuclear transcription factor kB activation and protein turn-over adaptations in skeletal muscle of patients with progressive stage of lung cancer cachexia

In **chapter 4**, it was verified whether mechanisms in control of muscle atrophy in experimental models of cancer cachexia could be appreciated in patients with NSCLC pre-cachexia and cachexia. The study confirmed that muscle atrophy in cachectic patients with lung cancer is accompanied by increased systemic and local muscle inflammation, whereas pre-cachectic patients show intermediate expression. Moreover, plasma transfer experiments revealed that factors contained within the circulation of both pre-cachectic and cachectic patients with lung cancer induce inflammatory signaling in skeletal muscle. Furthermore, it was shown that the balance between muscle protein synthesis and degradation was disturbed in favor of increased proteolysis by autophagy signaling, without alterations in indices of the anticipated ubiquitin proteasome system (UPS). Protein synthesis signaling was characterized by increased Akt phosphorylation without alterations in downstream Akt phosphosubstrates in cachectic patients. This finding implies impaired anabolic signaling that could, in combination with increased proteolysis, contribute to the net loss of muscle protein in cancer cachexia. These findings of increased proteolytic signaling and impaired anabolic signaling at the level of Akt provide further support for a more targeted nutritional modulation beyond merely macronutrient supplementation.

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

Physical performance is frequently affected in patients with cancer, even in early stages of body weight loss. In **chapter 5,** exercise endurance was assessed as determinant of muscle function in comprehensively phenotyped pre-cachectic patients with NSCLC. The exploratory study showed 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 Ubiquitin Proteasome System (UPS) proteolytic activity appears present in skeletal muscle. As patients in different stages of cachexia could benefit from unique intervention strategies, underlying mechanisms of the decreased exercise capacity was studied in **chapter 6**.

Preserved muscle oxidative metabolic phenotype in clinical cancer cachexia

Experimental evidence suggests altered metabolic signaling in cancer cachexia, which could explain the decreased exercise intolerance in pre-cachectic patients observed in **chapter 5**. Therefore, the study described in **chapter 6**, addressed whether alterations in muscle oxidative metabolism are present in patients with NSCLC pre-cachexia and cachexia. The study demonstrated oxidative metabolism appears mainly preserved in patients with lung cancer cachexia, despite significant increases in systemic pro-inflammatory mediators as putative triggers. This is illustrated by the absence of alterations in fiber type distribution, mRNA transcript levels of regulators of oxidative signaling, protein expression of mitochondrial complexes and oxidative enzyme activity, despite evident systemic inflammation. Accordingly, it is concluded that oxidative phenotype is not affected in patients with lung cancer cachexia.

Conclusion

In the current dissertation it was revealed that systemic and local (muscle) inflammation can be appreciated in NSCLC cachexia and that factors present in the circulation are capable of inducing the transition to local inflammatory signaling. With respect to muscle protein turnover, NSCLC cachexia proteolytic signaling was characterized by increased autophagy-related signaling, while no indications for sustained elevations in activity of the ubiquitin proteasome system (UPS) were found. The data on protein synthesis signaling showed increased Akt phosphorylation without activation of downstream targets such as mTOR or FOXO and points at a futile compensatory response to a catabolic milieu. Combined, the increased proteolytic signaling and impairment of anabolic signaling at the level of Akt can be the cause for the net loss of muscle mass in NSCLC cachexia.

In addition, exercise endurance was significantly declined in patients with NSCLC pre-cachexia. The decrease in muscle endurance does not correlate with adaptations of muscle oxidative or glycolytic metabolism, as none of these measures was altered in patients with pre-cachexia or cachexia. Furthermore, the current dissertation revealed that pre-cachexia can be considered an early stage of NSCLC cachexia with respect to phenotype, i.e. lean mass atrophy and muscle function and molecular signatures, i.e. systemic and local inflammatory signaling, and muscle protein turnover.

Samenvatting

Samenvatting

Kankercachexie is een paraneoplastisch fenomeen dat vaak optreedt bij niet-kleincellige longkanker. Het wordt gekenmerkt door progressief gewichtsverlies en buitenproportioneel spiermassaverlies. De aanwezigheid van kankercachexie heeft grote negatieve gevolgen voor patiënten met kanker. Zo is kankercachexie geassocieerd met een verhoogde toxiciteit van en een verlaagde respons op oncologische behandelingen, een verlaagde spierfunctie, een verminderde kwaliteit van leven en een verhoogde kanker-gerelateerde mortaliteit. Op dit moment zijn er geen effectieve behandelstrategieën die deze negatieve consequenties van kankercachexie kunnen voorkomen of reduceren. Hoewel bevindingen uit experimentele modellen het inzicht in de pathofysiologie van kankercachexie hebben vergroot, is het van essentieel belang om deze bevindingen te valideren in patiënten met verschillende stadia van kankercachexie. In dit proefschrift wordt een uitvoerig overzicht van het fenotype en de moleculaire karakteristieken van de verschillende stadia van kankercachexie beschreven in patiënten met niet-kleincellige longkanker.

Oesofagitis is niet de primaire oorzaak voor vroeg gewichtsverlies bij patiënten die behandeld worden met gelijktijdige chemoradiotherapie voor niet-kleincellige longkanker

Gelijktijdige behandeling met chemotherapie en radiotherapie is de voorkeursbehandeling voor patiënten met stadium III niet-kleincellige longkanker. Deze behandeling gaat gepaard met een hoge incidentie van ernstige ontsteking van de slokdarm (oesofagitis). Er wordt vaak gedacht dat het gewichtsverlies dat optreedt tijdens gelijktijdige chemoradiotherapie een gevolg is van verminderde voedingsinname door oesofagitis. Echter, in de klinische praktijk werd opgemerkt dat het gewichtsverlies tijdens gelijktijdige chemoradiotherapie al lijkt op te treden voordat er sprake is van oesofagitis. Dit impliceert dat ook andere oorzaken bijdragen aan vroeg gewichtsverlies tijdens gelijktijdige chemoradiotherapie. In **hoofdstuk 2**, werd data over gewichtsverlies en oesofagitis verzameld in een retrospectieve en in een prospectieve studie. Er werd gevonden dat gewichtsverlies frequent optreedt tijdens gelijktijdige chemoradiotherapie bij patiënten met niet-

kleincellige longkanker, vroeg begint na start van chemoradiotherapie en onafhankelijk is van (chemo)radiotherapie-oesofagitis in de eerste weken van concurrent chemoradiatie. Daarnaast werd gevonden dat de totale calorische voedingsinname (bestaande uit spontane inname en bijvoeding) niet verminderd was gedurende gelijktijdige chemoradiotherapie, terwijl de spierkracht wel snel afnam na start van de therapie. Dit kan mogelijk het gevolg zijn van actieve afbraak van spierweefsel. De bevindingen uit deze studie wijzen erop dat (chemo)radiotherapie-oesofagitis niet verantwoordelijk is voor 'vroeg' gewichtsverlies tijdens gelijktijdige chemoradiotherapie en impliceren dat een vroeg startende en intensieve voedingsinterventie, mogelijk in een multimodaal behandelplan, gerechtvaardigd zou zijn voor optimale ondersteuning tijdens gelijktijdige chemoradiotherapie.

Spiermassaverlies in cachexie: kan eiwitrijke voeding de balans herstellen?

Aangezien het een uitdaging is om kankercachexie te voorkomen of reduceren, is het belangrijk om de effectiviteit van interventies die op dit moment beschikbaar zijn te evalueren. In **hoofdstuk 3** wordt een literatuurstudie beschreven die de effectiviteit van eiwitrijke voeding op het reduceren van spieratrofie tijdens cachexie evalueert. Op basis van de beschikbare literatuur kan geconcludeerd worden dat eiwitrijke voeding (>1.5 g/kg per dag), eventueel in combinatie met anabole strategieën zoals fysieke training, spiermassa zou kunnen behouden of zelfs doen toenemen tijdens cachexie. Daarnaast werd geëvalueerd welk mechanisme mogelijk verantwoordelijk zou zijn voor een positief effect van eiwit of specifieke aminozuren op spiermassa. De literatuurstudie liet zien dat *in vitro* studies bewijs hebben gevonden voor een toename van spiereiwitaanmaak en afname van spiereiwitafbraak door (vertakte-keten) aminozuren in spiercellen. Echter, er zijn weinig gerandomiseerde studies zijn die het effect van een eiwitrijke voeding bij patiënten met cachexie hebben onderzocht.
De studies die het effect van eiwitrijke voeding op spierfunctie onderzochten lieten verschillende resultaten zien en er zijn tot nu toe geen studies die onderliggende moleculaire veranderingen in relatie tot spierfunctie hebben onderzocht. Om een definitieve conclusie te trekken over het effect van eiwitrijke voeding op spiermassa

en -functie tijdens (kanker) cachexie zijn aanvullende studies nodig in adequaat gekarakteriseerde patiëntengroepen.

Veranderingen in de spier tijdens de progressie van kankercachexie

Vanaf **hoofdstuk 4** is een overzicht gegeven van (moleculaire) veranderingen met betrekking tot de aanmaak, afbraak en stofwisseling van de spier bij patiënten met verschillende stadia van kankercachexie bij niet-kleincellige longkanker. De patiënten in **hoofdstuk 4 en 6** werden gestratificeerd volgens de recente, internationale kankercachexie consensus. Hierdoor draagt dit proefschrift bij als een instrument voor standaardisering van deze kankercachexie classificatie.

Nuclear transcription factor κB activatie en veranderingen in eiwitmetabolisme in skeletspieren van patiënten met verschillende stadia van cachexie bij niet-kleincellige longkanker

In **hoofdstuk 4** werd onderzocht of moleculaire veranderingen in eiwitstofwisseling die gevonden werden in experimentele modellen van kankercachexie ook geobjectiveerd konden worden in patiënten met (pre-) cachexie bij niet-kleincellige longkanker. De studie in **hoofdstuk 4** toonde dat spieratrofie in cachectische patiënten met longkanker geassocieerd is met een verhoogde systemische en lokale ontstekingsrespons, terwijl in patiënten met pre-cachexie intermediaire expressie van ontstekingsmarkers werd gevonden. Ook werd gevonden dat factoren aanwezig in het plasma van patiënten met zowel longkanker pre-cachexie als cachexie op spierniveau inflammatoire signalering kunnen stimuleren. Daarnaast werd gevonden dat de balans tussen spiereiwitaanmaak en –afbraak verstoord is, waarbij eiwitafbraak vergroot was als gevolg van verhoogde autofagie signalering, zonder veranderingen in het geanticipeerde proteolytische ubiquitine proteasoom systeem. Veranderingen in eiwitaanmaak werden gekarakteriseerd door een verhoogde fosforylering van het belangrijke anabole signaleringsmolecuul Akt. Er waren echter geen aanwijzingen voor een verhoogde fosforylering van fosfo-substraten van Akt, hetgeen wijst op een verstoorde respons in de spiereiwitaanmaak. Deze verstoorde respons in de spiereiwitaanmaak zou, in combinatie met de verhoogde

proteolytische activiteit, verantwoordelijk kunnen zijn voor een netto verlies aan spiermassa.

Pre-cachexie bij patiënten met stadium I–III niet-kleincellige longkanker: Systemische inflammatie en functieverlies zonder tekenen van activatie van het ubiquitine proteasoom systeem in de spier

De fysieke conditie van patiënten met kanker is vaak verminderd. In **hoofdstuk 5** werd middels een fietsergometrie test de inspanningscapaciteit bij patiënten met pre-cachexie bij niet-kleincellige longkanker onderzocht. Deze studie toonde dat de inspanningscapaciteit significant verlaagd is bij patiënten met pre-cachexie bij niet-kleincellige longkanker, zonder dat er veranderingen waren in lichaamssamenstelling. Daarnaast werd een verhoogde systemische inflammatoire respons gevonden zonder tekenen van activatie van het ubiquitine proteasoom systeem in de spier. In **hoofdstuk 6** werd onderzocht of een verandering in de energie stofwisseling ten grondslag ligt aan de verminderde inspanningstolerantie in patiënten met pre-cachexie.

Het oxidatieve metabole fenotype is niet veranderd in de skeletspier van patiënten met kanker(pre-)cachexie bij niet-kleincellige longkanker

In **hoofdstuk 6** werd onderzocht of de afname van oxidatief energie metabolisme dat werd gevonden in experimentele modellen van kankercachexie ook aanwezig is in patiënten met kanker(pre-)cachexie, en mogelijk verantwoordelijk is voor de verminderde inspanningscapaciteit die gevonden werd bij patiënten met pre-cachexie in **hoofdstuk 5**. In tegenstelling tot de bevindingen in experimentele studies werd in **hoofdstuk 6** gevonden dat er geen veranderingen zijn in het oxidatieve fenotype in patiënten met (pre-)cachexie bij niet-kleincellige longkanker, ondanks een duidelijk verhoogde inflammatoire respons. Zo werden er geen verschillen gevonden in zuurstofafhankelijke en niet-zuurstofafhankelijke vezelverdeling, de hoeveelheid mRNA transcripten van regulatoren van oxidatieve signalering, eiwitexpressie van mitochondriële complexen en activiteit van oxidatieve enzymen.

Conclusie

In het huidige proefschrift zijn karakteristieken en moleculaire mechanismen van (pre-) cachexie in patiënten met niet-kleincellig longkanker onderzocht. Er werd aangetoond dat systemische en lokale inflammatie verhoogd is in patiënten met cachexie als gevolg van niet-kleincellige longkanker en dat factoren aanwezig in het plasma van patiënten met (pre-) cachexie lokale inflammatoire signalering in de spier kan induceren.
Met betrekking tot eiwitmetabolisme in de spier zijn er aanwijzingen gevonden voor verhoogde eiwitafbraak via autofagie signalering, zonder bewijs voor activatie van het ubiquitine proteasoom systeem. De data met betrekking tot eiwitaanmaak toonde een verhoogde fosforylering van Akt zonder activatie van de geassocieerde fosfo-substraten, hetgeen zou kunnen wijzen op een verstoorde respons in de eiwitaanmaak. De verhoogde eiwitafbraak en verstoring van eiwitaanmaak kunnen verantwoordelijk zijn voor het netto verlies van spierweefsel in patiënten met niet-kleincellige longkanker.
De spierfunctie, bestaande uit spierkracht en inspanningscapaciteit, was verlaagd in patiënten met (pre-) cachexie. Het verlies van inspanningscapaciteit lijkt niet te berusten op een verandering in oxidatief metabolisme van de spier bij patiënten met (pre-)cachexie als gevolg van niet-kleincellige longkanker.

Het huidige proefschrift toont verder dat pre-cachexie beschouwd kan worden als een vroeg stadium van cachexie met betrekking tot fenotype (spieratrofie en spierfunctie) en moleculaire veranderingen die betrekking hebben op systemische en lokale inflammatie alsook eiwitmetabolisme.

Dankwoord

Dankwoord

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Publications

Publications

1. Op den Kamp, C.M., Langen, R.C., Haegens, A. & Schols, A.M. Muscle atrophy in cachexia: can dietary protein tip the balance? *Curr Opin Clin Nutr Metab Care* **12**, 611-616 (2009).

2. Verhees, K.J., Schols, A.M., Kelders, M.C., Op den Kamp, C.M., van der Velden, J.L. & Langen, R.C. Glycogen synthase kinase-3beta is required for the induction of skeletal muscle atrophy. *Am J Physiol Cell Physiol* **301**, C995-C1007 (2011).

3. Op den Kamp, C.M., Langen, R.C., Minnaard, R., Kelders, M.C., Snepvangers, F.J., Hesselink, M.K., Dingemans, A.C. & Schols, A.M. 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. *Lung Cancer* **76**, 112-117 (2012).

4. Op den Kamp, C.M., Langen, R.C., Snepvangers, F.J., de Theije, C.C., Schellekens, J.M., Laugs, F., Dingemans, A.M. & Schols, A.M. Nuclear transcription factor kappa B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. *Am J Clin Nutr* **98**, 738-748 (2013).

5. Op den Kamp, C.M., De Ruysscher, D.K., van den Heuvel, M., Elferink, M., Houben, R.M., Oberije, C.J., Bootsma, G.P., Geraedts, W.H., Pitz, C.C., Langen, R.C., Wouters, E.F., Schols, A.M. & Dingemans, A.M. Early body weight loss during concurrent chemo-radiotherapy for non-small cell lung cancer. *J Cachexia Sarcopenia Muscle* (2014).

6. Op den Kamp, C.M., Gosker, H.R., Lagarde, S., Tan, D.Y., Snepvangers, F.J., Dingemans, A.C., Langen, R.C. & Schols, A.M. Preserved muscle oxidative metabolic phenotype in clinical cancer cachexia (*Submitted)*

Curriculum vitae

Curriculum vitae

Céline Op den Kamp was born February 26th 1983 in Maasbree, the Netherlands. In 2001 she graduated at the Gymnasium of the Valuascollege (Venlo, the Netherlands). Following the graduation, she started her Medical Studies at the University of Maastricht. During her Medical Studies, she worked as a student-assistant at the department of Anatomy and Embryology (Maastricht University) and participated in courses at the Faculty of Law. She expanded her horizon by following some of her internships abroad (Nepal, Suriname and Australia). In her fourth year she started participating in oncology research at the department of Respiratory Medicine at Maastricht University Medical Centre+. She supported studies concerning angiogenesis in non-small cell lung cancer (NSCLC). In the last year of her medical studies, she joined the Laboratory of Respiratory Medicine for an internship. The title of her thesis was: “The role of the ubiquitin proteasome pathway (UPP) in muscle atrophy”. After obtaining her Medical Degree *cum laude*, she continued working as a PhD student at the department of Respiratory Medicine. Her PhD project focused at elucidating underlying mechanisms of cancer cachexia in patients with non-small cell lung cancer. After finishing her research years, she worked at the department of Internal Medicine at the Maastricht University Medical Centre+ for one year. Currently, she is working as general practitioner in training at Maastricht University.