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Disclosing the molecular mechanisms underlying cancer-induced WAT browning

Tese de Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, Ramo Molecular, realizada sob a orientação científica da Professora Doutora Rita Ferreira do Departamento de Química da Universidade de Aveiro e do Professor Doutor Lúcio Lara Santos do Instituto Português de Oncologia do Porto

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Palavras-chave Caquexia; cancro da cabeça e pescoço; catabolismo; lipólise; remodelação do tecido adiposo

Resumo

O cancro da cabeça e pescoço está associado a uma elevada mortalidade e morbilidade, e acredita-se que 50% dos pacientes com este tipo de cancro experienciem uma perda de peso significativa numa fase avançada de doença, uma condição designada de caquexia. A patofisiologia da caquexia associada ao cancro é complexa e envolve mediadores produzidos pelo tumor ou pelo hospedeiro em resposta ao tumor, que induzem alterações sistémicas que culminam na perda de peso e consequente perda da qualidade de vida. Com o objetivo de melhor compreender os mecanismos moleculares subjacentes à caquexia associada ao cancro da cabeça e pescoço, no presente trabalho colectamos dados clínicos e analisamos amostras de soro de 17 pacientes com carcinoma espinocelular na orofaringe ou hipofaringe, com e sem caquexia (determinada com base na perda de peso corporal superior a 5% nos 6 meses anteriores).

Os resultados obtidos permitiram verificar que o índice de massa corporal bem como o estadio do tumor (T3 e T4 em ambos os grupos) não se relacionam com a presença de caquexia, apesar de os índices nutricionais MUST e PG-SGA serem mais elevados nos doentes com perda de peso corporal superior a 5% em 6 meses. Mais ainda, não se observaram diferenças significativas de marcadores bioquímicos indicativos de alterações metabólicas associadas à remodelação do tecido adiposo. Os níveis séricos da citocina pró-inflamatória TWEAK e da proteína de fase aguda CRP não foram significativamente diferentes entre os 2 grupos de pacientes, bem como os níveis das adipocinas, leptina e adiponectina e da hormona gástrica grelina. No entanto, observaram-se níveis séricos da citocina catabólica miostatina significativamente mais elevados nos pacientes com caquexia, o que sugere que o catabolismo muscular contribui para o desenvolvimento desta síndrome paraneoplásico.

Em resumo, os resultados obtidos no presente estudo não suportam a contribuição da remodelação do tecido adiposo para o desenvolvimento de caquexia mas evidenciam a importância do catabolismo muscular para a perda de peso corporal nos doentes com cancro da cabeça e pescoço. Estudos futuros envolvendo mais grupos de doentes e mais doentes por grupo serão importantes para melhor compreender a contribuição da remodelação do tecido adiposo na patogénese da caquexia associada ao cancro da cabeça e pescoço.

Keywords Cachexia; head and neck cancer; wasting; lipolysis; WAT remodelling

A**bstract**

Head and neck cancer (HNC) is a significant cause of cancer morbidity and mortality worldwide and it is believed that 50% of HNC patients experience significant weight loss at an advanced stage of the disease, a condition known as cachexia. The pathophysiology of cancer cachexia is complex and involves several mediators produced by the tumour or the host that induce body weight loss and, consequently, impairs quality of life. In this thesis project we aimed to better comprehend the molecular mechanisms underlying cachexia in HNC by evaluating clinical and biochemical parameters from 17 patients with squamous cell carcinoma at oropharynge and hypopharing, with and without cachexia (assessed by body weight loss higher than 5% in 6 months).

Our results showed no association between body mass index and tumour staging with the establishment of cachexia in head and neck cancer, despite the higher scores of MUST and PG-SGA observed in patients with body weight loss higher than 5% in 6 months. No apparent metabolic changes associated with adipose tissue remodelling were detected among patients' groups. The levels of TWEAK and CRP were not significantly different among the 2 groups of patients, not supporting the contribution of inflammation to the development of cachexia. The serum levels of the adipokines leptin and adiponectin, and of the gastric hormone ghrelin do not evidence the contribution of WAT remodelling to the head and neck cancer-related body weight loss. However, the significantly higher serum levels of myostatin in the group of patients with body weight loss higher than 5% in 6 months highlights the contribution of muscle wasting to the cachexia phenotype in head and neck cancer.

Taken together, our results evidence the contribution of muscle wasting but not of WAT remodelling to the development of cachexia in the set of head and neck cancer. Future studies involving a higher number of patients and more groups of patients will be important to better follow WAT remodelling and its interplay with muscle wasting in the pathogenesis of head and neck cancer.

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List of Abbreviations

ActRIIB	Activin type IIB receptor (ActRIIB)
ALS	Autophagy-lysosomal system
ANO	Anorexia
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BWC	Body weight and composition
cAMP	Cyclic adenosine monophosphate
CASCO	Cachexia score
CRP	C-reactive protein
E1/2/3	Ubiquitin-activating enzyme
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
Fas	Free fatty acids
FGF21	Fibroblast growth factor-21
GM-CSF	Granulocyte macrophage-colony-stimulating factor
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSL	Hormone-sensitive lipase
IFN-γ	Interferon gamma
IkB	Inhibitory protein kBα
IL	Interleukin
IMD	Inflammation /metabolic disturbances/immunosuppression
IRS	Insulin Receptor Substrate Pathways
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
LMF	Lymphocyte mitogenic factor
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinases
mRNA	Messenger RNA

MMPs	Metalloproteases
MuRF1	Muscle RING-finger protein-1
MUST	Malnutrition Universal Screening Tool
NF-kB	Nuclear factor kappa B
NPYY1	Hypothalamic neuropeptide Y-Y1
OXPHOS	Oxidative phosphorylation
PGC-1α	Peroxisome proliferator–activated receptor gamma coactivator-1α
PGE2	Prostaglandin E_2
PG-SGA	Patient generated subjective global assessment
PHP	Physical performance
PIF	Proteolysis-inducing factor
PI3K/Akt	Phosphatidylinositol 3-kinase/proteinkinase B
PKR	RNA-dependent protein kinase
PPARγ	Peroxisome proliferator–activated receptor
PRDM16	pR domain containing 16
PTHrP	Parathyroid Hormone-Related Peptide
QOL	Quality of life
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SAA	Serum amyloid A
SNS	Sympathetic nervous system
STAT3	Signal transducer and activator of transcription 3
TNF	Tumour necrosis factor
T3	Triiodothyronine
T4	Thyroxine
TLR4	Toll-like receptor 4
TWEAK	TNF-like weak inducer of apoptosis
UCP	Uncoupling protein
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
ZAG	Zn-α2-glycoprotein

I. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a significant cause of morbidity and mortality by cancer worldwide with rates of incidence varying around the world and with a global incidence of 500,000 cases *per year* with the highest rates being found in Southeast Asia and Eastern Europe (Brennan et al., 1995; Hardisson, 2003; Kreimer et al., 2005). In Portugal, HNSCC is one of the most common cancers worldwide, constituting the fifth cause of death with cancer among men (Silveira et al., 2010). In the western world a decline of its incidence has been observed and attributed to the population's awareness of the risk factors, such as smoking and alcohol abuse. On the other hand an increase in oral tongue and oropharyngeal cancer has been noticed, which may be connected with an increase of human papilloma virus (HPV) related infections (Leemans et al., 2011).

HNSCC is a complex disease, characterized by clinical, pathological, phenotypical and biological heterogeneity with origins in genetic and epigenetic alterations that lead to hyperplasia of squamous epithelium, dysplasia, carcinoma *in situ* and, eventually, cancer (Haddad et al., 2008; Leemans et al., 2011). The current view of HNSCC onset relies in three main genetic alterations, that comprise the inactivation of the p53 tumour suppressor gene, the inactivation of the cyclin dependent kinase inhibitor 16 and the overexpression of epidermal growth factor receptor (EGFR) (Douglas et al., 2004).

In spite of the advances made in the clinical management of HNSCC, the survival rate after diagnosis is still low, being lower than in other types of cancer , and the major cause of death being cervical node and distant metastasis (John et al., 2009; Molinolo et al., 2009). The treatment and prognosis of HNSCC varies with the stage of the disease, anatomic site and, in the case of prognosis, with patient's response to the treatment. The therapeutic approaches usually involve surgery, radiotherapy and may also involve chemotherapy; however, the main treatment is surgical resection. The presence of positive pathologic lymph nodes strongly influences the prognosis (Brennan et al., 1995; Leemans et al., 2011; Rhys-Evans et al., 2001). Some molecular markers,

such as HPV infection and tumour markers, as well as genetic polymorphisms might also be used to predict the outcome of the disease (Brennan et al., 1995; Hopkins et al., 2008). The presence of body wasting also influences disease prognosis (Cabal-Manzano et al., 2001; Ebadi et al., 2014; Tisdale, 2002).

Cells of HNSCC develop molecular strategies that allow them to evade growh inhibitory effects of cytokines that are present in the microenvironment of the tumour, therefore, in this disease the ability of a tumour to become malign is associated with an altered response to cytokine stimulation (Pries et al., 2006). Recent studies report an increase in the production of inflammatory mediators, such as cytokines, induced by the tumour that can lead to increased tumour promotion and invasion, angiogenesis and metastasis (John et al., 2009 1). Among the HNSCC-derived pro-inflammatory mediators are interleukin IL-1, IL-4, IL-6, IL-8, granulocyte macrophage-colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), prostaglandin E_2 (PGE_2) and fibroblast growth factors (FGF) (Pries et al., 2006). IL-1 has been shown to play an important role by inducing the activation of signal transduction pathways, involved in the transcription of proinflammatory cytokines genes (John et al., 2009). Another major player in HNSCC cases is epidermal growth factor receptor (EGFR), since it has been demonstrated elevated levels of EGFR in this disease and elevated activity of polypeptide growth factor tyrosine kinase receptor. Consequently, many downstream intracellular targets of EGFR are activated, which stimulate tumour proliferation, apoptosis, angiogenesis and cell migration/invasion (John et al., 2009; Schmitz, 2010 ; Squarize et al., 2006).

Head and neck cancer patients experience a significant weight loss related with the disease and the treatment itself, that has been attributed to a multifactorial metabolic syndrome, best known as cachexia (Gorenc et al., 2015; Mason et al., 2016; Wang et al., 2016). Indeed it is believed that more than 50% of HNSCC patients at an advanced stage of the disease experience weight loss and possibly cachexia (Couch et al., 2007; Couch et al., 2015). The presence of cachexia greatly contributes to poor prognosis with a negative impact in the quality of life of cancer patients. Apart from this it is also associated with physical, psychological and social problems (Couch et al., 2015).To the best of our knowledge, cachexia is most of the times underestimated in the clinical

management of HNSCC. Nevertheless, cancer patients would certainly benefit from multimodal therapies also targeting cachexia.

1. Cancer associated cachexia: definition and staging

The term cachexia is derived from the Greek “kakos hexis”, which means bad condition and is, by definition, a metabolic syndrome associated with illness and characterized by loss of muscle and adipose tissue (Das et al., 2011; Tisdale, 2005; Evans, 2008). This paraneoplastic syndrome accounts for 20% of cancer deaths, with up to one third of the cachectic patients losing more than 5% of their original body weight (Mendes et al., 2015; Tisdale, 2002; Agustsson, 2012). Cachexia is usually associated with particular types of cancer, predominantly those of pancreas, gastrointestinal tract, non-Hodgkin´s lymphoma, prostate, lungs and head and neck. Patients with these tumours experience the greatest degree of weight loss (Mendes et al., 2015; Tisdale, 2002; Tisdale, 2005). This condition can arise in a patient with a tumour comprising less than 0.01% of the host weight, although some large tumours do not produce cachexia (Tisdale, 2003). Apart from the loss of skeletal muscle and adipose tissue, cachexia also includes symptoms such as anorexia, hypoglycaemia, anaemia and asthenia (Mondello et al., 2015). Symptoms like asthenia (or lack of muscular strength), reflect the muscle wasting that takes place in cachectic cancer patients (Argilés et al., 2003; Strassmann et al., 1992; Theologides, 1979). Recent findings suggest the involvement of other organs such as brain, liver, gut and heart (figure 1).

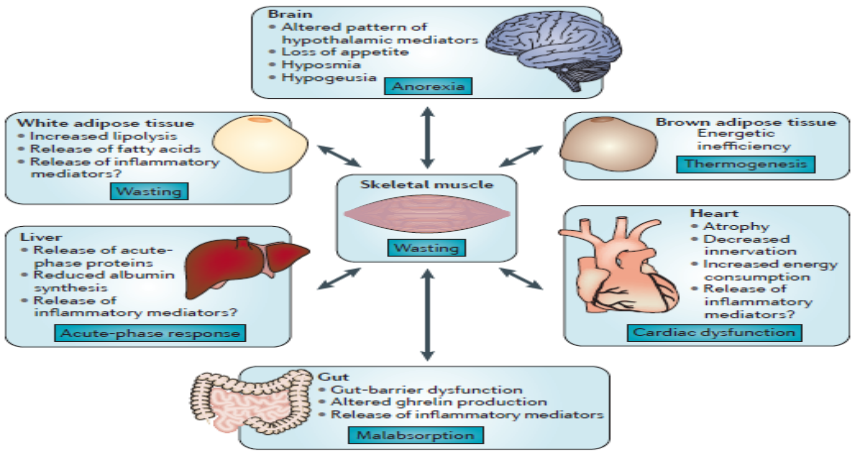

Figure 1. Cachexia as a multi-organ syndrome (Prieto-Hontoria et al., 2011).

Since cancer cachexia has become clinically relevant some authors proposed the staging of this syndrome in three clinically relevant stages: pre-cachexia, cachexia and refractory cachexia, even though not all patients experience all three stages of the disease. Pre-cachexia is characterized by early clinical and metabolic signs, such as anorexia, inflammation and metabolic alterations. It precedes a weight loss of approximately 5% or less (Argilés et al., 2011; Fearon, 2011). It includes patients with anorexia and chronic systemic inflammation. The presence of inflammation is indicated by elevated serum levels of C-reactive protein (CRP) (Muscaritoli et al., 2010). The risk of progression varies and depends on the cancer type and stage, the presence of systemic inflammation, low food intake and lack of response to anticancer therapy. A patient is characterized as having cachexia if presents a body weight loss greater than 5% over 6 months or has on-going weight loss of more than 2%. In the last stage, the cachexia can be clinically refractory as a result of very advanced cachexia or of rapidly progressive cancer, which is unresponsive to therapy (Fearon et al., 2011).

The reduced food intake experienced by cachectic patients has anorexia as a major factor and, in most cases, catabolic factors induced by an abnormal host response to tumour and/or tumour factors (Bosaeus, 2008). Apart from anorexia, the loss of lean tissue, also characteristic of cachexia might be difficult to detect because of the accumulation of water, which might disguise the early changes. This water retention may occur as a consequence of hypoalbuminemia and may account for an increase in body weight (Tisdale, 2002; Muscaritoli, 2010). The imbalance between anabolism and catabolism within skeletal muscle seems to be responsible for the accelerated muscle loss, leading to muscle weakness, fatigue, immobility and, ultimately, death due to loss of respiratory muscle function. Death normally occurs when weight loss is about 30% (Tisdale, 2002; Muscaritoli, 2010).

A methodology was recently proposed for the quantitative assessment of cancer cachexia and involves the use of a score known as cachexia score (CASCO). This score is calculated taking in consideration the level of weight loss and composition, inflammation, metabolic disturbances and immunosuppression, physical performance and quality of life (figure 2). Several biochemical parameters are considered for the assessment of inflammation and metabolic disturbance, such as plasma CRP, IL-6, lactate and triglycerides, whereas handgrip strength and validated questionnaires are used for the assessment of physical performance and quality of life. This staging of

cachexia envisions to improve its clinical management through targeted therapies (Blum et al., 2014; Tisdale, 2005; Argilés, 2011).

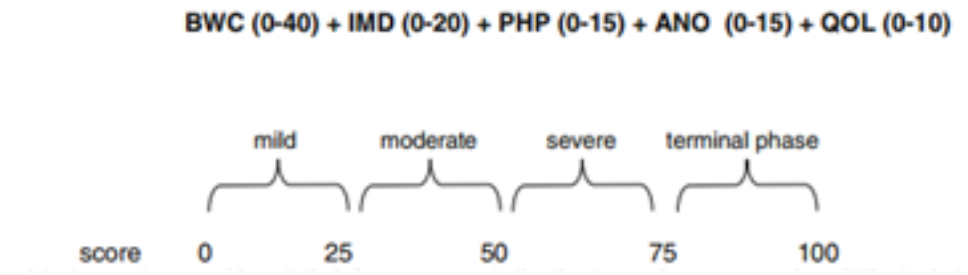

Figure 2. CASCO staging scale (Argilés et al., 2011).

The components of this score are body weight and composition (BWC) accounts for 40% of the score; Inflammation /metabolic disturbances/immunosuppression (IMD) accounts for 20% of the score; Physical performance (PHP) accounts for 15% of the score; Anorexia (ANO) accounts for 15% of the score; Quality of life (QOL) accounts for 10% of the score. The value calculated in the score allows the staging of the patient in mild, moderate, severe and terminal phase.

2. Tumour-host interplay in cancer cachexia

The pathophysiology of cancer cachexia is complex and seems to involve several chemical mediators. These mediators can be divided into two groups, produced by the host or/and by tumour cells, which includes cytokines such as tumour necrosis factor (TNF)-α, IL-1 and IL-6 (figure 3). The first group mostly leads to appetite suppression, although TNF-α has also been associated with increased lipolysis, by supressing the cleavage of lipoprotein lipase (LPL), and the induction of proteolysis, through the ubiquitin-proteasome proteolytic pathway (Tisdale, 2002; Ebadi, 2014). The other group includes catabolic products, secreted by tumour cells, such as lymphocyte mitogenic factor (LMF), known for acting on the adipose tissue through cyclic adenosine monophosphate (cAMP) signalling pathway and proteolysis-inducing factor (PIF) that induces proteolysis in skeletal muscle by up-regulating the ubiquitin-proteasome pathway (Hirai et al., 1998; Skipworth et al., 2007; Tisdale., 2002; Mendes., 2015). Mechanistically, PIF not only promotes protein degradation by increasing the levels of ubiquitin-carrier protein and proteasome subunits, but also inhibits protein

synthesis through, for example, the activation of the RNA-dependent protein kinase (PKR) (Eley et al., 2008; George et al., 2007; Mendes et al., 2015; Argilés, 2005). PIF was recently linked to the hepatic cytokine production and was shown to activate the nuclear factor-κB (NF-kB) in primary cultures of human hepatocytes, which lead to the increased production of IL-6, IL-8 and CRP and the decrease in transferrin production (George et al., 2007; Argilés, 2005; Tisdale, 2002). Other evidences suggest that PIF may be able to induce cellular apoptosis in murine myotubes through caspase activity (Skipworth et al., 2007).

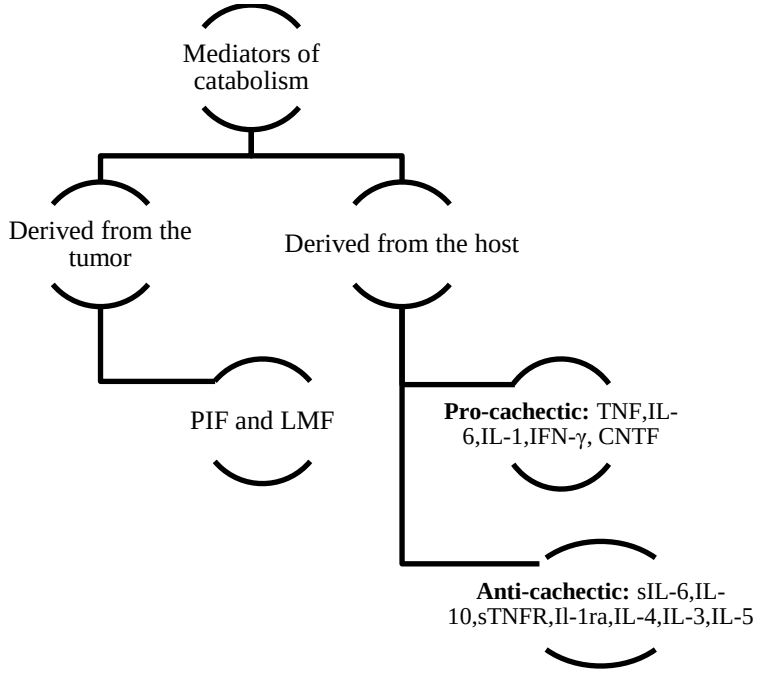

Figure 3. Mediators of cancer-induced body wasting (adapted from (Tisdale, 2002).
(IL – interleukin; sTNFR – soluble tumour necrosis factor receptor; INF-γ – interferon-γ; CNTF – ciliary neurotrophic factor; PIF – proteolysis-inducing factor. LMF – lipid-metabolizing factor).

Systemic inflammation is believed to play a key role in the pathogenesis of cachexia, considering the imbalance between pro-inflammatory cytokines, such as TNF-α and IL-6, and anti-inflammatory cytokines, such as IL-4 and IL-15 (Muscaritoli et al., 2010). Some of these cytokines have been proposed as mediators of the metabolic changes associated with cachexia, such as host and tumour cell derived TNF-α, because of its ability to suppress key metabolic enzymes and induce cachexia in pre-clinical models (Theologides, 1979; Fearon, 2006). Apart from this, TNF-α and IL-1 are able to stimulate PGE_2 production in macrophages, fibroblasts and endothelial cells. The presence of the tumour results in a persistent host inflammatory response, characterized

by the production of T helper 1 cytokines, such as TNF-α, and the induction of an acute-phase response, which is associated with hypermetabolism (Gordon et al., 1999 ; Noguchi et al., 1996).

This acute-phase response is a systemic reaction to local or systemic perturbations in the body homeostasis that could be caused by trauma, infection, injury, among others. In the presence of a tumour, local inflammatory cells secrete cytokines, into the bloodstream, leading to an increase in the production of positive acute phase proteins by the liver (Skipworth et al., 2007; Lelbach, 2007 ; Seelaender, 2012). The concentration of acute-phase proteins changes after inflammation increases or decreases depending if they are positive or negative proteins, respectively. The positive group includes CRP and fibrinogen, whereas the negative group involves albumin and transferrin (Fearon et al., 1999).

3.1 Cancer induced muscle wasting

During periods of diminished food intake, muscle proteins are degraded by the organism in order to provide the amino acids used in gluconeogenesis; however, if the period of starvation is prolonged, the organism reduces the protein breakdown to conserve nitrogen and lean body mass. In cancer patients this ability seems to be absent, leading to the depletion of host´s proteins (Argiles et al., 1997). The loss of myofibrillar proteins (actin, myosin and troponin) in muscle cells is of great relevance in cancer cachexia, as it results in muscle weakness and fatigue (Argilés et al., 2014a; Schiaflino, 1997). Many metabolic alterations are responsible for this loss of muscle mass, including the imbalance between protein synthesis and degradation, and amino acids metabolism, mainly related to their transport and to branched-chain amino acid oxidation, since the tumour has high demands for essential amino acids to support its growth. Furthermore, an increase in apoptosis and an impaired capacity for regeneration contributes to muscle wasting (Argilés et al., 2014a; Argilés, 1999).

Muscle mass depends on the balance between the rate of protein synthesis and degradation (Tisdale, 2002). The decrease in protein synthesis could result from reduced plasma insulin concentrations and insulin sensitivity of skeletal muscle or even from the

decline in the levels of protein translation, in amino acid supply or in the balance of amino acids that are required for protein synthesis. There are three proteolytic pathways that are responsible for protein catabolism in skeletal muscle: the lysosomal system, which is involved in the proteolysis of extracellular proteins and cell-surface receptors and has a key role in cellular function; the cytosolic calcium-regulated calpains, which has been demonstrated to be very important in the initial degradation of myofibrillar proteins; and the adenosine triphosphate (ATP) ubiquitin-dependent proteolytic pathway. Of these three systems, ubiquitin-dependent proteolysis is considered the most important for protein degradation in a range of catabolic conditions, including starvation, sepsis, metabolic acidosis, severe trauma as well as cancer cachexia, since it is believed to be involved in the degradation of abnormal proteins and in the breakdown of skeletal muscle proteins (figure 4) (Cataldo et al., 1996; George et al., 2007; Muscaritoli et al., 2006; Schiaflino et al., 1997; Tisdale, 2002; Temparis, 1994).

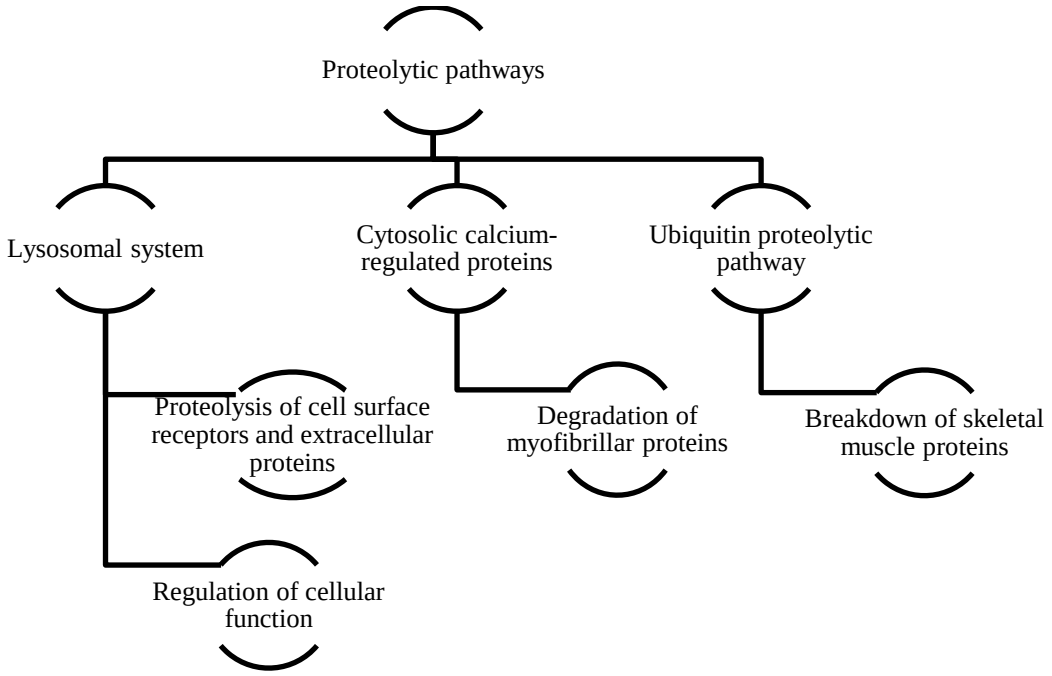

Figure 4. Schematic representation of the proteolytic pathways, which are divided in three main pathways: lysosomal system, cytosolic calcium-regulated proteins and the most important, the ubiquitin proteolytic pathway (adapted from Tisdale, 2002; Argilés, 2005).

Ubiquitin can be found free or conjugated with other cellular proteins. The presence of ubiquitin marks the protein for degradation by the 26S proteasome, in an ATP-dependent manner. Protein ubiquitination is possible due to the action of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (figure 5), which levels are closely related with the expression of

TNF-α and IL-1. The expression of E3 proteins is related with the activity of the ubiquitin-proteasome pathways, since these proteins are responsible for the transference of activated ubiquitin from ubiquitin carriers to a lysine residue in the protein target. In skeletal muscle, specific E3 ligases are known to regulate ubiquitination: atrogin1/MAFbx, and muscle RING-finger protein-1 (MuRF1) (figure 5). Some evidences suggest that TNF-α increases the expression of free or conjugated ubiquitin, connecting the action of this cytokine with skeletal muscle proteolysis (Argilés et al., 1999; George et al., 2007; Hasselgren, 1999; Li et al., 2005; Sakuma et al., 2012; Tisdale, 2005; Argilés, 2005).

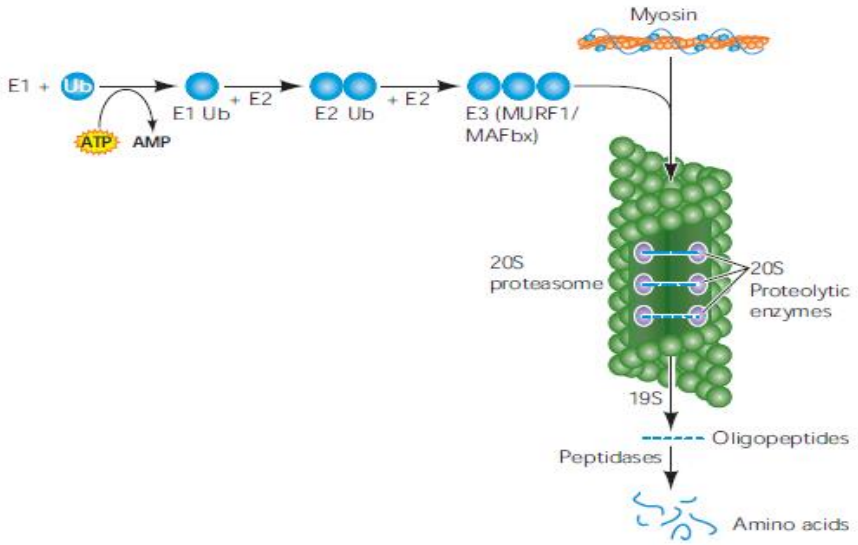

Figure 5. The ubiquitin proteasome pathway (DeWys, 1982).
Ub (ubiquitin); Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-protein ligase.

Inflammatory cytokines that are secreted by either immune cells or tumours, directly induce signalling pathways that up-regulate enzymes involved in skeletal muscle protein turnover (Tisdale, 2005). Current research has found elevated levels of proinflammatory cytokines, such as TNF-α, IL-1 and IL6, that leads to the suppression of some muscle genes, to the activation of the ubiquitin-proteasome mediated proteolysis and to the inhibition of myosin heavy chain genes (George et al., 2007). Two established signalling pathways were reported to be activated in skeletal muscle by pro-inflammatory mediators, the NF-κB and the p38 mitogen-activated protein kinase (MAPK) pathways, known for regulate the attaching of ubiquitin to targeted proteins for

elimination (Xu et al., 2011). In normal conditions, NF-kB is inactivated by its inhibitory protein kBα (IkB); however, when stimulated by TNF-α a signalling cascade is initiated leading to the phosphorylation and ubiquitination of its inhibitor, releasing NF-kB to travel to the nucleus and promoting the up-regulation of E3 ligases (Tisdale, 2005).

Recently, a new member of the TNF superfamily has been described, TNF-like weak inducer of apoptosis (TWEAK), that together with its receptor Fn14 (fibroblast growth factor inducible 14) have been identified as important regulators of skeletal muscle mass (Kumar, 2012). TWEAK binds to Fn14 receptor and together they induce the proliferation of myoblasts and inhibit their differentiation into myotubes, regulate cell survival, wound repair, inflammation, angiogenesis and apoptosis. TWEAK induces proinflammatory responses and stimulates the expression of chemokines, cytokines, adhesion molecules and metalloproteases (MMPs). TWEAK induces muscle atrophy through the augmentation of the expression of the ubiquitin E3 ligase MuRF1 and the consequent activation of the ubiquitin proteasome pathway. It was also found that TWEAK can induce the expression of the components of autophagy-lysosomal system (ALS) and activate caspases, especially caspase-3, in cultured myotubes (Dogra et al., 2007; Tisdale, 2003; Kumar, 2012).

3.2. Cancer cachexia- related fat remodelling

In cancer cachexia, skeletal muscle loss is accompanied by the loss of white adipose tissue (WAT) (Argilés et al., 2014a). It has been suggested that mobilization of fatty acids often occurs before evidences of weight loss (Esper et al., 2005). Indeed, the breakdown of fat seems to precede that of skeletal muscle proteins, and it seems that some signals generated during the breakdown of WAT triglycerides may be responsible for the activation of muscle proteolysis (Argilés et al., 2014b).

Adipose tissue is an active secretory organ and a major repository of energy, which is responsible for the energy balance, homeostasis, appetite, inflammation, insulin sensitivity and angiogenesis. In periods of excessive energy, the adipose tissue stores energy in the form of triglycerides and, in periods of lack of energy, they release the stored triglycerides in the form of non-esterified fatty acids (or free fatty acids,

FFAs). The mass of WAT is regulated by two major pathways: lipolysis (fat breakdown) and lipogenesis (fat synthesis) (Arner et al., 2014; Ebadi et al., 2014; Tisdale, 2005). The dissolution of fat mass results from three different altered processes: i) increase in lipolytic activity and consequently in hyperlipemia; ii) decrease in the activity of lipoprotein lipase (LPL), the enzyme responsible for the cleavage of both endogenous and exogenous triglyceride into glycerol and FFAs which impairs lipid uptake in WAT; iii) reduced *de novo* lipogenesis in adipose tissue resulting in decreased esterification and decrease triglyceride deposition (Argiles et al., 1997; Argilés et al., 2014a). Some studies have shown that plasma glycerol concentrations during fasting are much higher in cancer patients who are experiencing weight loss compared with weight-stable individuals, providing further evidence for an increase in lipolysis (Tisdale, 2002; Das, 2011). Other mechanisms, including impairment in adipogenesis, elevated fat oxidation and decreased lipid deposition have also been attributed to fat loss in cancer (Ebadi et al., 2014).

Elevated lipolysis has been reported to be the main cause of adipose tissue loss in cancer patients, even though the underlying specific mechanisms have not been clearly defined (Ebadi et al., 2014). An increased expression and activity of the enzyme hormone sensitive lipase (HSL) has been reported in cancer cachexia as well as a decrease in LPL activity, presumably due to the combined activity of cytokines such as TNF-α, IL-6 and IFN-γ, which leads to a decrease in the uptake of exogenous lipids and an increase in circulating triglycerides. The infiltration of inflammatory cells, primarily macrophages, into WAT promotes the local production of inflammatory mediators, initiating a negative set of effects in the adipose tissue function and also may induce fat cell death (Rydén et al., 2008). HSL and adipose triglyceride lipase (ATGL), which catalyses the first step in triglyceride hydrolysis and formation of diacylglycerol, are major enzymes that contribute to triglyceride breakdown in adipose tissue (Ebadi et al., 2014; Das, 2011). Indeed, elevated expression of HSL, either messenger RNA (mRNA) or protein, has been reported in cancer cachectic patients compared to weight stable ones. HSL activity is regulated by hormones, such as catecholamines and glucagon, through a cAMP-mediated process. Binding of hormones to G-protein coupled receptors results in the up-regulation of adenylate cyclase, which leads to an increase of intracellular cAMP concentrations (Ebadi et al., 2014).

The increased mobilization of fat is thought to be related to a specific tumour-produced compound, lipid-mobilizing factor (LMF), which is mediated by β3 adrenoceptors. LMF is very similar to a zinc-α-2-glycoprotein (also known as ZAG), which can be found both in WAT and in brown adipose tissue (BAT) (Esper et al., 2005; Sanders, 2004). This glycoprotein can sensitize adipocytes to lipolytic stimuli, by increasing the stimulation of G proteins, Gαs, and inhibiting Gαi, and has a direct lipolytic effect in WAT, which is mediated by a cyclic AMP-dependent mechanism. LMF is responsible for the release of FFAs and glycerol, since it stimulates the hydrolysis of triglycerides by increasing the production of adenylate cyclase. The glycerol produced goes towards the liver, where it is used in gluconeogenesis, whereas FFAs are used by other tissues as an alternative substrate to glucose. ZAG is an adipokine and induces lipid utilization, increasing fat oxidation and is also responsible for the increased production of the uncoupling protein (UCP)-1 in BAT (Argilés et al., 2006; Esper et al., 2005; Islam-Alim et al., 2001; Mendes et al., 2015; Argilés, 2014 ; Muscaritoli et al., 2006; Sanders et al., 2004; Tisdale, 2009). LMF and ZAG act through β-adrenoreceptor, and since β-agonists, such as β2-adrenergenic, are able to stimulate hypertrophy in the muscle, it was discovered that LMF can stimulate protein synthesis in the myotubes and decrease protein degradation. The main effect was centred on the inhibition of the ubiquitin-proteasome pathway, suggesting that the combined action of LMF and ZAG are able to protect the skeletal muscle from atrophy and explain why loss of fat mass precedes loss of skeletal muscle (Tisdale, 2009). LMF causes a specific loss of fat mass, along with a decrease in plasma leptin levels and a significant increase of uncoupling proteins UCP-1, UCP-2 and UCP-3 levels in BAT (Esper et al., 2005; Sanders, 2004).

The alterations in adipose tissue metabolism include changes in the expression of genes involved in the browning of WAT (Agustsson, 2012). Some authors highlight the important role of WAT browning in the development and progression of cancer cachexia. The activation of thermogenesis in the interscapular BAT has been reported and seems to contribute to the hypermetabolic state of cachexia (Petruzzelli et al., 2014). Brown adipocytes have a large number of mitochondria and consequently of UCP-1, which modulates the oxidative phosphorylation (OXPHOS). The presence of UCP-1 renders the inner membrane of the mitochondria permeable; therefore the proton gradient is disrupted, and is released as heat, without production of ATP. So, this mitochondrial protein switches mitochondrial respiration from ATP generation to

thermogenesis (figure 6). UCP-1 serves as a marker for brown fat activation, which can be connected with the energy deficiency found in cachectic patients (Argilés et al., 2005; Argilés, 1999 ; Cypess et al., 2009; Jiménez-Aranda et al., 2013; Quarta et al., 2013). Several studies demonstrated that molecular regulators such as peroxisome proliferator-activated receptor gama (PPARγ), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α) and pR domain containing 16 (PRDM16) are involved in WAT browning. PGC1α is a key transcription factor activated by cold adaptation and promotes mitochondrial biogenesis, oxidative phosphorylation and directly regulates UCP1 expression. PRDM16 is a transcriptional co-regulator that controls the fate of precursor cells between skeletal muscle cells and brown adipocytes. When the levels are low, it promotes muscle differentiation whereas its presence in brown adipocytes regulates the expression of UCP1 (Tsoli et al., 2016).

Usually BAT is activated through the exposure to low temperatures that leads to the stimulation of the β-adrenergic pathway and to the activation of cAMP/PKA signalling that in turn, modulate PGC1 levels. However, there are other ways to activate BAT, such as through the stimulation of transcription factor forkhead box protein C2 (FOXC2) that is responsible for the increased mitochondrial biogenesis and thermogenesis, through regulation of mitochondrial transcription factor A (TFAM). Another ways is through the activation of bone morphogenic protein 7 (BMP7) that triggers MAPK p38 pathways and fibroblast growth factor 21 (FGF21), which increases thermogenesis in BAT. Furthermore, irisin, an hormone produced by exercised skeletal muscle acts on white adipocyte precursors through MAPKs ERK1/2 and p38 pathways (Tsoli et al., 2016).

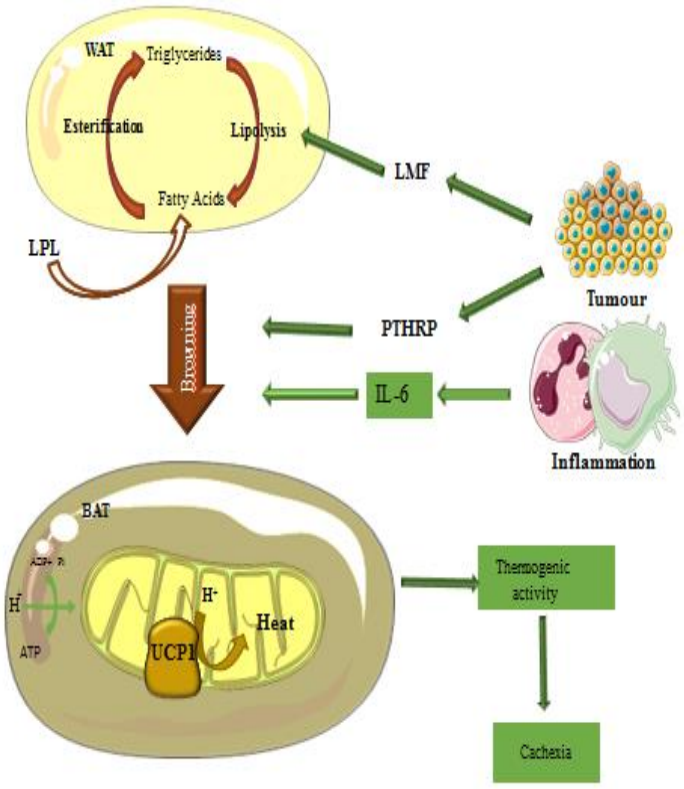

Figure 6. Browning of white adipose tissue in cachexia (based on Argilés, 2014a).

In addition to elevated lipolysis, decreased lipogenesis and reduced entry of fatty acids owing to decreased activity of LPL are responsible for adipose tissue wasting. This represents the "browning" process of white cells, in which UCP-1 is expressed. UCP-1 promotes heat production and energetic inefficiency. This cell conversion can be triggered by both humoral inflammatory mediators, such as IL-6 and tumour-derived compounds, such as PTHRP. This figure was made with Servier Medical Art.

WAT browning in cancer was highlighted by the introduction of staging with ^{18}F-fluorodeoxyglucose positron emission tomography (PET) scanning (Petruzzelli et al., 2014) (figure 7). Some tumour-induced factors were suggested to be involved in this fat remodelling, such as inflammatory cytokines, EGF family members, parathyroid-hormone-related protein (PTHRP), the irisin and cyclooxygenase (COX2) (Jiménez-Aranda et al., 2013). The phenotypic switch of WAT to BAT and the increased energy expenditure was reported to proceed skeletal muscle atrophy in many mouse models of cancer cachexia (Petruzzelli et al., 2014).

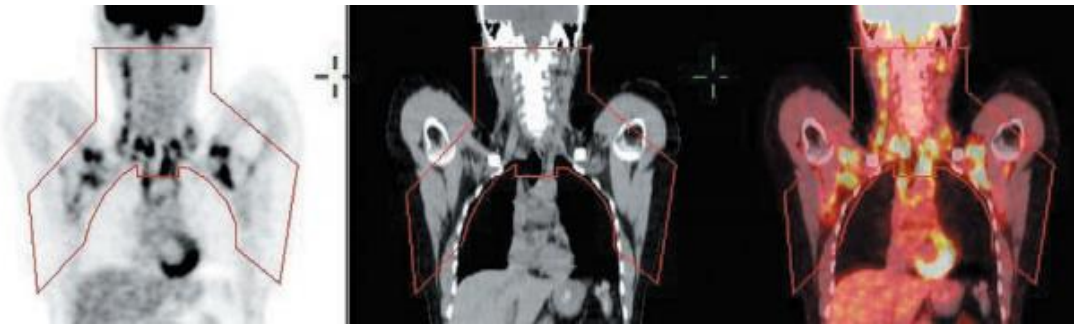

Figure 7. The figure shows the quantification of the amount and activity of brown adipose in the cervical, supraclavicular, and superior mediastinal depots of brown adipose tissue. (PET (left), computed tomography (CT, center), and combined PET–CT (right) (Cypess et al., 2009).

Brown adipocytes induced in WAT are also known as ‘‘beige’’ cells, which are derived from a population distinct from mature, white and brown, adipocytes (figure 8) (Petruzzelli et al., 2014). Unlike WAT, which stores energy as intracellular lipid droplets, brown and beige adipocytes are metabolically active and promote energy expenditure (Martz, 2014).

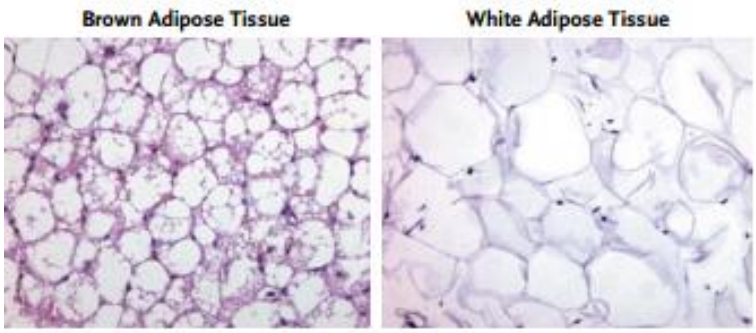

Figure 8. Adipose tissue samples stained with hematoxylin and eosin show the histological differences between brown and white adipose tissue. In brown adipose tissue granular cytoplasm containing mitochondria and multiple fat vacuoles are observable (Cypess et al., 2009).

Other findings have linked WAT browning to the thyroid system. This enzyme is controlled by norepinephrine and is capable of generating triiodothyronine (T3) from thyroxine (T4). Intracellular T3 is capable of inducing the transcription of the UCP1 gene. However, this new finding not only have shed a light in the role of the thyroid in this process, but also highlighted a link between the thyroid and the sympathetic nervous system (SNS). The presence of 5’-deiodinase, which is produced after meals, in BAT is regulated by bile acids. The liver also releases a fibroblast growth factor-21

(FGF21) that interacts with FGF receptor/β-Klotho complexes at the cell surface, inducing mitochondrial uncoupled respiration and glucose oxidation. Therefore FGF21 directly activates heat production by BAT and promotes the WAT browning depots, highlighting the importance of liver in this process. The thyroid effects on the hypothalamus activates peripheral BAT through the induction of AMP-kinase, leading to enhanced activation of the SNS (Villarroya et al., 2013). Moreover, activated macrophages are able to control the thermogenic action of BAT via local release of catecholamines. This process seems to be similar to the reported in the exposure to cold. The exposure to cold was reported to activate the IL-4/IL-13-mediated pathway of macrophage activation, within BAT. These activated macrophages produce norepinephrine, which was previously referred to as having an important role in the thyroid (Villarroya et al., 2013).

3.3 Adipokine pathway associated with adipose tissue alterations in cancer

Adipokines are cytokines produced by adipose tissue that have an important role in energy balance, metabolism and inflammatory responses. Some adipokines such as leptin, adiponectin and resistin, participate in systemic inflammatory response, and may influence the action of other cytokines, such as TNF-α and IL-6, which makes them able not only to regulate inflammation but also angiogenesis, cell proliferation, differentiation and migration (Karapanagiotou et al., 2008). Leptin is predominantly produced by the adipose tissue but also by the placenta and bone marrow and acts in the central nervous system to suppress food intake and regulate energy homeostasis. The ability to control energy homeostasis derives from the control of energy intake and energy expenditure and also has additional effects (Zhou et al., 2013). Apart from this it also plays a role in the endocrine and immune systems, including reproduction and glucose homeostasis. Some studies suggest that leptin plays an important role in the pathophysiology of cancer cachexia, since it is a proinflammatory cytokine that increases when infection is present and plays a role in $CD4^+$ lymphocyte proliferation, macrophage phagocytosis and the secretion of IL-1 and TNF-α. Leptin binds to its receptor and activates different signalling pathways, such as the Janus Kinase/Signal

Transducer and Activator of Transcription (JAK/STAT), MAPK, phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), 5' AMP-activated protein kinase (AMPK) and insulin receptor substrate pathways (IRS), which affect cell proliferation and survival (Mantovani et al., 2000; Paz-Filho et al., 2011) .

Another cytokine with relevance in the regulation of WAT is ghrelin, a hormone mainly produced by the stomach and released into circulation in its acetylated and active form. Ghrelin has an important role in the induction of the growth hormone release, induction of adiposity, food intake and inhibition of the pro-inflammatory cytokines. In its active form it stimulates food intake by binding to the growth hormone secratagogue receptor (GHSR), located in the neurons of the hypothalamus, decreasing vomiting and nausea. This cytokine plays a role in the modulation of blood glucose levels and glucose disposal in the skeletal muscle and adipose tissue and is known to regulate lipid metabolism and lipid storage. Ghrelin also regulates the consumption of energy, through the inhibition of WAT browning and consequently, thermogenesis. Ghrelin levels correlate positively with various cachectic states, such as anorexia nervosa and severe congestive heart failure, and elevated levels of this hormone were recently reported to be associated with several types of cancer (Esposito et al., 2015; Sever et al., 2014).

Adiponectin also plays a role in the pathogenesis of cachexia. The most important identified functions of adiponectin are anti-atherogenic, anti-inflammatory and insulin-sensitivity effects. There is increasing evidence that this adipokine is able to increase oxygen consumption and thermogenesis, leading to weight loss (Kubota et al., 2007). It decreases lipid synthesis and the production of glucose in the liver (by decreasing gluconeogenesis), leading to a decrease in the blood concentrations of glucose and free fatty acids (Kubota et al., 2007).

The pathway that involves adiponectin and its receptors, AdipoR1 and AdipoR2 mediates the activation of AMPK that plays a major role in the regulation of growth arrest and apoptosis. Activated AMPK works by stimulating p53 and p21. Independent of AMPK activation, adiponectin decreases the production of reactive oxygen species (ROS), leading to a reduced activation of mitogen-activated protein kinases (MAPK) and thereby inhibition of cell proliferation (Prieto-Hontoria et al., 2011; Candore, 2010).

3. Aims

Cancer associated cachexia is recognized as a negative prognostic indicator. The metabolic changes that occur lead to the depletion of fat stores and skeletal muscle, resulting in body weight loss with negative impact on patient´s treatment response and quality of life. Aiming to add new insights on the mechanisms underlying cachexia in head and neck cancer, in the present thesis we intended to:

i) Evaluate the potential association between BMI, nutritional status, tumour stage and location, and the incidence of cachexia;

ii) Analyse the levels of metabolic parameters in head and neck cancer patients with and without cachexia;

iii) Study the relation between inflammation and body wasting in head and neck cancer;

iv) Analyse how the profile of hormones involved in the regulation of adipose tissue remodelling changes with cachexia;

v) Study the contribution of the catabolic cytokine myostatin to the cachectic phenotype.

II. Materials and Methods

II.I. Patient Selection

Seventeen male patients diagnosed with head and neck cancer were enrolled in the present study, which was conducted between March and September 2016. Study protocol was approved by the *Instituto Português de Oncologia do Porto* Ethics Committee. The nature and purpose of the study was explained to participants before written informed consent was obtained. The eligibility criteria included patients: i) no obese and no diabetic; ii) no prior history of cancer; iii) with spinocellular tumors; iv) not submitted to prior cancer treatment; v) able to do unrestricted physical activity. Clinicopathological data was obtained from patients' clinical records. Among the information gathered by physicians from the *Instituto Português de Oncologia do Porto* was age, smoking and drinking habits, medication and tumors' stage. HNC tumours were histologically classified according to morphologic characteristic, concerning morphologically identifiable cell types and histological patterns that allow the identification of histogenesis of the tumor as epidermoid / squamous (Sobin, 1981). Tumour staging was done according to the International Union Against Cancer's (UICC) classification system for oral cancer (Sobin et al., 2011). All patients underwent nutritional evaluation and information regarding body mass index (BMI), Malnutrition Universal Screening Tool (MUST) and Patient-Generated Subjective Global Assessment (PG-SGA) scores were collected. MUST score uses several parameters to determine the risk of malnutrition in cancer patients, such as weight loss, BMI, serum albumin concentration, questions about food intake, being completed by professionals. The PG-SGA evaluation comprises medical history, weight loss, nutrition, food intake, among others, and is completed by the patient (Gorenc et al., 2015). This tool was specifically designed to assess malnutrition in oncology and results from the adaptation of the Subjective Global Assessment (SGA or Detsky index), which allows a simple and reproducible classification of patients into three groups: (A) well nourished, (B) moderate or suspected malnutrition, (C) severe malnutrition (Detsky et al., 1987). One of the strong points of the PG-SGA tool is that, in addition to recent weight loss, assessment of nutritional status includes symptoms such as loss of appetite, nausea,

swallowing difficulties, etc., the patient's dietary intake and functional capacities (Detsky et al., 1987).

Patients were assigned to one of two groups according to the percentage of body weight loss reported in the last 6 months. Patients that reported a weight loss higher than 5% were included in the head and neck cancer + cancer cachexia (HNC+CC) group whereas patients with no weight loss or weight loss lower than 5% in 6 months were included in the head and neck cancer with no cachexia (HNC) group. At the end, six patients were enrolled in the HNC group and 11 subjects in the HNC+CC group.

II.II Blood Collection and biochemical measurements

Blood samples were collected in the morning at IPO-Porto. No fasting was required to patients. Blood samples were allowed to clot for one hour and then centrifuged at 4000g during 10 minutes. The supernatant was collected and stored at -20ºC until analysis.

The biochemical parameters glucose, urea, cholesterol, triglycerides, total protein and albumin were measured using an automated analyzer (AU500 Clinical Chemistry Analyzer, Beckman Coulter, Inc) and C-reactive protein was determined by an immune-turbodimetric technique using an automated analyzer (Beckman Coulter AU). These analyses were performed at IPO-Porto. The levels of cytokines and hormones were assessed by immunoblot as described in the following subsection.

II.III. Immunoblot assessment of serum levels of cytokines

Serum samples were diluted in Tris buffered saline (TBS; 100 mM Tris, 1.5 mM NaCl, pH 8.0) and 100 µL was slot-blotted into a nitrocellulose membrane (Whatman, Protan) under vacuum after membrane activation in 10% methanol. The effectiveness of this procedure was confirmed by membrane staining with Ponceau S. Then, membranes were incubated with 5% (w/v) dry nonfat milk in TBS-T (TBS with 0.5% Tween 20) to avoid nonspecific binding. The membranes were then incubated with a primary

antibody, diluted 1:1000 in 5% (w/v) dry nonfat milk in TBS-T (mouse monoclonal anti-adiponectin, ab22554, Abcam; rabbit monoclonal anti-leptin, ab16227, Abcam; mouse monoclonal anti-ghrelin, ab64325, Abcam; mouse monoclonal anti-TWEAK, ab37170l, Abcam; rabbit polyclonal anti-GDF8 (myostatin), ab996, Abcam) at room temperature for two hours. The membranes were then washed with TBS-T (3 times, 10 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody, conjugated with horseradish peroxidase (GE Healthcare), depending on the primary antibody. Chemiluminescence ECL (Amersham Pharmacia Biotech) was used to detect immune-reactive bands, according with the manufacturer´s instructions. The images were then recorded using X-ray films (Kodak Biomax Light Film, Sigma, St. Louis, MO,USA), a procedure performed in a dark room. Films were scanned in Molecular Imager Gel Doc XR+System (Bio-rad) and analyzed with ImageLab (v 5.0 Bio-Rad).

II.I.V. Statistics

An exploratory data analysis was initially conducted using graphical techniques (bar charts, box and scatter plots) and a quantitative analysis (statistical measures) was performed in order to characterize each group, detect possible extreme outliers and measurement error. In order to identify the alterations between patients with and without cachexia, tests of the equality of means for independent samples were conducted: Mann-Whitney test (the assumptions of the tests were performed). Statistical analysis was conducted using IBM SPSS Statistics Software 22. Results were considered significantly different when $p<0.05$. Values are presented as mean ± standard deviation for all variables.

III. Results and discussion

In order to add new insights on the molecular mechanisms underlying HNC cachexia, two groups of cancer patients were considered in the present study. The information regarding age, body mass index (BMI), lifestyle and disease stage in each group of patients is overviewed in table 1.

As can be depicted in table 1, patients from both groups present BMI higher than 20 Kg/m^2 but lower than 25 Kg/m^2 suggesting that all cancer patients are lean. The BMI values in the group HNC+CC was somehow unexpected once according to Fearon (Fearon et al., 2011) cachexia is characterized by a BMI inferior to 20 Kg/m^2. However, water accumulation as a consequence of hypoalbuminemia might affect BMI (Muscaritoli et al., 2010; Tisdale, 2002). Indeed, lower levels of serum albumin were noticed in HNC+CC patients (figure 10). So, our data suggest that BMI might not discriminate cachexia in HNC patients.

The risk of cachexia´s progression was previously suggested to be dependent on cancer type and stage (Fearon et al., 2011). All cancer patients enrolled in the present study evidenced squamous cell carcinoma mostly located at oropharynx and hypopharynx, at T3 and T4 stages of disease, with staging being done according to the International Union Against Cancer's (UICC) classification system for oral cancer (Sobin et al., 2011). Squamous cell cancer of the head and neck is one of the most common cancers worldwide, constituting the fifth cause of death with cancer among men in Portugal (Silveira et al., 2010). It has been reported that patients with hypopharyngeal cancer had the worst health related quality of life score, compared with tumours at other sites within the head and neck, and that stage had the strongest impact (Sanderson et al., 2002). In the present study, the percentage of patients with tumours of greater dimensions and advanced local disease (T4 stage) was higher in HNC+CC than in HNC group (91 vs 67%, respectively; table 1). Curiously, the percentage of patients with lymph nodes metastasis (N2 and N3) was lower in HNC +CC than in HNC group. However, the number of patients in each group, particularly in HNC group, is low which might biased the association between disease stage and cachexia risk.

Table 1. Baseline patient characteristics in each group

	Group	
Patients' characteristics	**HNC+CC** (n=11)	**HNC** (n=6)
Age (years)	52.0 ± 5.37	55.7 ± 3.61
Body mass index (Kg/m^2)	21.64 ± 1.52	22.33 ± 4.41
Tumour location (%)	Oral cavity (27) Oropharynx (37) Hypopharynx (27) Larynx (9) Nasopharynx (0)	Oral cavity (0) Oropharynx (33) Hypopharynx (33) Larynx (17) Nasopharynx (17)
Disease stage (%) **T** **N** **M**	 T2 (0); T3 (9); T4 (91) N0 (25); N1 (17); N2 (50); N3 (8) M0 (91); M1 (9	 T2 (0); T3 (33); T4 (67) N0 (0); N1 (16); N2 (67); N3 (17) M0 (100); M1 (0)
Smoking habits	Over 20 cigarettes/day	Over 20 cigarettes/day
Drinking habits (%)	Moderate (0) Heavy (100)	Moderate (80) Heavy (20)
Medication (n=17)	Angiotensin converting enzyme inhibitors (n=2) Proton pump inhibitors (n=1) Semisynthetic opioid (n=1) Nonsteroidal antiflammatory goup Statins group Benzodiazepines group (n=2) Angiotensin II receptor antagonis (n=1) Calcium channel blocker (n=1)	Angiotensin converting enzyme inhibitors (n=1) Proton pump inhibitors (n=1) Synthetic opioid (aminocyclohexanol group) (n=1) Calcium channel blocker (n=1) Miscellaneous analgesic (n=2)

There is evidence of a marked association between smoking and drinking habits and the development of head and neck cancer (Leemans et al., 2011). The results obtained support this association by demonstrating that both groups present strong drinking and smoking habits. People who use both tobacco and alcohol are at greater risk of developing these cancers than people who use either tobacco or alcohol alone (Pelucchi et al., 2006). Tobacco smoke and alcohol consumption are associated with oxidative damage of DNA. The tobacco carcinogen benzo[α]pyrene diol epoxide (BPDE) seems to promote genetic damage by forming covalently bound DNA adducts throughout the genome, including p53 (Serpi, 2003). Damage promoted by tobacco carcinogens might be repaired by the nucleotide excision repair (NER) system and also by the base excision repair (BER) system. So, individual variations in NER/BER might influence tobacco smoking related cancer risks (Health & Services, 2010). The oxidative damage of DNA induced by exposure to carcinogenic factors and not repaired by NER or BER systems may lead to the abnormal expression of tumour suppressor genes and/or proto-oncogenes, which in turn, activate pathways that lead to the malignant transformation of cells (Reuter, 2010). Even though implicated in the aetiology of head and neck cancer, there is no evidence of the association between smoking and drinking habits with the development and progression of cachexia.

Not all patients were taking medication at the time, only 3 patients in each group were medicated. In the HNC+CC group the medicated patients were being treated for elevated levels of cholesterol and triglycerides, depression and anxiety, gastric problems, hypertension and addictions, namely alcohol. In the HNC group they were being treated for hypertension, gastric problems and pain. A study of the literature showed that the medication that could interfere with cachexia is the calcium chain blockers and angiotensin converting enzyme inhibitors that are known to suppress sympathetic activity, and to lower leptin levels. Therefore, patients who were under this medication might present lower leptin levels than expected (Masuo et al., 2001).

III.I. Nutritional parameters evaluation

HNC patients are frequently malnourished at the time of diagnosis and prior to the beginning of treatment, which determines the patient's tolerance to curative treatment. So, nutritional counselling is usually considered in the multidisciplinary standard of care of these patients (Bossola, 2015). Many screening tools for nutritional risk have been published (Green et al., 2005), but no consensus has been reached concerning their use. In IPO-Porto, MUST (Malnutrition Universal Screening Tool) score and PG-SGA (Patient-Generates Subjective Global Assessment) are the tools implemented in the nutritional counselling. MUST score uses several parameters to determine the risk of malnutrition in cancer patients, such as weight loss, BMI, serum albumin concentration, questions about food intake, being completed by professionals. The PG-SGA evaluation comprises medical history, weight loss, nutrition, food intake, among others, and is completed by the patient (Gorenc et al., 2015). This tool was specifically designed to assess malnutrition in oncology and results from the adaptation of the Subjective Global Assessment (SGA or Detsky index), which allows a simple and reproducible classification of patients into three groups: (A) well nourished, (B) moderate or suspected malnutrition, (C) severe malnutrition (Detsky et al., 1987). One of the strong points of the PG-SGA tool is that, in addition to recent weight loss, assessment of nutritional status includes symptoms such as loss of appetite, nausea, swallowing difficulties, etc., the patient's dietary intake and functional capacities (Detsky et al., 1987).

Figure 9 shows a clear association between the MUST score and PG-SGA results. An elevated risk of malnutrition was observed in the HNC+CC group, with a MUST score superior to 2 and a PG-SGA score of B and C. In contrast, the HNC group showed that all the patients present a low risk of malnutrition, with a MUST score of 0 or 1 and a PG-SGA score of A. Head and neck cancer patients have one of the highest malnutrition rates (25-50%) in the oncologic set, even before starting treatment (Mason et al., 2016). The tumour location and the progression of the disease might cause reduced food intake and malnutrition due to dysphagia and xerostomia (Gorenc, 2015). The comparison of our results given by these tools with BMI data, suggests that other factors must influence BMI among cachectic patients, since there is a significant difference between BMI and MUST score and PG-SGA results.

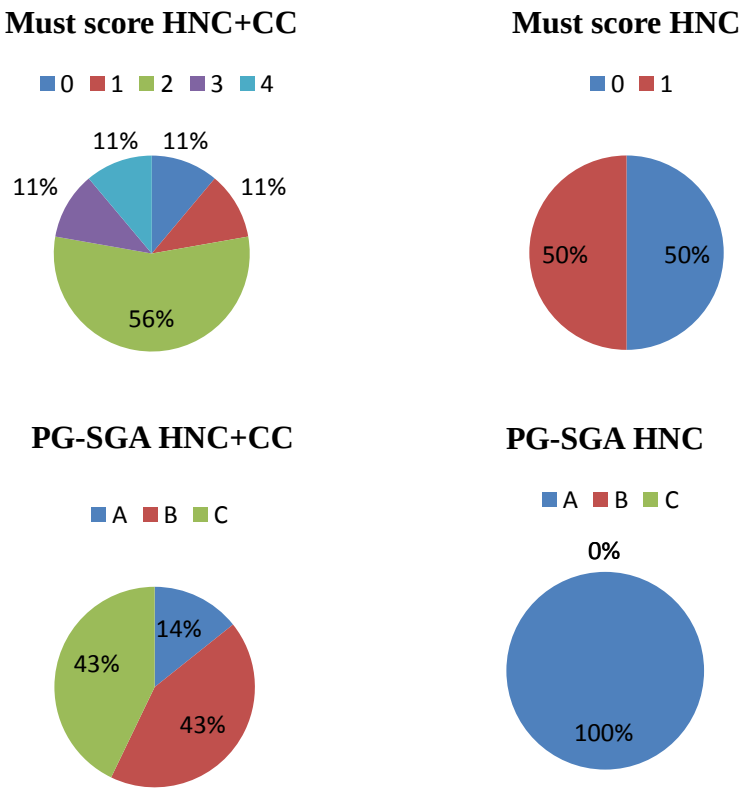

Figure 9. MUST score evaluation of the HNC+CC (n=9) and HNC (n=4) groups; score =0 represents low risk of malnutrition; score = 1 represents a medium risk of malnutrition; score≥ 2 represents high risk of malnutrition. PS-SGA global evaluation of the patients in the HNC+ CC (n=7) and HNC (n=3) groups: A score = well nourished; B score = slightly malnourished; C score = highly malnourished.

III.II Analysis of biochemical parameters

In order to better characterize the metabolic alterations underlying CC in HNC we analysed serum levels of glucose, total protein, total cholesterol and triglycerides and the results obtained are presented in table 2. All the results were in the normal range (compared to reference values).

Table 2. Evaluation of serum levels of glucose, total protein, total cholesterol and triglycerides in HNC+CC and HNC groups.

	HNC+CC (n=11)	**HNC** (n=6)
Glucose **(mmol/L)**	5.57 ± 0.53	4.98 ± 0.69
Total protein **(g/L)**	74.83 ± 4.78	72.16 ± 3.13
Total cholesterol **(mmol/L)**	4.32 ± 0.77	5.24 ± 1.27
Triglycerides **(mmol/L)**	1.16 ± 0.27	1.66 ± 0.91

No differences in the levels of these biochemical parameters were observed among groups, which do not support the metabolic alterations reported in CC. Even so, the trend to higher serum glucose levels noticed in HNC + CC patients (table 2), might reflect the Cori cycle increased activity between the host and the tumour, in order to maintain the metabolic needs (Friesen, 2015; Esper, 2005; Keller, 1993). Glucose is metabolized by the tumour via glycolysis, and a major consequence of this metabolism is the release of lactate into circulation. Once in circulation, the lactate is transported to the liver where the carbon skeleton is used to synthesize glucose through gluconeogenesis that might be released from the liver to support tumor's metabolic needs, in the so called Cori cycle (DeWys, 1982; Giordano et al., 2003). There is evidence that, in the presence of cachexia, the Cori cycle metabolizes half of all glucose available and 60% of all lactate disposals (Esper et al., 2005). In addition to lactate, also alanine and glycerol serum levels are expected to be elevated in cachexia, due to the metabolic changes in protein metabolism. These metabolites are used to produce glucose in the liver through gluconeogenesis (Fearon, 2012).

Focusing on cholesterol and triglycerides serum levels, data obtained (table 2) suggests no cachexia-related lipolysis of the adipose tissue as previously suggested (Bing et al., 2004; Das et al., 2011; Ebadi et al., 2015; Legaspi et al., 1987). Indeed, when there is lack of energy, the adipose tissue releases free fatty acids derived from stored triglycerides (Ebadi et al., 2014), as a consequence of increased ATGL and HSL

activities (Bing et al., 2004; Das et al., 2011; Ebadi et al., 2015). Lipid mobilization is prompted by the increased circulation of several factors, such as the adipokine ZAG, LMF, IL-1, IL-6 and TNFα (Porporato, 2016). Both LMF and ZAG produce specific loss of body fat with a tendency to increase lean body mass, this effect appears to be due to interaction with a β-adrenergic receptor. Loss of adipose tissue was coupled with an increase in expression of UCP1 in BAT and consequent increase in energy expenditure (Tisdale, 2005). Also the released fatty acids serve as an energy source for heat production in BAT, showing a correlation between the remodelling of adipose tissue with increased energy expenditure and WAT browning (Tisdale, 2005).

In order to evaluate the contribution of inflammation to the metabolic profile of head and neck cancer patients we measured the serum levels of albumin, CRP and TWEAK (figure 10). Inflammation has a major role in the development of cancer cachexia. It is known that in response to tissue injury an acute-phase response begins, leading to an increased production of acute-phase proteins (Argilés et al., 2005). In the present study no alterations of serum CRP levels were observed among groups ($p=0.291$), which might explain the BMI variation among groups, considering the positive correlation between BMI and CRP levels previously reported (Md, 2007). Nevertheless, lower levels of serum albumin were observed in HNC+CC group, though not statistically significant ($p= 0.067$; figure 10). So our data do not apparently support the association between inflammatory markers and weight loss, due to the spill over effects of excessive cytokine production by tumours (Tsoli et al., 2013).

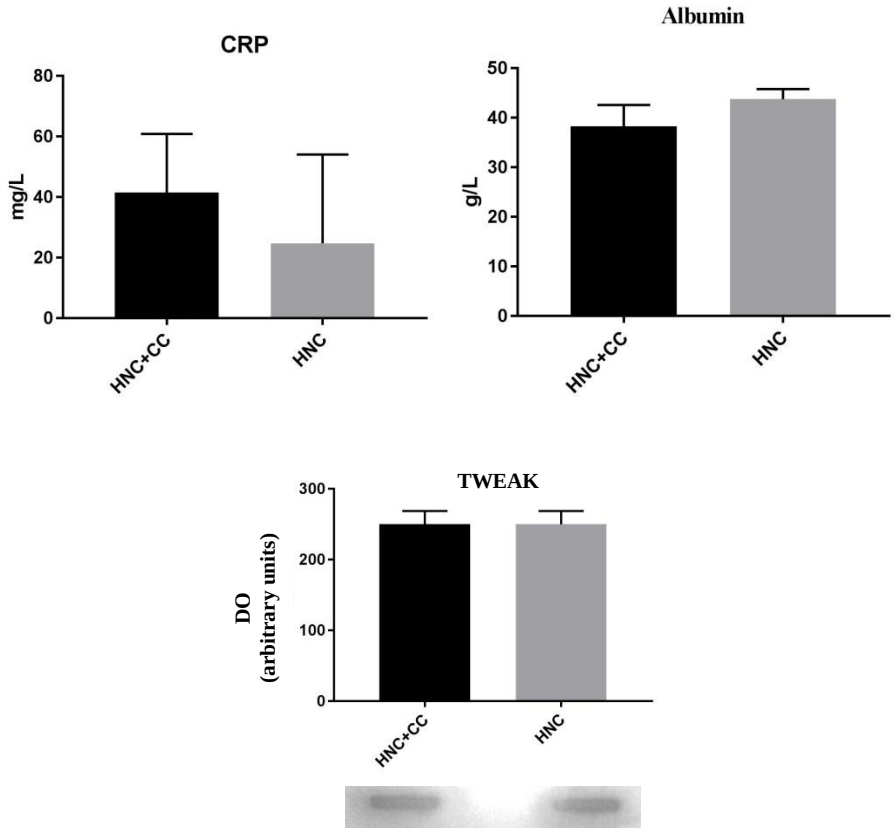

Figure 10. Variation of C-reactive protein and albumin levels in serum samples from both groups. Variation of TWEAK levels in serum samples evaluated by immunoblot from patients in both groups. Under the graph is shown a representative image of immunoblot data. The values (mean± SD) are expressed in mg/L, g/L and arbitrary units of optical density (OD).

The presence of tumour is expected to be associated with persistent host inflammatory response, which is associated with hypermetabolism (Noguchi et al., 1996). Among the alterations that cachexia induces in the body, the liver exhibits some marked changes in the pattern of protein synthesis. This changes include increased production of acute-phase proteins, including CRP, and a decrease in the production of albumin, leading to a state of hypoalbuminemia that must be due to increased transcapillary escape and increased degradation (Argilés et al., 2015; Fearon et al., 2012). The production of acute-phase proteins leads to a mismatch in amino acid composition between skeletal muscle and acute phase proteins and it has been suggested that during a low food intake this may amplify the need for muscle mobilization, therefore the acute-phase response may accelerate muscle wasting in cachectic cancer patients (Fearon et al., 2012).

Curiously, no differences of TWEAK levels were noticed among groups. TWEAK is a pro-inflammatory cytokine from the TNF superfamily that acts by binding

to Fn14 (Schiaffino et al., 2013). TWEAK and its ligand Fn14 induce pro-inflammatory responses by stimulating the expression of chemokines, cytokines, adhesion molecules and MMPs. Muscle wasting involves the degradation of selective muscle proteins, such as myosin heavy chain (MHC). TWEAK was found to increase the expression of muscle-specific E3 ubiquitin ligases MuRF1 and MAFbx and stimulates the conjugation of ubiquitin with MyHC, suggesting that TWEAK causes degradation of MHC through the activation of UPS (Bhatnagar et al., 2012). There is also evidence of TWEAK cooperating with TNF-α to increase the inflammatory response and TWEAK also activates both the classical and alternative NF-kB signalling pathways and induces the expression of NF-kB-regulated proinflammatory cytokines and cell adhesion molecules, suggesting that TWEAK might mediate inflammatory responses (Dogra et al., 2007; Londhe et al., 2015). Our results do not apparently support the inflammation in the development and progression of cachexia in head and neck cancer and its connection with the metabolic changes that cachectic patients undergo.

In order to evaluate the contribution of adipose tissue remodelling to CC, we evaluated the serum levels of the hormones leptin, ghrelin and adiponectin (figure 11). Adipokines yield significant effects on metabolism and lipogenesis as well as in the regulation of human inflammatory responses (Karapanagiotou et al., 2008).

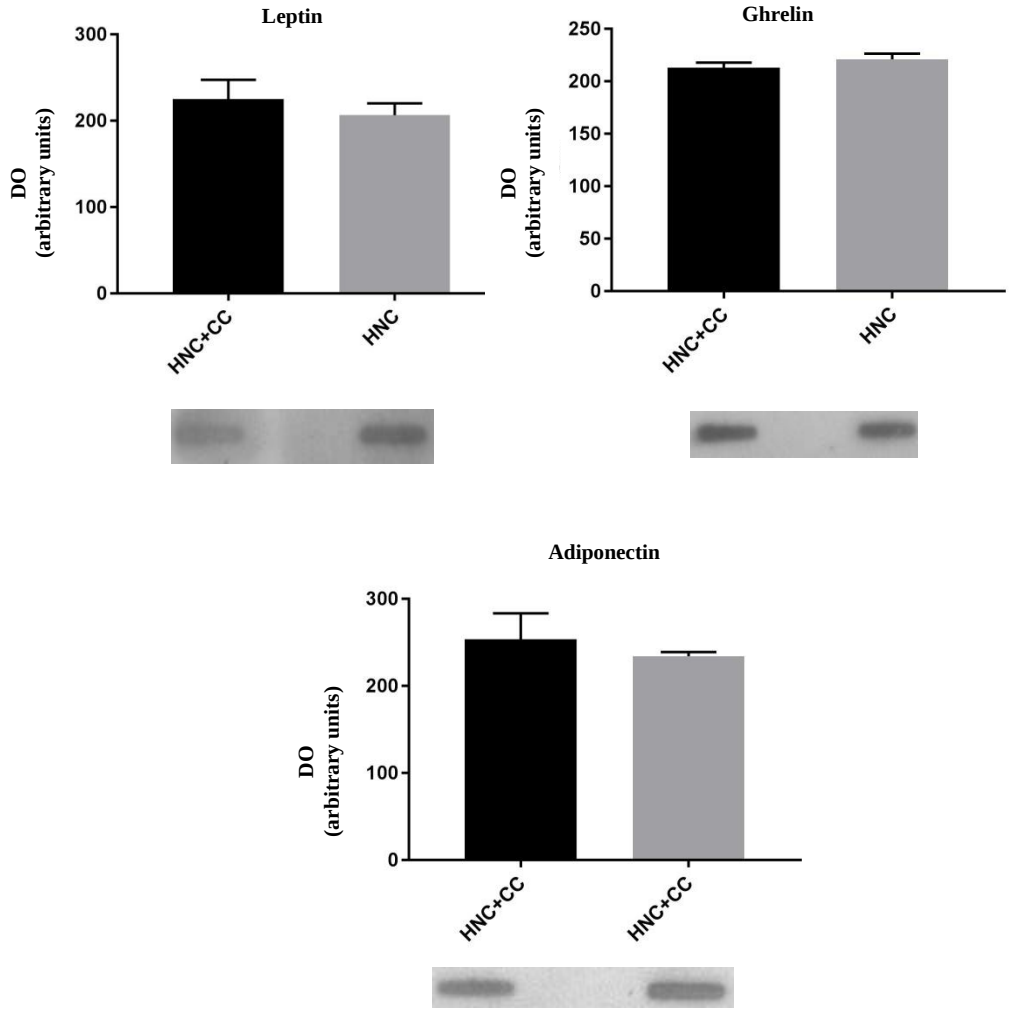

Figure 11. Variation of leptin, adiponectin and ghrelin levels in serum samples from both groups, evaluated with immunoblot. Under the graph is shown a representative image of immunoblot data. The values (mean± SD) are expressed in units of optical density (OD).

Our results do not evidence differences of serum leptin levels among groups (p =0.078; figure 14), neither of serum ghrelin content (p=0.151). Leptin is a member of the cytokine family and is an important signalling molecule in the energy regulation (Begenik et al., 2015). Leptin is predominantly expressed in the adipose tissue and signals in the hypothalamus the status of fat stores, playing a significant role in energy homeostasis (Begenik et al., 2015; Mak, 2014). Despite the importance of hypothalamic mediation of leptin actions, there is evidence that, at least at high concentrations, leptin acts directly on white adipocytes, by stimulating lipolysis and inhibiting lipogenesis (Orci et al., 2004; Balistreri, 2010). There is also evidence of leptin´s involvement in the inflammatory response, carcinogenesis and to the impaired glucose metabolism (Prieto-Hontoria et al., 2011; Morton, 2007). Ghrelin exerts

antagonistic effects on the leptin-induced decrease in food intake through activation of the hypothalamic neuropeptide Y-Y1 (NPYY1) pathway (Takahashi et al., 2009). It is known for regulating fat mass by decreasing the level of neuropeptide Y in the hypothalamus, and consequently decreasing food intake (Takahashi et al., 2009). It has been reported that ghrelin levels increase under conditions of fasting or low BMI (Sever et al., 2016). Under fasting conditions, the stomach secretes ghrelin into the bloodstream to act a "hunger" mediator that signals the gastrointestinal fuel status from the periphery to the central nervous system in order to stimulate food intake and to adjust energy balance through decreasing energy expenditure. This decrease in energy expenditure is achieved by activating white adipocytes (Müller et al., 2010). Ghrelin has also been shown to cause a positive energy balance and weight gain, by decreasing fat utilization and increasing carbohydrate utilization, and to have anti-inflammatory effects since it decreased serum levels of IL-6 (DeBoer et al., 2007; Shimizu, 2003). The absence of an association between CC and ghrelin levels in our study might reflect the similarities observed in BMI among groups (table 1).

We also found no differences in the serum levels of adiponectin among groups. Adiponectin is produced and secreted exclusively by the adipose tissue. Therefore, alterations in adipose tissue, a compensatory response to inflammation and the development of insulin resistance can lead to alterations in the expression levels of this adipokine (Kemik et al., 2012; Donatto, 2013 ; Smiechowska et al., 2010). There is growing evidence that adiponectin modulates the production of other cytokines, and is able to activate AMP kinase to stimulate glucose uptake and fatty acids oxidation in the skeletal muscle, decreasing the influx of fatty acids to the liver and the total triglyceride content and decrease vascular inflammation (Bing et al., 2010; Dalamaga et al., 2012). In CC, it has been reported decreased levels of adiponectin as a reflex of adipose tissue wasting (Diakowska, 2014). So, our data regarding serum levels of cytokines involved in WAT browning (figure 11) do not support adipose tissue remodelling in head and neck cancer cachexia.

WAT remodelling seems to precede skeletal muscle catabolism in cancer cachexia (Petruzzelli, 2016). So, we analysed the levels of serum myostatin in order to verify if at this stage of disease muscle catabolism has a major contribution to CC than

WAT remodelling. In figure 12 we can see that serum myostatin levels are significantly higher in HNC+CC patients (p =0.037).

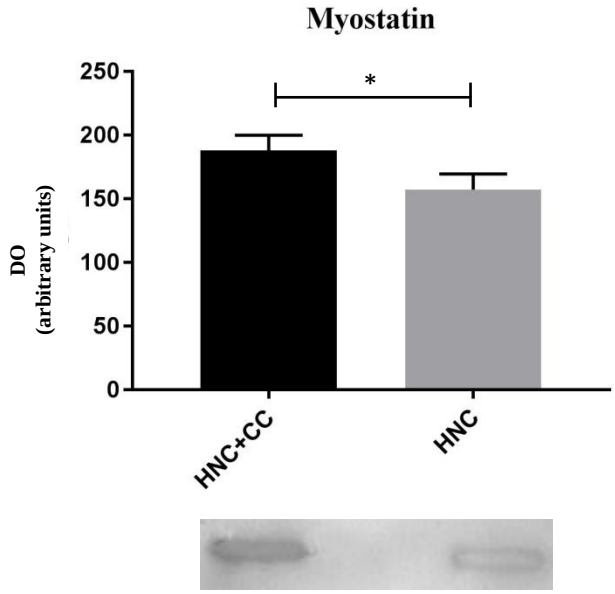

Figure 12. Variation of myostatin levels in serum samples from both groups, evaluated immunoblot. Under the graph is shown a representative image of immunoblot data. The values (mean± SD) are expressed in arbitrary units of optical density (OD). (*p<0.05)

Myostatin, a member of the transforming growth factor-β superfamily, is almost exclusively expressed in the skeletal muscle, where it negatively regulates myocyte differentiation/growth and determines muscle size (Liu et al., 2008; Skipworth, 2007). It has been implicated in several forms of muscle wasting, including cancer cachexia and has been proposed to negatively regulate skeletal muscle mass (Mueller et al., 2016; Costelli, 2008). Binding of free myostatin to the Activin type IIB receptor (ActRIIB) results in the activation of transcription factors belonging to the SMAD family, although myostatin might also signals through other pathways, such as the Extracellular signal-Regulated Kinase (ERK)/Mitogen Activated Protein Kinase (MAPK) cascade (Costelli et al., 2008). Myostatin is increased upon inflammatory signalling, whereas it inhibits myoblast differentiation and increased FOXO3 activation (Zhou et al., 2010; Petruzzelli, 2016). The overexpression of this catabolic cytokine also affects the adipose tissue, where both reductions and increases in the size of adipocyte have been reported (Costelli et al., 2008). The absence of myostatin, as for instance in knockout mice ($Mstn^{-/-}$) leads to increased muscle mass and drives browning of WAT indirectly

through the AMPK-peroxisome proliferator-activated receptor g co-activator 1a-fibronectin type III domain-containing 5 signalling (Shan, 2013). This increase in myostatin levels supports cachexia-related muscle wasting, and precludes changes in adipose tissue metabolism, though not supported by alterations in adipokines' levels.

IV. Conclusions and future perspectives

In the present study, we set out to disclose the molecular mechanisms underlying cachexia in head neck cancer through the analysis of serum samples and its association with anthropometric data and tumour stage. Our results allowed us to conclude that:

i. BMI and tumour stage do not apparently relates with the establishment of cachexia in head and neck cancer, based on weight loss higher than 5% in 6 months reported by patients. Nevertheless, MUST and PG-SGA scores support a higher risk of malnourishment in patients from HNC+CC group.
ii. There are no apparent metabolic changes associated with HNC cachexia despite a trend to higher glucose levels, not supporting cachexia-related adipose tissue remodelling and muscle wasting.
iii. Inflammation does not seem to distinguish cachectic from non-cachectic patients in the set of head and neck cancer, given by CRP, TWEAK and albumin levels.
iv. The serum levels of the adipokines leptin and adiponectin and of the gastric hormone ghrelin do not evidence the contribution of WAT remodelling to head and neck cancer-related body weight loss.
v. The significantly higher serum levels of myostatin in the HNC+CC group supports the contribution of muscle wasting to the cachexia phenotype in head and neck cancer patients.

Taken together, our results support the contribution of muscle wasting but not of WAT remodelling to the development of cachexia in the set of head and neck cancer. Nevertheless, future studies should be performed involving a higher number of patients to better support our molecular and antropometric findings envisioning to improve the clinical management of patients in risk of cachexia. We believe that it will also be important to include information regarding body composition, such as evaluation of adipose folds and DEXA, and other experimental groups, such as a group of patients with pre-neoplasic lesions and a group of smokers with no cancer to better follow WAT remodelling and its interplay with muscle wasting in the pathogenesis of head and neck cancer. Focus should continue to be given to the molecular pathways underlying cancer cachexia envisioning to improve patients' response to therapeutics, quality of life and survival.

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VI. Appendix

Table 3. Table with the clinical information, concerning patient number, weight loss, tumour stage and location, MUST score, and serum levels of glucose, cholesterol, triglycerides, total protein, albumin and C-reactive protein of HNC+CC and HNC patients

	Patient Number	Weight Loss (Kg)	Tumour stage and location	Must score	Glucose (mmol/L)	Cholesterol (mmol/L)	Triglycerides (mmol/L)	Total protein (g/L)	Albumin (g/L)	CRP (mg/L)
HNC+CC	4	9 (in 5 months)	pT4N2c tongue	3	5.9	4.55	1.14	68	45	1
	1	7 (in 3 months)	cT4N3M0 oropharynx	0	5.2	4.92	1.20	73	43	40.1
	2	14 (in 2 months)	cT4N2M0 nasopharynx	2	5.3	3.48	1.71	76	40	9.30
	8	12 (in 6 months)	cT4N1M0 base of the tongue	0	6.1	No data	No data	76	43	71.7
	9	6 (in 6 months)	cT2N2M0 piriform sinus	1	5.5	3.89	0.78	74	34	145.9
	11	5 (in 5 months)	cT4N2M1 SUPRAGLOTE	No data	6.1	4.69	1.30	73	41	17.7
	12	6 (in 6 months)	cT3N2M1 Posterior pharynx wall	2	5.5	5.92	1.25	75	43	14.9
	5	10 (in 6 months)	cT4N0M0 oropharynx	No data	5.9	3.59	0.91	80	28	34.1
	15	27 (in 4 months)	cT4N2M0 tongue	4	5.3	3.80	1.31	66	36	55.7
	17	3 (in 1 month)	cT4N0M0 piriform sinus	1	4.4	5.47	0.91	82	37	4.40
	18	8 (in 3 months)	cT3N2M0 base of the tongue	No data	6.3	3.53	0.87	81	31	5.40
	10	1 (in 2 months)	T3N2M0 pharynx	0	6.3	5.57	1.01	73	42	7.82
HNC	6	2 (in 6 months)	cT4N2M0 pharyngolaryngeal	0	4.7	6.66	3.38	71	46	1.90
	13	0 (in 8 meses)	cT2N1M0 SUPRAGLOTE	0	4.8	6.00	1.76	77	47	2.10
	7	0.5 (in 7 months)	cT4N2M0 nasopharynx	No data	4.3	5.60	0.89	69	45	0.80
	3	1 (in 3 months)	cT4N3M0 h hypopharynx	1	4.8	4.49	1.32	74	42	47
	14	0 (in 6 months)	cT3N2M0 amygdala	1	5	3.09	1.59	69	36	58.2