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Cachexia in patients with head and neck cancer undergoing radiotherapy or concurrent chemoradiotherapy: Characterization, molecular mechanisms and relationships

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pela Professora Doutora Isabel Marques Carreira e pelo Professor Doutor Luís Pereira de Almeida e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2016

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UNIVERSIDADE DE COIMBRA

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Mestrado em Biotecnologia Farmacêutica

Cachexia in patients with head and neck cancer undergoing radiotherapy or concurrent chemoradiotherapy: Characterization, molecular mechanisms and relationships

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre, realizada sob orientação científica da Professora Doutora Isabel Marques Carreira, Professora Associada com Agregação da Faculdade de Medicina da Universidade de Coimbra e co-orientação do Professor Doutor Luís Pereira de Almeida, Professor Auxiliar da Faculdade de Farmácia da Universidade de Coimbra.

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Abstract

Head and neck cancer (HNC) is a type of neoplasm that comprises a heterogeneous group of biologically similar tumors. Those patients usually suffer from cachexia, responsible for unintentional weight loss in HNC patients with impact on quality of life (QoL) and survival rate. It is strongly associated to dysfunctional pathways in skeletal muscle and inflammatory response. Nowadays, there is increasing evidence that physical activity (PA) can modulate the inflammatory response in cancer, already established in colon, prostate, ovarian and breast cancer. The aim of this study was to characterize alterations on body composition, QoL, PA and cachectic biomarkers in HNC patients before, during and after the treatment. Additionally, it is also intended to correlate systemic inflammation markers and changes in expression of genes associated to the dysfunctional pathways with body composition, QoL and PA. Twenty-three HNC patients were recruited from Maxillofacial Surgery Department, Coimbra Hospital and University Centre before any treatment. The treatment lasted about 12 weeks and the assessment of the outcome measures were before, during and at the end of the treatment and also in the first follow-up consultation. The outcome measures include demographic and medical variables, body composition (assessed by bioimpedance), daily PA (International PA questionnaire), QoL (QLQ30–H&N35 module) and plasma levels of various biomarkers. The analysis of correlations between all the variables of the patients at their baseline state, before any treatment and the analysis of changes in each variable throughout the treatment regimen of each patient, were performed. It was possible to withdrawn from this project that cachexia was associated with higher inflammation states, worst QoL and higher sedentary lifestyles. These were still preliminary results, however it can be concluded that PA can modulate the inflammatory response in cancer, preventing the muscle degradation observed in cachectic patients.

Resumo

O cancro da cabeça e pescoço (CCP) é um tipo de neoplasia agressiva e clinicamente heterogénea. Doentes com este tumor experienciam perda de peso bastante significativa, resultado da radioterapia ou da quimioradioterapia concorrente com um grande impacto na qualidade de vida e taxa de sobrevivência destes doentes. A atividade física surge como um tratamento viável e efetivo, que poderá ter um impacto positivo na composição corporal e na qualidade de vida geral dos doentes de CCP. O objetivo deste estudo é caracterizar a alteração da composição corporal total, qualidade de vida, atividade física diária e biomarcadores de caquexia durante e após o tratamento. Também pretendemos estudar o impacto do tratamento nas vias moleculares envolvidas na regulação da caquexia, de forma a desenvolver novos modelos clínicos que poderão melhorar a qualidade de vida e sobrevivência destes doentes. Vinte e três doentes com CCP, diagnosticados nos últimos dois anos foram recrutados, antes de iniciarem o tratamento, para participar no estudo. O tratamento dura cerca de 12 semanas com avaliações antes do início do tratamento (semana 0), durante (semana 6), no fim do tratamento (semana 12) e 2 meses após o tratamento (consulta de acompanhamento). Estas avaliações incluem variáveis demográficas e médicas, composições corporais, qualidade de vida, atividade física, biomarcadores inflamatórios e de caquexia, e expressão génica. Foram analisadas correlações entre as diferentes variáveis antes do início do tratamento e alterações em cada variável ao longo do tratamento para cada doente. Foi possível averiguar que a caquexia está associada a maiores estados inflamatórios, pior qualidade de vida e estilos de vida sedentários. Este estudo ainda se encontra numa fase preliminar, mas pode-se concluir que o exercício físico pode modular a resposta inflamatória associada ao cancro e a degradação de massa muscular que lhe está associada.

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Index of Abbreviations

Alb	Albumin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
BMI	Body mass index
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CRP	C - reactive protein
CRT	Chemoradiotherapy
CT	Chemotherapy
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EORTC QLQ C30	European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire core 30
FBXO32	F-box protein 32
FFM	Free fat mass
FFMI	Free fat mass index
FM	Fat mass
FMI	Fat mass index
Fn14	Fibroblast growth factor-inducible 14
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	Gamma-glutamyl transpeptidase
GUSB	Glucuronidase beta
HADS	Hospital anxiety and depression scale
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell cancer
HPRT1	Hypoxanthine phosphoribosyl transferase 1

HPV	Human papillomavirus
IFN-γ	Inteferon gamma
IL-1	Interleukin 1
IL-Ira	Interleukin-Ira
IL-4	Interleukin-4
IL-6	Interleukin 6
IL-10	Interleukin-10
IL-15	Interleukin-15
IPAQ-SF	International Physical Activity Questionnaire – Short Form
LDH	Lactate Dehydrogenase
MET	Multiples of the rest metabolic rate
MuRF1	Muscle RING-finger protein-1
NCBI	National Center for Biotechnology Information
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NF-κb	Nuclear factor κb
P	P-value
PA	Physical activity
PF	Primer forward
PR	Primer reverse
QLQ30–H&N35	Quality of Life Questionnaire for Head and Neck Cancer module
QoL	Quality of life
qPCR	Real-time quantitative polymerase chain reaction
R	Pearson correlation value
RT	Radiotherapy
sIL-6R	Soluble interleukin-6 receptor
SMM	Skeletal muscle mass
sTNFR	Soluble tumor necrosis factor receptors

SX	Surgery
T.BIL	Total bilirubin
TBW	Total body water
TNFSF12	Tumor necrosis factor (ligand) superfamily, member 12
TNF-α	Tumor necrosis factor alpha
TRIM63	Tripartite motif containing 63
TWEAK	TNF-like weak inducer of apoptosis
UPS	Ubiquitin-proteasome system
Z	Z statistics value
β-Actin	Beta-actin

1. Introduction

1.1. Head and Neck cancer

Head and neck cancer (HNC) is a type of cancer that comprises a heterogeneous group of biologically similar tumors, that arise from the upper aerodigestive tract, the salivary glands, thyroid and parathyroid glands, paranasal sinuses and the skin of the head and neck (Figure 1) (Safdari [et al.], 2014, Sammut [et al.], 2014). Moreover, often these cancers occur within the mucosal membrane of the throat, mouth and nose and are designated head and neck squamous cell cancer (HNSCC) (Marur and Forastiere, 2008, Safdari [et al.], 2014). HNSCC displays severe malignant phenotypes, which translates into an extensive invasion of surrounding tissues. This acute spreading leads to metastization of distant organs, even at an early stage of the cancer (Safdari [et al.], 2014).

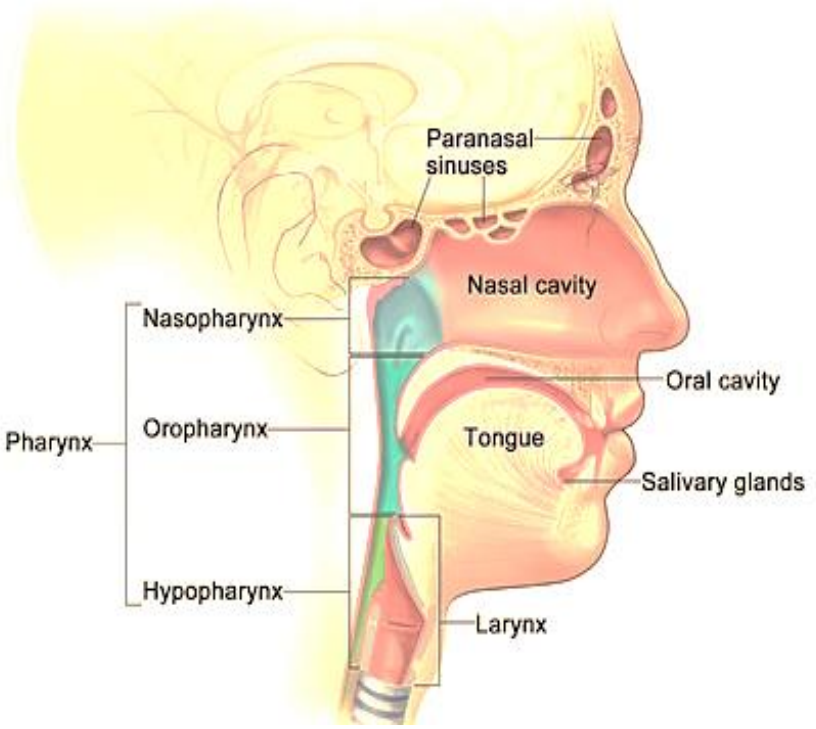

Figure 1 Illustration of HNC regions. Location of paranasal sinuses, nasal cavity, oral cavity, tongue, salivary glands, larynx, and pharynx (including the nasopharynx, oropharynx, and hypopharynx). Adapted from http://www.cancer.gov/types/head-and-neck/head.

1.1.1. Epidemiology

Globally, the annual incidence of HNC is more than 550.000 cases with around 300.000 deaths each year, with HNSCC being the most abundant, around 90% of all cases (Argiris [et al.], 2008, Safdari [et al.], 2014). Two-thirds of HNC cases are found in developing countries, with the higher incidence rates in India, Australia, Central and Western Europe and South Africa (Parkin and Bray, 2006). The higher mortality rates for oral cancer in Europe are in Hungary and Slovakia (Garavello [et al.], 2010). In Portugal, 2233 new cases were diagnosed in 2008, with a male/female ratio of 5:1 (**Table 1**). When broken down, 37% of the cancer cases are on the oral cavity, 28% on the pharynx, 25% on the larynx and 10% on the nasal cavity and glands. It occurs more frequently in men than women, about 2 to 3 times, and the incidence increases with age in all countries. This relates to the fact that man are known to consume more alcohol and tobacco and to be more exposed to occupational hazard. In etiological terms the consumption of alcohol and tobacco probably explain 90% of HNC cases (Johnson, 2001).

Table 1 Number of new cases, by topography, in Portugal, 2012. Data retrieved by Globocan.

Site	Males	Females	All	M:F ratio
Lip	124	47	171	3:1
Tongue	272	86	358	3:1
Mouth	227	65	292	3:1
Salivary Glands	81	60	141	1:1
Oropharynx	232	19	251	12:1
Nasopharynx	75	28	103	3:1
Hypopharynx	204	6	210	34:1
Pharynx	44	10	54	4:1
Nasal cavity and middle ear	56	35	91	2:1
Larynx	530	32	562	16:1
Head and neck cancer	**1845**	**388**	**2233**	**5:1**

1.1.2. Etiology and risk factors

1.1.2.1. Alcohol and tobacco

Alcohol and tobacco are responsible for at least 75% of HNC in Europe and United States (Hashibe [et al.], 2007, Marron [et al.], 2010). Several studies have established a link between cancer of the oral cavity and tobacco and/or alcohol use, however the risk associated with smoking is higher than with the consumption of alcohol (Hashibe [et al.], 2009). In the tobacco and alcohol area, there are many factors that can affect HNC tumor development, such as site, sex, amount of smoking/drinking, years of smoking/drinking, type of tobacco/alcoholic beverage (Castellsague [et al.], 2004, Marron [et al.], 2010, Reidy [et al.], 2011).

1.1.2.2. Comorbidity

Aging, as it is widely known, is the most important reason for cancer development, mostly because of the higher risk of developing other diseases (Rowland and Yancik, 2006). Because of tobacco use, the most often comorbidities within HNC patients are cerebrovascular, pulmonary and cardiovascular disease, and it is reported that the high frequency of comorbidities can be linked to poor survival rate (Reid [et al.], 2001). It is known that patients with later stage disease have a higher dead risk, so the presence of additional comorbidities can limit treatment options and outcomes (Gourin [et al.], 2005).

1.1.2.3. Diet

Dietary and nutritional determinants have been suggested as etiological agents, despite being less well understood and less significant (Grobbelaar [et al.], 2004). Fruits and vegetables have been associated with beneficial effects, whereas the high consumption of animal fats may be a risk factor in developing HNC (Lucenteforte [et al.], 2009). The Mediterranean diet and diets with high intake of essential nutrients, such as iron, beta carotene, vitamin C, vitamin E, zinc, have been associated with protective effects against HNC (Li [et al.], 2014).

1.1.2.4. Oral health

There have been an increasing number of studies that correlates patients' oral health with their overall health. For HNC patients, they have already found a relationship between some dental diseases and some types of HNC, independent of tobacco use (Divaris [et al.],

2010, Guha [et al.], 2007). *Homann et al.* also suggested a link between certain salivary enzymes with poor dental hygiene, which may interact with alcohol and increase the risk of cancer in the oral cavity (Homann [et al.], 2000).

1.1.2.5. Virus and bacteria

The Human papillomavirus (HPV) is the most important virus involved in the development of HNC. In 1975, Byers was the first to report a new subset of HNC patients, younger than 30 years and with tongue cancer (Byers, 1975). Then, Shantz and Yu analyzed the incidence of tongue cancer in individuals with less than 31 years between 1973 and 1997 and found a growth of 60%, whereas there was no rate change in the other age classes (Schantz and Yu, 2002). Only in 2005 were these cases linked to positive oral HPV, mainly the 16 and 18 strains (Kreimer [et al.], 2005). HPV is a sexually transmitted disease, most well-known for its role in cervical cancer, which can be spread through oral sex (Ragin [et al.], 2007). With the increase of oral sex between younger populations, it is predicted an escalation of the incidence of HPV-related oral cancer in the upcoming decades (Campisi and Giovannelli, 2009). The Epstein-Barr virus (Kang [et al.], 2015), human immunodeficiency virus (D'souza [et al.], 2014), gastroesophageal reflux disease (Langevin [et al.], 2013) and oral lichen planus (Barnard [et al.], 1993) are also potential biological and viral factors, mostly because of immunosuppression and inflammation of the esophagus and larynx region (O'sullivan [et al.], 2014).

1.1.2.6. Genetic background

Only a small portion of individuals exposed to carcinogens develop the disease, so it is clear that an intrinsic susceptibility has an important role, especially in younger patients. There is evidence that family inheritance plays a decisive role in its onset (Warnakulasuriya, 2009). However, in oral cancer, the relative risk in patients who reported cases in the family is smaller than in the pharynx or larynx cancer. The biggest problem in the interpretation of this data is to distinguish if the disease occurs due to heredity or due to similar lifestyle among family members (Negri [et al.], 2009). There are no known inheritable polymorphism that may contribute to the development of oral cancer. However, the exposure to carcinogens lead to various molecular events which promote cumulative changes in specific genes. Genomic instability has been repeatedly established as an early sign of malignant transformation (Warnakulasuriya, 2009).

Cabanillas and colleagues have already described the relevance of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and its role in the development of HNSCC and melanomas when it has a germline mutation. Despite germline mutations in *CDKN2A* only have been reported in the literature in no more than 10 families, this author highly recommends the screening of *CDKN2A* mutations for differential diagnosis of familial HNSCC and the need to sensibilize the carriers of these mutations (Cabanillas [et al.], 2013). Boedeker and colleagues also suggested a molecular genetic screening of all patients with head and neck paragangliomas for germline mutations, after their research showed that up to one third of these are associated with various tumor syndromes, and 90% of those were associated with mutations on various succinate dehydrogenase genes (Boedeker [et al.], 2014).

1.1.3. Treatments

The management of all cancer patients poses a significant challenge for clinicians. For those with HNSCC, the critical goal in cancer treatment is the survival of the individual and the quality of patient's life, however preservation of the voice is also taken into consideration in the selection of the treatment. If possible, it is also important to preserve other organisms' key functions, such as swallowing, salivating and breathing (Gourin [et al.], 2009, Sammut [et al.], 2014). The treatment will depend on the specific sub-site of the primary tumor and on tumor stage. Patient performance status, age, other co-existing diseases, patient's wishes and potential for recurrences after treatment are other factors to take into consideration (Gelbard [et al.], 2006, Gourin [et al.], 2005). HNSCC treatment usually involves either surgery (SX), radiotherapy (RT), chemotherapy (CT) or any combination of the above.

1.1.3.1. Clinical Work-up

Throughout all treatment, a multidisciplinary team of health care professionals made of a head and neck surgeon, a clinical oncologist, a radiologist, a pathologist, a dietician, a reconstructive plastic surgeon, a maxillofacial surgeon and a dentist should supervise all procedures and treatment decisions. HNC patients should undergo a computerized tomography scan or a magnetic resonance imaging to determine the extent of the tumor, starting at the base of the skull, over the neck, and stopping at the thorax, to look for metastases. This is followed by a thorough clinical examination with biopsies, to exclude other tumors, and an ultrasound-guides aspirations to look for regional metastasis (NCCN,

2009). The patients are then staged according to the TNM staging system and a treatment plan is established.

TNM staging system:

- T (1–4): size or direct extent of the primary tumor:
  - Tis: carcinoma in situ;
  - T0: no evidence of primary tumor.
- N (0–3): degree of spread to regional lymph nodes:
  - N0: tumor cells absent from regional lymph nodes;
  - N1: metastasis in a single lymph node (same-side), 3 cm or less in size;
  - N2a: metastasis in a single lymph node (same side), more than 3 cm but not more than 6 cm in greatest dimension;
  - N2b: metastasis in multiple lymph nodes (same-side), none more than 6 cm in greatest dimension;
  - N2c: metastasis in bilateral or opposite-side lymph nodes, none more than 6 cm in greatest dimension;
  - N3: metastasis in a lymph node more than 6 cm in greatest dimension.
- M (0/1): presence of metastasis:
  - M0: no distant metastasis;
  - M1: metastasis to distant organs.

Use of an "X" instead of a number means that the parameter was not assessed (NCCN, 2009).

1.1.3.2. Surgery

SX was the first available treatment for HNC patients, but the morbidity and disfigurement rates were high, and patients would have, more often than not, difficulties swallowing, breathing and speaking, which greatly affected the patient's quality of life (QoL) (Safdari [et al.], 2014). Over the last two decades, SX has progressed in efficacy and sophistication, mainly because of micro-vascular techniques, with reconstruction of soft-tissues, bone, and only the removal of the tumor and enough surrounding tissues (Hanasono, 2014, Novakovic [et al.], 2009). These new techniques lessen the aesthetic and functional limitations that SX can cause.

SX applied as therapeutics has two main goals, the resection of the tumor mass and involved tissue (lymph nodes) and the removal of endocrine organs that may affect the spreading of the disease. SX was the only treatment available for HNC patients until RT was

introduced, and RT was initially intended to replace SX as treatment. This has not been the case, instead more often than not, the two modalities are used together (Colledge, 1938).

1.1.3.3. Radiotherapy

Radiation therapy is the use of ionizing radiation as a therapy agent to destroy tumor cells. The dose of radiation being employed is calculated to the volume of tumor tissue and applied for a determined period of time. It is necessary to take care not to damage the surrounding tissues, for future tissue regeneration shall be at the expense of these (Baskar [et al.], 2014). Ionizing radiation may be corpuscular (electrons, protons and neutrons) or electromagnetic (X and gamma photons), the latest being used in most RT treatments. This radiation will interact with the cells' DNA, in a direct and/or indirect way. In the direct form, the DNA molecule is cleaved by the radiation, interfering directly in the cell replication process. The indirect way occurs through hydrolysis of water, which leads to disruption of DNA strands and, finally, to cell death or inability to replicate. The indirect way ends up being the most frequent process and the most important, since water is the element in larger quantities in cells (Jham and Da Silva Freire, 2006).

RT can be of two types, external and internal, and the choice between the two types is performed depending of the characteristics and location of the tumor and the treatment goal. In more advanced cases, when SX is not possible, and in which the prognosis is severe, CT combined with RT is applied (Russo [et al.], 2008).

1.1.3.4. Chemotherapy

Chemotherapeutic agents are chemical compounds used to destroy or prevent the proliferation of cancer cells, but that also cause toxicity in normal tissues with high cell turnover rate. The chemical compounds more effective in CT are methotrexate, bleomycin, cisplatin, fluorouracil, among others (Wen and Grandis, 2015). Cisplatin, said to be the most effective compounds, is usually administered for 3 to 4 weeks at a dose of 80 to 100mg / m^2 (Savvides, 2010).

Originally, CT was given to patients who could not be submitted to SX and where conventional RT was unlikely to be curative. Nowadays, CT by itself is usually used only in palliative treatment. In curative treatment, it is always given in combination with RT either before RT (as induction or neoadjuvant), at the same time as RT (as concomitant or concurrent), or after surgery (as adjuvant) (Choong and Vokes, 2008). The combination of RT and CT is reported to improve loco-regional control and survival rates, at least in

patients with advanced disease. There is also an increase of the use of chemoradiotherapy (CRT), since it was reported to reduce distant relapse (Brizel and Vokes, 2009, Malone and Robbins, 2010).

The limitations of CT are its side effects, in particular the immediate side effects, but there is growing evidence of higher rates of late toxicity side effects as well (Bentzen and Trotti, 2007).

1.1.3.5. Emerging drugs

Therapies that specifically target cellular pathways associated with carcinogenesis are now routinely used in many areas of oncology, as they increase survival and reduce treatment toxicity. Both RT and CT as treatment for HNC, have improved over the years, however they have almost reached their limit of effectiveness when balancing toxicity and efficacy (Bentzen and Trotti, 2007). Consequently, the focus has now turned to the development of molecular targeted therapies specific to the biology of HNC. Already well documented are the therapies associated with epidermal growth factor receptor (EGFR), but it is necessary to develop new mechanisms of action for novel clinical therapies (Fung and Grandis, 2010). In table 2 are depicted current FDA-approved drugs for the treatment of HNSCC, including five conventional CT drugs (cisplatin, methotrexate, 5-flurouracil, bleomycin, and docetaxel) and one targeted agent (cetuximab). Emerging investigated drugs in clinical trials are mostly related to targeting a variety of downstream molecular pathways, which aberrant activation are associated with tumor growth and angiogenesis. It also comprises gene therapy immunotherapy agents (Wen and Grandis, 2015).

Table 2 Currently applied drugs and compounds for HNSCC treatment (Wen and Grandis, 2015).

Compound	Mechanism of action
Cisplatin	DNA synthesis inhibitor
Methotrexate	Thymidylate synthase inhibitor
5-Flurouracil	DNA and RNA synthesis inhibitor
Bleomycin	DNA inhibitor
Docetaxel	Microtubule stimulant
Cetuximab	EGFR mAb

1.1.4. Complications and side effects

It is evident that this disease have the potential impact of impairing some of the most basic and personal functions for patients. Vital structures are likely to be damaged and will, as a consequence, compromise patients with far-reaching disease and treatment side-effects. Due to the location of the tumor, patients will often have difficulty swallowing, breathing and speaking, which will affect greatly the patient's QoL (Sammut [et al.], 2014). Not only will it lead to various side effects, such as xerostomia (dry mouth), dysphagia (difficulty of swallowing), weight loss and fatigue, but also challenge their ability to perform daily physical, social, emotional and psychological functions (Denaro [et al.], 2013, Sammut [et al.], 2014).

SX might affect the patient's ability to chew, swallow or talk, and the neck may become swollen (Kerawala and Heliotos, 2009). Shoulder disability, shoulder pain, reduced cervical mobility, carotid blow-out bleeding and lymphedema are other complication with a later onset (Kerawala and Heliotos, 2009, Van Wilgen [et al.], 2004). The type of SX performed and the preoperative status of the patient may affect the degree of postoperative complications (Proctor [et al.], 2004, Sanabria [et al.], 2008).

Patients who receive radiation or CT may experience redness, irritation, dry mouth or thickened saliva, difficulty in swallowing and nausea. Loss of taste and stiff jaw are also problems that often occur after this type of therapy, which may decrease appetite and affect nutrition (Wall [et al.], 2013). CT used to treat HNC can cause many of the same complications as radiation particularly if the treatment is prolonged and in high dosage. Some are acute, such as mucositis, dermatitis, infections, hyposalivation and taste dysfunction while others are late, such as fibrosis of soft tissues and necrosis (Glastonbury [et al.], 2010). The adverse events are graded on different scales according to their severity. However, unlike CT the radiation therapy is anatomically site-specific, and the risk of oral complications tends to be permanent, due of the unavoidable tissue damage (NIH, 2016).

The most critical side effect caused by this cancer and/or its treatment is the involuntary and severe weight loss, suffered by 38% to 82% of patients with HNSCC. It may occur as a consequence of physical disability, but also due to a metabolic disorder (Silver [et al.], 2007).

1.1.5. Poor prognosis

Recurrence rates are high in these tumors. Metastatic or recurrent HNC have a poor prognosis, with an average survival of 6-10 months (Lopez [et al.], 2014, Richey [et al.],

2007). The high incidence of distant metastasis, second primary tumors, and co-morbidities are the reason for the poor survival rates of HNC patients (Gleich [et al.], 2003).

The heterogeneity of both the patient and disease presentation means that definitive prognostic information is difficult, because it all depends on various factors, variation of tumor site, size and pathological classification, diversity of treatment options, the presence of co morbidities (Omura [et al.], 2016). It is necessary a precise interpretation of such factors as a unit, and that is a difficult task to accomplish.

Predictions about treatment success are not amenable to percentage answers. There are, however, some studies that allow us to predict the relative treatment success, according to some factors. One and two-year survival is better in patients who present tumors in the larynx rather than oral cavity or pharynx. Persistent hoarseness, a feature of laryngeal cord cancer, is amenable to early detection, so it is much easier to identify cancers in its initial stage (Exarchos [et al.], 2012). A better outlook has been found for patients diagnosed with oral cancer HPV 16 positive which seems more susceptible to induction CT compared to tumors that are HPV negative. There is also better long-term survival because it appears to be less likely of recurrence from tumors fitting into this pathological classification (Fakhry [et al.], 2008).

1.2. Cancer Cachexia

The referred weight loss, which occurs despite adequate caloric intake, is known as cachexia syndrome. This syndrome is not well understood and the underlying mechanisms are still being explored. It was, consequently, essential for researchers and clinicians to agree upon a universal definition for cachexia (Evans [et al.], 2008). Therefore, in Cachexia Consensus Conference held in Washington DC in December 2006, the agreed definition was:

> *"Cachexia, is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity."*

1.2.1. Signs and symptoms

Cancer cachexia is a condition that is associated with many forms of cancer (Vaughan [et al.], 2013). It is characterized by weight loss related to muscle wasting, but also disturbances in energy balance, and changes in carbohydrate, lipid and protein metabolism (Battaglini [et al.], 2012, Silver [et al.], 2007). These effects have been associated with increased morbidity and significant lower survival rate, becoming an independent mortality predictor in patients with cancer in stage III and IV (Silver [et al.], 2007). In its final form, it may eventually lead to progressive physical disability and impairment of respiratory function (Von Haehling and Anker, 2010). It is been reported a prevalence up to 80% in patients with cancer in an advanced stage, and that approximately 25% of all cancer deaths are resultant of cachexia alone, (Richey [et al.], 2007, Von Haehling and Anker, 2010).

In HNC patients, it is often difficult to determine accurately the reason for weight loss (Denaro [et al.], 2013). Due to the location of the tumors, the patients may be experiencing weight loss because of mechanical obstruction, which will lead to starvation (Von Haehling and Anker, 2010). However, weight loss due to cancer cachexia appears to be the result of dysfunctional cellular pathways, such as the ubiquitin-proteasome system (UPS), a muscle cellular component and the dystrophin glycoprotein complex, which facilitates muscle catabolism (Macdonald [et al.], 2003); Also, tumor-derived catabolic factors and circulating proinflammatory cytokines have been reported to increase muscle and adipose tissue catabolism (Macdonald [et al.], 2003, Seruga [et al.], 2008). Recent research suggest that the loss of muscle protein is mediated not only by increased protein catabolism, but also decreased protein anabolism. It may appear that the dynamic of both processes will ultimately account for the progressive muscle wasting seen in cancer cachexia (Glass, 2005, Macdonald [et al.], 2003).

1.2.1.1. Inflammation

Both researchers and clinicians stress the importance of the study of links between tumor-derived factors and muscle wasting (Dodson [et al.], 2011). Recent studies associated anorexia and weight loss with animal and human models, suggesting a role for pro-inflammatory cytokines in cancer cachexia with emphases in interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (Fearon [et al.], 2012, Gelin [et al.], 1991).

C - reactive protein (CRP) is also correlated to systemic inflammation, and its presence is typical in acute phase response (Deans and Wigmore, 2005). Its synthesis is

regulated by IL-6 and occurs mostly in the liver, however a small part of CRP is produced in the smooth muscle cells of diseased blood vessels (Bassuk [et al.], 2004). CRP intervenes in the inflammatory response by acting as an opsonin, which enhances phagocytosis. Additionally, it activates part of the complement pathway, but limits the activation of the more prominent inflammatory responses (Black [et al.], 2004). CRP levels are shown to temporarily increase with the practice of intense physical activity, due to the inflammatory response against muscle damage and has been positively correlated with weight loss (Barber [et al.], 1999, Kasapis and Thompson, 2005).

As a negative acute phase protein, albumin (Alb) is a clear indicator of the inflammatory state in many types of disorders (Don and Kaysen, 2004). Alb is the major constituent of plasma, representing around 60% of plasma protein composition (Vincent, 2009). It is synthetized in the liver and then released into the vascular system where it plays a role in maintaining the membrane's permeability and osmotic pressure (Soni and Margarson, 2004). It is also involved in the transportation of circulating substances and it intervenes in the coagulation pathway, acting as an anticoagulant (Soni and Margarson, 2004, Vincent, 2009). Albumin's beneficial effects are said to be proportional to its plasma concentration, however no reports have yet uncovered the involved function (Doumas and Peters, 1997). Moreover, various authors state that the levels of serum Alb may act as indicators of the nutritional status and predictors of mortality in cancer patients, even if these studies are still limited when pertaining HNC (Danan [et al.], 2016, Doumas and Peters, 1997).

Lactate dehydrogenase (LDH) is an enzyme involved in the glycolysis and the Cori cycle and is normally associated to a poor prognosis in various types of cancer (Jin [et al.], 2013, Miao [et al.], 2013). It is mostly present in the heart, kidneys, liver and muscle. In blood plasma LDH levels are relatively low, however they tend to increase in diseased states (Jin [et al.], 2013). More specifically, in cancer situations the high energetic needs of cancer cells raise the protein levels in the blood flow. Inflammation and tissue damage are also related to higher levels of circulating LDH (Miao [et al.], 2013).

1.2.1.2. Muscle Wasting

The main reason for cachexia development is the increase in energy expenditure due to an elevated basal metabolic rate, which leads to an raised rate of protein turnover without an appropriately significant increase in protein synthesis (Barber [et al.], 1999). The pathway regulating protein breakdown is the adenosine triphosphate ubiquitin-dependent pathway. This has been shown to be upregulated in cancer cachexia and can be modulated

by the presence of pro-inflammatory cytokines (Fearon [et al.], 2012, Tsoli and Robertson, 2013). Titin, the third most abundant protein present in skeletal muscle, has been associated with muscle atrophy and cachexia (Peng [et al.], 2005).

1.2.1.3. The ubiquitin-proteosome system

The UPS is an important pathway for protein degradation, namely in the muscle, and it seems to be up-regulated in the presence of cancer cachexia (Macdonald [et al.], 2003). In normal situations, ubiquitin, with the help of some enzymes, will recognize and connect to the protein, such as myosin's heavy chains, to be degraded by the proteasome (Bonaldo and Sandri, 2013, Macdonald [et al.], 2003). With the manifestation of cachexia, the presence of abnormal quantities of pro-inflammatory cytokines will induce skeletal muscle proteolysis through the suppression of muscle genes and the activation of UPS. Therefore, the myosin's heavy chains expression will be inhibited, leading to the dissociating of myosin from the muscle. This free myosin will also be degraded by the cytokines-activated UPS (Battaglini [et al.], 2012, Macdonald [et al.], 2003). Recent evidence also suggests that the cytokine TNF-like weak inducer of apoptosis (TWEAK), mediates skeletal muscle atrophy that occurs under denervation conditions. The TWEAK fibroblast growth factor-inducible receptor 14 (Fn14) is overexpressed in muscle, which will lead to the activation of nuclear factor κB (NF-κB), a major pro-inflammatory transcription factor that is strongly linked to skeletal muscle wasting (**Figure 2**). The activation of NF-κB is enough to trigger the expression of Muscle RING-finger protein-1(MuRF1) and atrogin-1, an ubiquitin ligase that breaks down the heavy chain of myosin, which is required for the UPS pathway (**Figure 3**) (Battaglini [et al.], 2012, Kumar [et al.], 2012, Mittal [et al.], 2010).

1.2.1.4. Expression of genes in skeletal muscle

The impact of RT or concurrent chemoradiation therapy on the molecular pathways involved in the regulation of lean muscle mass can be studied through the changes in expression of genes associated to atrophy and hypertrophy pathways in skeletal muscle. In this project, the focus will be on F-box protein 32 (*FBXO32*); tumor necrosis factor (ligand) superfamily, member 12 (*TNFSF12*); tripartite motif containing 63 (*TRIM63*); and nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*).

1.2.1.5. TNFSF12

TWEAK is the protein encoded by *TNFSF12* and is a cytokine that belongs to the TNF ligand family (Kumar [et al.], 2012). It has overlapping signaling functions with TNF, however TWEAK has a more wide presence in tissues and can induce multiple apoptosis pathways when binding with the Fibroblast growth factor-inducible 14 (Fn14), a TWEAK receptor. Both TWEAK and the Fn14 receptor have been associated to the regulation of muscle mass and have been implicated in the progression of muscle atrophy (**Figure 2**) (Kumar [et al.], 2012, Mittal [et al.], 2010).

1.2.1.6. NFKB1

The *NFKB1* gene encodes a protein from the NF-κB protein complex. NF-κB is a transcription regulator activated by various cytokine. When incorrectly activated, NF-κB can trigger many inflammatory diseases while continuous inhibition of NF-κB leads to disorders in the immune cell development or irregular cell growth (**Figure 2**) (Cai [et al.], 2004, Mittal [et al.], 2010, Moore-Carrasco [et al.], 2007).

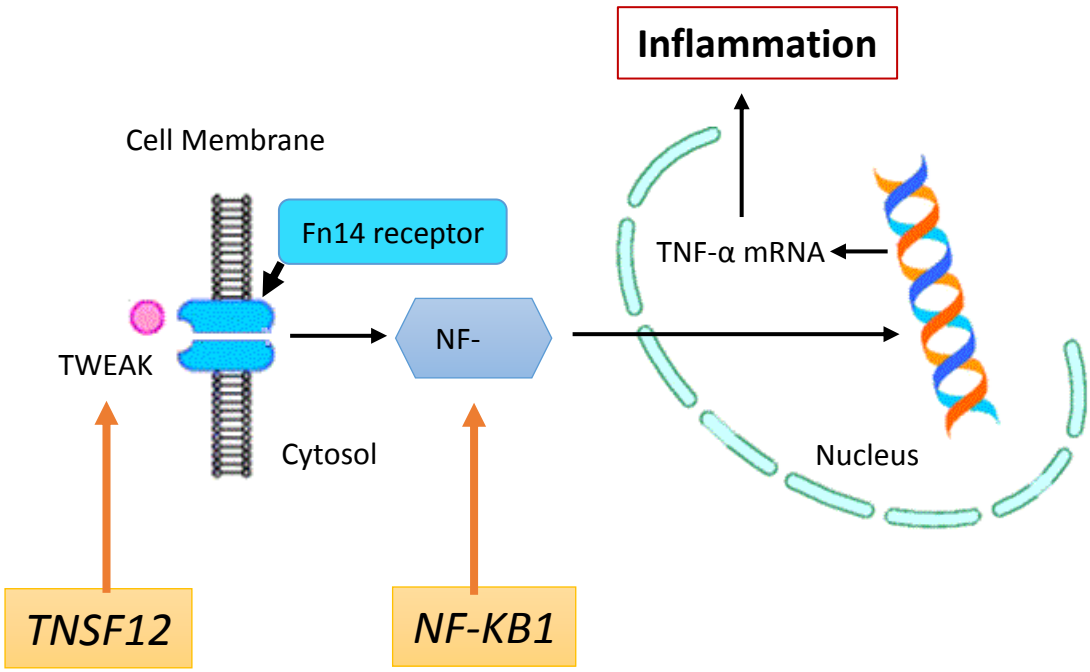

Figure 2 The TWEAK Fn14 receptor activates NF-κB, a transcription factor involved in the activation of the transcription of TNF-α, a pro-inflammatory cytokine. Adapted from (Kung [et al.], 2011).

1.2.1.6.1. *FBXO32*

The *FBXO32* gene encodes for the F-box 32 protein, also known as atrogin-1. It integrates the SKP1-cullin-F-box, of the ubiquitin protein ligase complex and is responsible for the phosphorylation-dependent ubiquitination. In situations of muscle atrophy this protein is reported to be highly expressed. Furthermore, some authors implied that mice deficient in this gene developed resistance to atrophy (**Figure 3**) (Guo [et al.], 2014).

1.2.1.6.2. *TRIM63*

The *TRIM63* gene is specially expressed in striated muscles. It encodes the E3 ubiquitin ligase, part of the RING zinc finger protein family, that plays an essential role in the UPS. It locates the Z-line and M-line of myofibrils, and it guides the myosin proteins for degradation (**Figure 3)** (Mittal [et al.], 2010, Su [et al.], 2014).

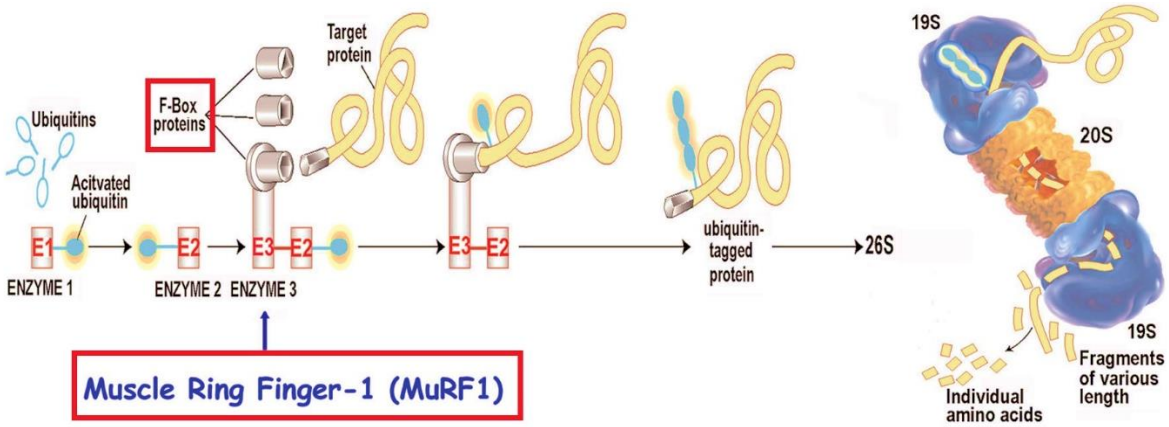

Figure 3 The ubiquitin proteasome pathway, with particular relevance of the involved genes analyzed in this study, namely *FBXO32* and MuRF1 (*TRIM63*) (Outlined in red). Adapted from: (Rajan and Mitch, 2008, Sassoon and Caiozzo, 2009).

1.2.2. Treatment

There is no optimum treatment available for cancer cachexia and treatment until now has been focused on treating the signs and symptoms. However, treating cachexia symptoms has been somewhat difficult as it is only detected in more advanced situations and by then the available therapy nowadays is unable to fully treat its symptoms. Therefore, new detection methodologies are needed in order to intervene in this syndrome in its early stages (Seelaender [et al.], 2015). Presently, the most used treatments modalities for cachexia are dietary consultation and emotional support. The treatments that are being now

designed are attempting to target the pathophysiological pathways that cause cancer cachexia as these become better understood, however most of them are still experimental and should only be used in clinical trials (Evans [et al.], 2008).

Currently, only two drugs can be prescribed in Europe, namely pro-gestational agents, such as megestrol acetate or medroxyprogesterone acetate, and corticosteroids. Of the new drugs that are currently undergoing clinical trials, we can categorize them in one of the following treatment modalities:

- Anabolic androgens;
- Ghrelin;
- Nonsteroidal anti-inflammatory drugs;
- Corticosteroids;
- Anti-cytokines;
- Omega-3 fatty acids;
- Pro-gestational agents;
- Antioxidant agents.

In the moment, there are 18 clinical trials in development, designed to test interventions for cancer cachexia. However, given that this syndrome is complex and involves a myriad of dysfunctional pathways, new modes of therapy may evolve a simultaneously inhibition of the catabolic and stimulation of the anabolic pathways in skeletal muscle (Couch [et al.], 2015).

1.2.3. Cachexia Modulation

In order to improve health-related QoL, physical functioning status, and potentially to improve overall prognosis, understanding the changes that occur during and after HNC treatment are essential to develop clinical models, help his avert weight loss and changes in total body composition of HNC patients.

1.2.3.1. Physical Activity

Nowadays, there is increasing evidence that physical activity (PA) can modulate the inflammatory response in cancer, already established in colon, prostate, ovarian and breast cancer. The decreased risk of mortality was up to 41%, also with an improvement in QoL (Sammut [et al.], 2014). It is recommended by the American Cancer Society that all cancer survivors undergo PA for at least 150 min/week and strength training exercises at least

twice a week (Kushi [et al.], 2012). However, after diagnosis in HNC patients, because of treatment-related symptoms, such as fatigue, swallowing difficulties, shortness of breath and pain, only approximately 8.5% of the patients are meeting the current guidelines (Sammut [et al.], 2014).

Recent studies suggest that PA induces the production of anti-inflammatory cytokines, such as interleukin-1ra (IL-1ra), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-15 (IL-15), soluble tumor necrosis factor receptors (sTNFR) and soluble interleukin-6 receptor (sIL-6R), which will induce protein synthesis in the muscle (Battaglini [et al.], 2012, Duffy [et al.], 2013, Sammut [et al.], 2014). Some studies also suggest that cancer patients are able to correct the metabolic imbalance caused by cachexia through regular exercise training. (Battaglini [et al.], 2012). In Figure 4 is described a hypothetical model of how physical exercise promotes such events. Cancer and some of the treatments can promote an increase in the production of pro-inflammatory cytokines, inducing pathways in muscle tissue losses (Battaglini [et al.], 2012, Nicolotti [et al.], 2011). This continuous muscle loss will lead to a decrease in functional state and QoL, with an ultimately loss of health in the patient. On the other hand, a regular regimen of physical exercise is able to promote the production of anti- inflammatory cytokines and reduce the expression of pro-inflammatory cytokines (Battaglini [et al.], 2012).

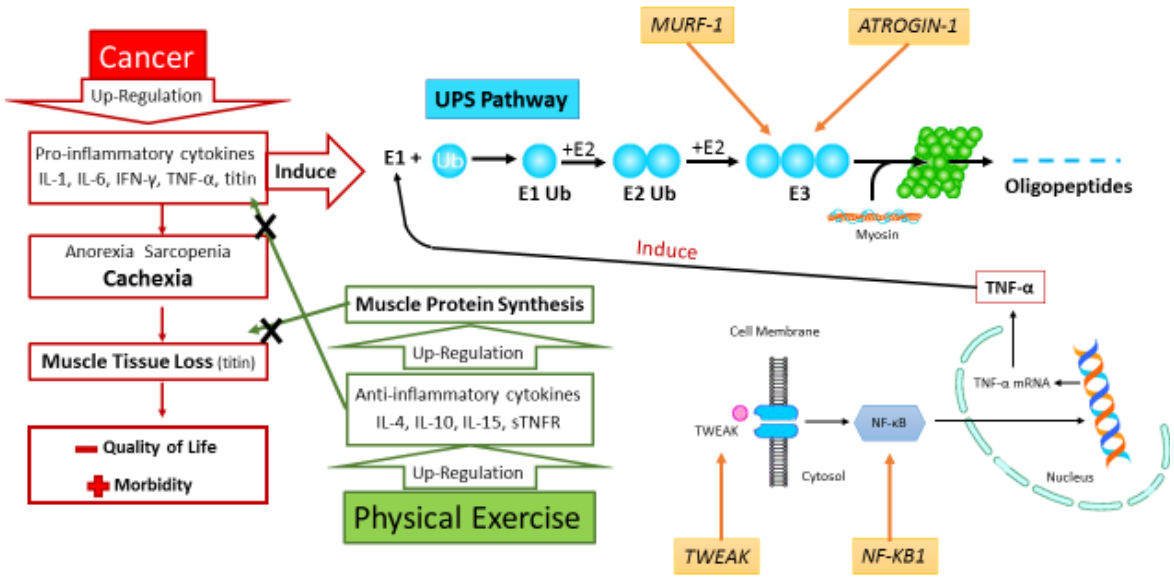

Figure 4 Physical Activity as a modulator of inflammatory response in cancer and the related genes of the ubiquitin-proteasome system (UPS). Adapted from: (Battaglini [et al.], 2012)

1.2.3.2. Diet

Cytokines also act in the hypothalamus to inhibit orexigenic and stimulate anorexigenic regulatory pathways, so the peripheral signals of hunger reach the hypothalamus but fail to produce a response because of the effects of cytokines, propagating the cachectic process (Couch [et al.], 2015).

Over 50% of patients with cancer complain of decreased appetite, which can be the result of cancer treatments or the cachexia syndrome. A specific cancer treatment symptom that can affect food intake in patients with HNC is the presence of dysphagia. Nevertheless, the manifestation of dysphagia does not seem to increase the incidence of cachexia in HNC patients (George [et al.], 2007). Unlike starvation, nutrition alone cannot correct the metabolic changes that accompany cachexia. Especially with HNC patients, the risk of nutritional deterioration increases because of the proximity of the tumor and require close nutritional follow-up. These patients should undergo nutritional counseling, and, if needed, nutritional intervention and supplements, with the aim of counteracting both the hypermetabolism and reduced dietary intake associated with cachexia. So far, clinical trials have not shown promising results with nutritional intervention, as it might treat malnutrition in HNC patients, but do not improve the signs and symptoms associated with cachexia. They do not demonstrate any increase in lean body mass, even when there is a significant increase in weight (Couch [et al.], 2007, Couch [et al.], 2015).

2.Aims of Study

The primary aim of the work described in this thesis is to characterize changes in total body composition for patients undergoing RT or concurrent chemoradiation therapy for HNSCC. In order to better organize and achieve the proposed aim, it has been divided into two main objectives:

1. The analysis of correlations between all the variables of the patients at their baseline state, before any treatment;
2. The analysis of changes in each variable throughout the treatment regimen of each patient.

We expect to reinforce and shed new light on the understanding of the weight loss during concurrent chemoradiation therapy and the potential role of PA and as an anti-cachectic modulators.

This project englobes the analysis of biochemical data, lean body mass, fat mass and body water. Enquiries about QoL and daily PA were made through specific questionnaires. The aim is to analyze the potential positive impact of PA by correlating the changes in lean body mass, QoL and cachexia biomarkers to the pattern of daily PA. The impact of therapy on the molecular pathways involved in the regulation of lean muscle mass was evaluated through the assessment of circulating levels of inflammatory biomarkers (TWEAK and titin) and through the study of changes in expression of genes associated to atrophy and hypertrophy pathways in skeletal muscle (*FBXO32*, *TNFSF12*, *TRIM63* and *NFKB1*).

Thus, this project comprises a pilot study, being these preliminary results used to develop in the future a larger study in HNC patients submitted to a specific PA program in order to assess through transcriptomic and genomic analysis the molecular processes associated to the improvement in their QoL and survival rates. This may allow us to develop novel clinical care models that will improve the patient's QoL in cancer patients.

3.Materials and Methods

3.1. HNC Patients

Twenty-three consecutive patients with HNC and before any treatment were recruited from the Maxillofacial Surgery Department, Coimbra Hospital and University Centre. The treatment modalities applied in this study group were: Group A) Surgery (SX), followed by radiotherapy (RT); Group B) Chemotherapy (CT), followed by chemoradiotherapy (CRT), Group C) CT followed by SX, followed by RT. The time point for sample collection and the number of time points varied for each treatment modality, and in each time point various outcome measures were assessed. Twelve-hour fasting blood samples were also collected in two blood vials, a 3 mL RNA preservative tube and a 9 mL EDTA tube **(Figure 5).**

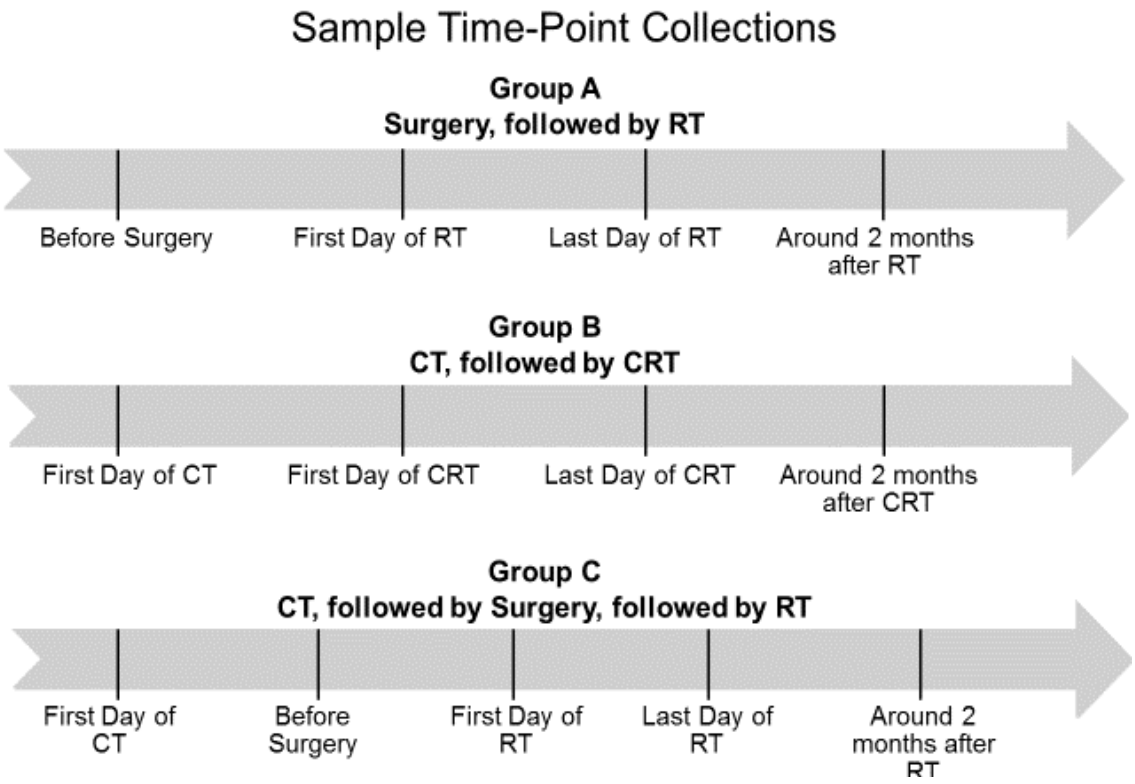

Figure 5 Sample time point collections for each treatment modality.

3.2. Patient data

Throughout the entirety of this project, data regarding many variables were assessed. Body composition variables were obtained through bioimpendance, with the purpose of evaluating the variation of lean body mass throughout the treatment. These variations will be correlated with the amount of daily physical activity (PA) and quality of life (QoL) of the patients, analyzed through specific questionnaires. Additionally, correlations between body composition, PA and QoL were made with the circulating levels of a variety of proteins and

changes in expression of genes associated to atrophy and hypertrophy pathways in skeletal muscle (*FBXO32*, *TNFSF12*, *TRIM63* and *NFKB1*).

3.2.1. Demographic and medical variables

Demographic and medical variables were obtained directly from the hospital process of these patients. These variables included age, sex, tumor features (localization, stage, TMN score), smoking and drinking habits, exposure to carcinogenic, chronic medication and family medical history.

3.2.2. Body composition

Body composition was assessed by bioimpedance, a noninvasive instrument commonly used by clinicians to evaluate body composition measurements and assess the health conditions of patients. The variables obtained by this instrument are height, weight, body mass index (BMI), percentage of fat mass (FM) and free fat mass (FFM), fat mass index (FMI) and free fat mass index (FFMI), skeletal muscle mass (SMM) and total body water (TBW). This parameter was evaluated in each treatment time point.

3.2.3. Physical activity

The amount PA practiced daily by each patient was evaluated through the International PA questionnaire, the 9 item short form (IPAQ-SF). The type of PA assessed by this form is walking, moderate-intensity activities and vigorous-intensity activities, expressed in MET-min per week. The MET values are multiples of the rest metabolic rate and the formula for the calculation of MET-minutes per week were obtained from the IPAQ scoring protocol. The MET-values defined for each activity were: walking = 3.3 MET's, moderate-intensity activities = 4 MET's, vigorous-intensity activities = 8 MET's; and for each activity, the formula used to calculate the MET-minute/week score was: MET-minute/week = Activity MET's / activity minutes * activity days. The value of total PA performed is the sum of the MET-minute/week values of each type of activity (walking, moderate-intensity activity and vigorous-intensity activity). This parameter was evaluated in each treatment time point.

3.2.4. Quality of life

For the assessment of QoL for each patient they answered the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire core 30 (EORTC QLQ-C30) questionnaire, specially developed to assess the QoL of cancer patients.

More exactly, this questionnaire evaluates the global health status, the functional scales and the symptom scales of the patients. However, this questionnaire is of a more generic nature, therefore a modular approach was combined for HNC-specific treatment measurements. For that we included the Quality of Life Questionnaire for Head and Neck Cancer module (QLQ30–H&N35), specific to HNC patients. This parameter was evaluated in the first, third and fifth treatment time point.

3.2.5. Hospital Anxiety and Depression Scale

The hospital anxiety and depression scale (HADS) was developed to detect states of depression, anxiety and emotional distress amongst non-psychiatric patients who were being treated for a variety of clinical problems. It is comprised of 14 questions, 7 of them related to anxiety symptoms and the other 7 to signs of depression. From a score of 7 or less the patient is considered normal, from 8 to 10 the patient is a borderline case and with more than 11 the patient is considered an abnormal case. This parameter was evaluated in the first, third and fifth treatment time point.

3.2.6. Plasma analysis

Plasma levels of selected proteins are obtained from the hospital, on each time-point. The analysis pertained the following proteins: albumin (Alb), C-reactive protein (CRP), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), aspartate transaminase (AST), Alanine transaminase (ALT) and total bilirubin (TBIL). This parameter was evaluated in each treatment time point.

3.3. Sample processing procedures

At each treatment time point two blood vials, a 3 mL RNA preservative tube and a 9 mL EDTA tube were also collected. Upon arrival, these samples were processed as described below, and then stored or used for other downstream applications.

For plasma recovery, the blood from the 9 mL EDTA tube had to be processed within 30 minutes after its collection. All blood tubes were centrifuged (Hettich Zentrifugen, Universal 320R) upon arrival (3000rpm, 10 minutes, 4°C) followed by the removal of all plasma into 2 mL Eppendorf's tubes. The 2 mL tubes were again centrifuged (12000rpm, 10 minutes, 4°C) and the supernatant was transferred to new 2 mL Eppendorf's tubes, properly identified and stored at -80°C. These samples would then be used for the extraction of plasmic RNA and for the enzyme-linked immunosorbent assay (ELISA).

The 9 mL EDTA tubes were again centrifuged (1600 g, 10 minutes, RT) and the buffy coat, approximately 800 µL, was collected into a 2 mL Eppendorf's tube, properly identified and stored at -20°C. These samples would then be used for DNA extraction.

The 3 mL RNA preservative tubes (*Blood RNA Preservative Tubes* from *Norgen Biotek Corporation*) upon arrival were properly identified and stored at 4°C in a dark place and avoid exposure to direct light. These samples were used for RNA extraction.

3.4. RNA extraction from Blood

The blood sample is collected to 3 mL *Blood RNA Preservative Tubes* from *Norgen Biotek Corporation,* which was followed by the extraction of RNA from blood using the *Preserved Blood RNA Purification Kit* from *Norgen Biotek Corporation*. This kit is intended for the isolation of total RNA from blood, through the use of RNA binding columns and a series of centrifugations and washing steps. All steps were made according to manufactures instruction. In the end, the extracted RNA was stored at -80°C for long-term storage.

3.5. RNA extraction from cultured cells

The RNA of the U87 cell line, a grade IV glioma cell line was extracted to construct the standard curve for the quantification step of the real-time quantitative polymerase chain reaction (qPCR). The *RNeasy Mini kit* from *Qiagen* was used for the extraction according to manufactures instruction. In the end, the extracted RNA was stored at -80°C for long-term storage.

3.6. DNA extraction from Blood

Genomic DNA from blood samples at each time point was extracted, using the *The JetQuick® Blood & Cell Culture DNA Midiprep Kit* from *Genomed*. For this extraction, the 800 µL of the buffy coat was used and PBS 1X was added to make up the initial 3 mL of sample needed. It is based on the use of DNA binding columns and a series of centrifugations and washing steps. The procedure was made according to the protocol included with the kit. The extracted DNA was stored at -20ªC for long-term storage.

3.7. Quantification

The quantification of the extracted DNA and RNA was performed by spectrophotometry, using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). For each measurement there was used 2 µL of DNA/RNA

sample, and the concentration was expressed in ng/μL. The absorbance of nucleic acids is around 260 nm and the contaminants absorb around 280 nm (proteins, phenols) and 230 nm (phenols, guanidine salts). The purity levels were assessed through the absorbance ratio 260/280 nm and 260/230 nm. The DNA is considered pure if the 260/280 nm ratio should be around 1.8 and the 260/230 nm ratio should range between 2.0 and 2.2. For RNA, both ratios should be around 2.0.

3.8. RNA extraction from Plasma

For the extraction of RNA from plasma two kits were used, the *Plasma/Serum Circulating Nucleic Acid Purification Maxi Kit (Slurry Format)* from *Norgen Biotek Corporation* and *MagMAX™ Viral RNA Isolation Kit* from *Applied Biosystems™* and within those kits various alterations of the respective protocol were also tested. The extraction with the *Plasma/Serum Circulating Nucleic Acid Purification Maxi Kit (Slurry Format)* is intended for the isolation of all nucleic acids present in plasma, through the use of resin columns. This kit was also always combined with the *RNase-Free DNase I Kit* from *Norgen Biotek Corporation*, which removes all traces of genomic DNA. The *MagMAX™ Viral RNA Isolation Kit* uses magnetic beads that capture the RNA. The tested samples were extracted with both kits on their own, but also with the *RNA Clean-Up and Concentration Micro-Elute Kit* from *Norgen Biotek Corporation.* The various tests are displayed on table 3. The extracted RNA was converted to cDNA or stored at -80°C.

3.9. Complementary DNA (cDNA) synthesis

The RNA extracted from plasma was then converted to cDNA, using the *Tetro cDNA Synthesis Kit* from *Bioline*. The Priming Mix added to each sample was prepared according to manufacter's instructions. The volumes of Oligo dT and Random Hexamers primers are to be adapted in order to improve the reverse transcription efficiency, as they target different types of RNA species. For these RNA templates, the defined volume for the Oligo dT and Random Hexamers primers were of 0.5 μL each. The reaction condition was followed according to the procedure included with the kit. After the conversion, the cDNA was stored at -20°C.

3.10. Real-time quantitative polymerase chain reaction (qPCR)

For the study of changes in expression of genes, we resorted to qPCR, a technique based on the polymerase chain reaction (PCR). In this technique, the amplification of the

targeted cDNA is monitored throughout the qPCR, and is lately quantified through the fluorescent measurement of fluorescent dyes that intercalate with any double-stranded DNA formed during the qPCR. The master mix used in this technique was the *5x HOT FIREPol EvaGreen qPCR Mix Plus (no ROX)* from *Solis BioDyne* and the 96 well plates were purchased from *Non-Skirted PCR Plates, Low-Profile* from *Starlab*. The *C1000 Touch™ Thermal cycle CFX96™ Real-Time PCR Detection System* and the *CFX Manager 3.1 program* from *Bio-Rad* were used in all of the assays.

3.10.1. Primers design

For the selection of the housekeeping gene, it was taken into consideration the if it was a constitutive gene, if they were reported as expressed in the control cell line used in this experiment (U87 cell line) and more importantly, if there is no reported alteration of the expression of the gene in disease states. In the end, four possible housekeeping genes were tested: beta-actin (*β-Actin*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), glucuronidase beta (*GUSB*) and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*). The genes of interest in study are *TNFSF12*, *TRIM63*, *NFKB1* and *FBXO32*. To design all the

Table 3 List of all the RNA extraction tests made with the extraction kits.

Kit	Test
Plasma/Serum Circulating Nucleic Acid Purification Maxi Kit (Slurry Format)	Elution volume: 35 µL
	Elution volume: 50 µL
	Elution volume: 100 µL
MagMAX™ Viral RNA Isolation Kit	Elution volume: 20 µL
	Elution volume: 35 µL
	Elution volume: 50 µL
	Elution volume: 100 µL
Plasma/Serum Circulating Nucleic Acid Purification Maxi Kit (Slurry Format) + RNA Clean-Up and Concentration Micro-Elute Kit	Initial plasma volume: 1 mL
	Initial plasma volume: 1.5 mL
	Initial plasma volume: 2 mL
	Final Elution volume: 15 µL
	Final Elution volume: 2 X 10 µL
MagMAX™ Viral RNA Isolation Kit + RNA Clean-Up and Concentration Micro-Elute Kit	

primers housekeepings and the four genes used in this study (*TNFSF12*; *TRIM63*; *NFKBI; FBXO32*), the program used was the *Beacon Designer™ version 8.0* from *Premier Biosoft*. The mRNA code for each gene was obtained from the National Center for Biotechnology Information (NCBI) online database, and in the end, all primers were subjected to the BLAST program of NCBI, to analyze the areas of similarity and make sure the primers were specific to the region of interest (the mRNA of each gene). The primers forward (PF) and reverse (PR) relative to each gene are represented in table 4. The primers for *β-Actin*, *GAPDH*, *GUSB*, *TNFSF12*, *TRIM63* and *FBXO32* were purchased from *Invitrogen* and the primers for *HPRT1* and *NFKB* were purchased from *Sigma-Aldrich*. Upon arrival, the primers were reconstituted to a concentration of 100 μmol with nuclease-free water (*Promega*) and stayed mixing overnight on a tube rotator at -4°C. For the qPCR reaction, a working solution for all primers was prepared, where the initial solutions are diluted to 10 μmol.

Table 4 Primers forward and reverse of all genes.

Gene	Primer Forward	Primer Reverse
β -Actin	5' CTCACCATGGATGATGATATCGC 3'	5' AGGAATCCTTCTGACCCATGC 3'
GAPDH	5' CCGCTTCGCTCTCTGCTCCT 3'	5' CCGTTGACTCCGACCTTCACCTT 3'
GUSB	5' ACTGGCGTCTGCGGCATT 3'	5' CCAATCCTCAGCACCACTCTTGT 3'
HPRT1	5' CTTGGTCAGGCAGTATAATCC 3'	5' GGGCATATCCTACAACAAACT 3'
TNFSF12	5' AGCCAGAATCAACAGCTCCAG 3'	5' ACAGCCTTCCCCTCATCAAAG
TRIM63	TCAGAGAGCAGGGACTAGGG	CACCCAACGACCAGGCATTA
NFKB1	5' AGGCAGCACTACTTCTTGAC 3'	5' ACCAGCAGCAGCAAACAT 3'
FBXO32	5' CACAGAGCCTTACCCTACAGAGAT 3'	5' AGACACTGCCTACTGGATCACAT 3'

3.10.2. Standard Curve

The control cell line used was the U87 cell line, a grade IV glioma cell line, since it reported expression of all the genes that are under study. The first standard is a 1:10 dilution of the cell line cDNA, followed by a series of dilutions with the same constant factor. Each standard curve has four standards with decreasing concentration and a no template control. The obtained standard curve is a regression line through these points, which will be used to determine the concentration of unknown samples from their CT-values.

3.10.3. Optimizations

For the qPCR reactions many conditions must be refined so that the assay is as efficient and accurate as possible. The volume of cDNA, the annealing temperature, the number of cycles and the primer concentration are some of the many conditions that have to be tested to achieve the best performance on the assays. Some of these conditions are tested with the standard curve and the housekeeping gene, being then adapted to the other genes. In other cases, because each gene has its own set of conditions, such optimizations must be made individually for each gene.

The amount of cDNA can range between 1 μL and 5 μL, depending on the initial RNA concentration. In this study, the designated amount of cDNA was 3 μL, since it was enough volume to present amplification. The standard number of cycles for the qPCR is 40, however, since our samples had low RNA concentration, it was opted to increase the number of cycles to 50.

The annealing temperature differed from gene to gene, according to the specifications of each set of primers (**Table 5**). In some cases where efficiency was a little lower than expected, different annealing temperatures were tested for the same gene, with a range from 60°C to 65°C. After the qPCR reaction, the cDNA was submitted to an agarose gel electrophoresis, at 3% (Agarose, *Nzytech, Routine grade*) and stained with Midori Green Advance DNA Stain (*Nippon Genetics Europe GmbH*), to make sure the amplified fragments were specific to our gene.

Four different primer concentrations were also tested to decrease non-specific

Table 5 qPCR protocol **A)** Annealing temperature for each set of primers and **B)** general reaction conditions for the qPCR.

A

Gene	Tm
β-Actin	65°C
GAPDH	65°C
GUSB	65°C
HPRT1	60°C
TNFSF12	63°C
TRIM63	61°C
NFKB1	60°C
FBXO32	60°C

B

Reaction conditions			
Reaction steps	**Temperature**	**Time**	**Cycles**
Initial Activation	95°C	15 minutes	1
Denaturation	95°C	15 seconds	50
Annealing	*	20 seconds	
Elongation	72°C	20 seconds	
Melting Curve	65°C –> 95°C	5 seconds	1

* Annealing temperature differs from gene to gene (Table 6A).

amplification: [PF/PR] = 200/200 nmol, [PF/PR] = 200/150 nmol, [PF/PR] = 150/200 nmol and [PF/PR] = 150/150 nmol for *GAPDH* and *GUSB*, at 65°C.

3.11. The enzyme-linked immunosorbent assay (ELISA)

The ELISA is a technique used for the detection and quantification of substances such as proteins and hormones. It is a plate-based assay, and it can analyze around 40 samples at a time. In this study, the substances in study were titin and TWEAK. The commercial kits used for each assay were the *Human Titin (TTN) ELISA* kit from *MyBiosource* and the *Human TWEAK (TNFSF12) ELISA Kit* from *Thermo Fisher Scientific*. All plates were analysed with the *Synergy multi-mode microplate reader* from *BioTek* and the data analysis was made with the *Gen5 Microplate Reader and Imager Software*, also from *BioTek*.

3.12. Data Analysis

Statistical analyses were performed using IBM SPSS statistics version 21.0 (IBM Corporation, Chicago, IL, USA). The normality of the data distribution was tested with the Shapiro-Wilk test. At baseline (n=23), variables were normally distributed. Variables for the second purpose were not normally distributed and, in this sense, non-parametric tests were used in these analyses. At baseline, associations between the variables were tested with Pearson Correlation. Friedman test followed by Wilcoxon signed-rank tests were performed for within-group comparisons in order to evaluate the effects of the intervention in the assessed variables. Variables normally and not normally distributed are reported as mean ± SD and median (Interquartile range), respectively. A p-value of 0.05 was considered statistically significant.

4.Results and Discussion

4.1. Baseline Analysis

The baseline analysis was made for a cohort of 23 patients (age: 57.83±12.93 years, range 32 – 77, 19 male and 4 female). This analysis includes information obtained from these patients before any treatment (1st evaluation), comprising a set of data pertaining the body composition (weight, lean body mass, body water, etc.), biochemical data, quality of life, signs of anxiety and depression, and the amount of daily physical activity (PA) practiced by each patient. This data will be used to evaluate the correlations between all of these variables, more importantly the influence of PA in the patients' lean body mass. The 23 patients were distributed through 3 treatment groups: Group A) 16 patients were submitted to chemotherapy (CT), followed by chemoradiotherapy (CRT); Group B) 6 patients were submitted to surgery (SX), followed by radiotherapy (RT); Group C) 1 patient was subjected to CT, followed by SX, followed by RT (**Table 6**).

Table 6 Demographic and medical variables.

Characteristics	(N)
Sex	
Male	19 (82%)
Female	4 (18%)
Tumor site	
Oral Cavity	23 (100%)
Tumor stage	
I	1 (4%)
II	2 (9%)
IVA	12 (52%)
IVB	6 (26%)
Unknown	2 (9%)
Tobacco history	
None	4 (18%)
Past	7 (30%)
Actual	12 (52%)
Alcohol history	
None	1 (4%)
Past	5 (22%)
Actual	17 (74%)
Treatment modality	
Group A: SX, RT	6 (26%)
Group B: CT, CRT	16 (70%)
Group C: CT, SX, RT	1 (4%)
Comorbidities	
No	13 (57%)
Yes	10 (43%)
Family history	
No	16 (70%)
Yes	7 (30%)

SX – Surgery; RT – Radiotherapy;
CT – Chemotherapy;
CRT – Chemoradiotherapy.

4.1.1. Demographic and medical variables

The demographic and medical variables from the patients evaluated in this study are represented in Table 6. All of the displayed tumors are on the oral cavity, with a higher prevalence of late stage disease (stage IV). Since oral cancer is the most prevalent of all HNC types, it is not unusual for the cohort to consist only on this type of cancer. In spite of the apparent easy accessibility of the oral cavity, most of these tumors are only diagnosed on later stages because of the actual detection methods. Unless perceived through an exam by a dentist or doctor, these cancers

are only detected when the patient is presenting signs and symptoms of the disease, which occur in the late stages. By then, SX is no longer an option and only CT or RT are available, which are the treatments associated with more complications and side effects (Garg and Karjodkar, 2012).

Most of the subjects had a history of alcohol and/or tobacco consumption, with the majority still presently undertaken those habits. As tobacco and alcohol are the most important risk factors, responsible for 75% of all HNC, the high amount of patients with at least one of these risk factors is not unexpected (Hashibe [et al.], 2007). Additionally, the high average age of the cohort (57.83±12.93) is also an indication that in these patients the development of HNC is mostly associated to a higher exposure to risk factors. In fact, when analyzed as a whole, all of the patients in this study presented at least one of the risk factors.

Other risk factor accessed in this study was the presence of comorbidities. Of the 10 (43%) patients that presented comorbidities, the most frequent were hypercholesterolemia, hypertriglyceridemia, hypertension and diabetes. These are the diseases more associated with aging, the primary cause of cancer development (Rowland and Yancik, 2006).

Only seven of the 23 patients (30%) affirmed having a family history of cancer. However, there are yet to be discovered inheritable polymorphisms involved in oral cancer pathogenesis. Most likely, these cases are associated to similar lifestyles among family members (Negri [et al.], 2009).

These variables are important to assess the global state of the cohort, regarding age, sex, tumor features and the exposure to risk factors. However, for this project, more relevant parameters were required. As such, additionally to the demographic and medical variables, numerous body composition parameters were evaluated, in order to assess variations in lean body mass and muscle mass, among others, which were relevant for the study of cachexia. QoL, depression and anxiety values were important to examine the overall health status of the patients. Biochemical data, such as plasma levels of inflammatory biomarkers, are essential to evaluate the physiologic state of the patients, as inflammation is strongly associated with cachexia. At least, the amount of physical activity practiced by each patient was compared with all of the above parameters in order to characterize its influence in these cachectic variables. The results obtained from all of these parameters are described in the following pages.

4.1.2. Body Composition

Body composition variables at baseline are present in Table 7. In 2 patients it was not possible to get this evaluation, one of them because he had a pacemaker and the other because the results were not available from the hospital. As such, weight (63.92±13.32 kg), height (1.67±0.08 m) and BMI were assessed for 22 patients while the other variables were measured for only 21. From this evaluation, the results obtained from these patients showed that, according to the average BMI value (22.94±5.45 kg/m^2), the patients BMI is within the normal weight range (20 – 25 kg/m^2), which indicates that the majority of our patients were not overweight at study entry. As cachexia is associated with loss of lean body mass, through bioimpendance, the relevant variables analyzed in this project were related with lean body mass (FFM%, FFMI and SMM) and fat body mass (FM%, FMI). TBW, as an indicator of treatment-associated toxicity is also present in this study.

Table 7 Body composition variables at baseline of the 22 patients.

Variables	mean ± SD	Range
Weight (kg)	63.92±13.32	40.70 – 88.15
Height (m)	1.67±0.08	1.51 – 1.84
BMI (kg/m^2)	22.94±5.45	15.90 – 34.01
FM %	22.12±13.11	2.00 – 45.00
FFM%	78.35±12.40	58.00 – 98.00
FMI (kg/m^2)	5.58±4.29	0.40 – 14.20
FFMI (kg/m^2)	17.45±2.69	11.60 – 22.30
SMM (kg)	23.15±5.11	12.40 – 32.10
TBW (L)	53.43±11.13	33.00 – 69.72

BMI – Body Mass Index; FM – Fat Mass; FFM – Free-fat Mass; FMI – Fat Mass Index; FFMI – Free-fat mass index; SMM – Skeletal Muscle Mass; TBW – Total Body Water.

4.1.3. Physical Activity (PA)

PA variables at baseline are presented in Table 8. The type of PA variables assessed by this form were walking, moderate-intensity activities and vigorous-intensity activities and were calculated using the predefined MET values (multiples of the rest metabolic rate – see page 25). All variables were analyzed for all 23 patients. Total PA (4888.65±7199.26 MET-min/week) ranges from 0.00 to 27516.00 MET-min/week, which indicates that are sample size exhibits patients with high levels of PA and patients with almost no PA. From this evaluation, the results obtained from these patients showed that, there is an ample variability in the practice of PA for all of the variables. The range values for walking, moderate-intensity activities and vigorous-intensity activities all start at 0.00, which indicates no PA. Some of the

patients may be physically inactive due to the development of the disease, and may already be displaying the more devastating side-effects. Additionally, as the patients are in the majority of old age, this lack of PA may be associated to the decline in their physical state due to age or other comorbidities. On the other hand, there are also patients that display high levels of PA, more significantly in the moderate and vigorous types of PA. Overall, this high deviation in PA values was beneficial to the study, because in spite of having a small cohort, it will allow correlations between PA and other variables, such as body composition and inflammatory biomarkers.

Table 8 Physical Activity (PA) variables at baseline for the 23 patients.

Variables	mean ± SD	Range
Total PA (MET-min/week)	4888.65±7199.26	0.00 – 27516.00
Walking (MET-min/week)	1209.52±1856.25	0.00 – 8316.00
Moderate PA (MET-min/week)	1049.57±2455.21	0.00 – 11760.00
Vigorous PA (MET-min/week)	2629.57±6017.20	0.00 – 19200.00
Time sitting (s)	253.04±187.97	60.00 – 660.00

MET - Multiples of the rest metabolic rate.

4.1.4. Quality of Life (QoL)

QoL variables at baseline are presented in Table 9. The European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire core 30 (EORTC QLQ-C30) assesses the QoL of cancer patients while the Quality of Life Questionnaire for Head and Neck Cancer module (QLQ30–H&N35) is specific for HNC patients. These questionnaires combined analyze 35 different items, however in the EORTC QLQ-C30 questionnaire, these items are divided into three main variables: global health status, functional scales and symptom scales. For the QLQ30–H&N35 questionnaire there is only one main variable measured. These variables are the most important and are represented in Table 10. Additionally, fatigue was also added since it is somehow displayed interesting correlations. The values for these parameters can oscillate between 0 and 100, where 0 indicates no outcome in the corresponding item and 100 express the highest level of that item. Specifically, the higher the Global Health Status value, the better is the overall health of the patient. The same applies for the remaining variables. However, the interpretation of those results may differ from variable to variable. The QLQ-H&N35 and the functional scales should be inferred in the same manner as the Global Health Status, as they are positive items. As such, the higher the value, the better the conditions of the patient. The symptom scales and fatigue, as negative items, should be understood in a contrary manner: the higher

the value, the worse the condition of the patient. Since it is a comparative questionnaire, results for this evaluation are dependent of multiples analysis of the same patient, as only alterations within the same patient can be considered reliable.

Table 9 Quality of Life (QoL) variables at baseline of the 22 patients.

Variables	mean ± SD	Range
EORTC QLQ-C30		
Global Health Status	46.97±27.04	0.00 – 100.00
Functional Scales	80.66±15.91	35.30 – 100.00
Symptom Scales	17.68±9.36	1.90 – 35.20
Fatigue	27.25±24.90	0.00 – 100.00
QLQ-H&N35	37.12±22.53	0.00 – 75.00

4.1.5. Hospital Anxiety and Depression Scales (HADS)

HADS variables were analyzed for all 23 patients. From a score of 7 or less the patient is considered normal, from 8 to 10 the patient is a borderline case and with more than 11 the patient is considered an abnormal case. For both anxiety (7.13±4.04) and depression (5.70±3.47) the average points indicate that the majority of the patients do not exhibit any of the emotional distress symptoms. Since this pertains pre-treatment conditions, these results are to be expected. However, as the anxiety range (2.00 – 17.00) reaches 17.00 points, a clear abnormal condition, it is possible that some of the patients are already experiencing anxiety problems. From the analyses of the 23 patients, it was observed that 3 (13%) patients presented anxiety symptoms and 1 (4%) patient presented depression symptoms. These results indicate that at the beginning of this project and before any treatment, the majority of these patients did not present anxiety or depression. The emotional distress presented by 4 patients may be due to the psychological or social stress, as well as the patient's own perception of their health status and incoming treatment (Couch [et al.], 2007).

4.1.6. Plasma Levels of proteins

Protein levels on plasma at baseline are presented in Table 10. All variables were analyzed for all 23 patients, however T.BIL levels for one patient were impossible to obtain due to hemolyzed plasma. Titin (0.68±0.65 ng/mL) and TWEAK (748.11±347.30 pg/mL) levels were obtained through ELISA and the remaining results were sent from the hospital. Both titin and TWEAK normal levels range were not available. Abnormal levels of ALP

(87.61±33.46 U/L), GGT (214.30±778.44 U/L), AST (32.96±38.41 U/L), ALT (21.43±20.04 U/L) and T.BIL (0.52±0.31 mg/dL) are usually associated to disorders on the liver (Korver [et al.], 1995, Vroon and Israili, 1990a, Vroon and Israili, 1990b). Alb (3.93±0.69 g/dL) and CRP (1.95±3.14 mg/L) are biomarkers for the inflammatory response, with Alb acting as a negative acute phase protein, with lower levels in inflammatory situations and CRP acting as a positive acute phase protein, with higher levels in inflammatory states (Black [et al.], 2004, Don and Kaysen, 2004). High levels of LDH (243.35±98.21 U/L) are usually associated a diseased states, such as cancer, inflammation and tissue damage (Miao [et al.], 2013). From this evaluation, the results obtained from these patients showed that the cancer-associated inflammatory state was not yet set in this group of patients, due to the normal levels of both CRP and Alb. Only the average levels of GGT were above the normal levels, which can indicate liver damage. Analyzing table 10 in more detail, it can be observe that, while the lower range values of many proteins (CRP, ALP, GGT, LDH, AST, ALT) do not diverge much from within normal levels, for the majority of these proteins the highest range values are distinctly higher than the normal levels. The high levels of the highest range value of ALP, AST and ALT associated with the above the normal levels of GGT could be related to liver disorders, due to the high percentage of patients in this study with a history of alcohol consumption (94%) (**Table 6)** High levels of the highest range value of CRP and LDH are associated to inflammation, which may indicate that some of the patients may be developing an inflammatory state. The fact that Alb, as a negative acute phase protein, presents a lower range value much lower than the normal levels, corroborates the information obtained from CRP and LDH.

Table 10 Protein levels on plasma at baseline.

Variables	Normal levels range	mean ± SD	Range
Alb (g/dL)	3.40 - 5.40	3.93±0.69	1.40 - 4.70
CRP (mg/L)	1.00 - 3.00	1.95±3.14	0.14 - 10.78
ALP (U/L)	44.00 - 147.00	87.61±33.46	43.00 - 210.00
GGT (U/L)	0.00 - 45.00	214.30±778.44	15.00 - 3778.00
LDH (U/L)	140.00 - 280.00	243.35±98.21	135.00 - 423.00
AST (U/L)	10.00 - 40.00	32.96±38.41	15.00 - 204.00
ALT (U/L)	7.00 - 56.00	21.43±20.04	4.00 - 99.00
T.BIL (mg/dL)	0.20 - 1.50	0.52±0.31	0.10 - 1.60
Titin (ng/mL)	-	0.68±0.65	0.15 - 2.83
TWEAK (pg/mL)	-	748.11±347.30	225.88 - 1407.99

Alb – Albumin; CRP – C-reactive protein; ALP – Alkaline Phosphatase; GGT – Gamma-glutamyl transpeptidase; LDH – Lactate Dehydrogenase; AST – Aspartate transaminase; ALT – Alanine transaminase; TWEAK – TNF-like weak inducer of apoptosis.

4.1.7. RNA extraction from plasma and qPCR

For the study of changes in the expression of the genes of interest (TNFSF12; TRIM63; NFKB1; FBXO32) we resorted to the qPCR experiment. It was necessary to extract the RNA from plasma and, as such, it required defining which RNA extraction protocols to use and which housekeeping gene was more appropriate for these samples.

4.1.7.1. RNA extraction from plasma tests

For the extraction of RNA from plasma, which would be converted to cDNA and used in the qPCR, two kits were used, and within those kits various alterations of the respective protocol were also tested. Quantification of the RNA samples was not possible due to the low amount of RNA, however it was possible to obtain amplification on the qPCR experiment on some of the tested samples (**Table 11**). It was decided that all extractions for each sample were to be made in duplicate, one with the *Plasma/Serum*

Table 11 List of all the RNA extraction tests made with the extraction kits and the result regarding qPCR amplification. The extraction tests underlined were the chosen for this project.

Kit	Test	qPCR Amplification
Plasma/Serum Circulating Nucleic Acid Purification Maxi Kit (Slurry Format)	Elution volume: 35 µL	Yes
	Elution volume: 50 µL	Yes
	Elution volume: 100 µL	No
MagMAX™ Viral RNA Isolation Kit	Elution volume: 20 µL	Yes
	Elution volume: 35 µL	Yes
	Elution volume: 50 µL	Yes
	Elution volume: 100 µL	No
Plasma/Serum Circulating Nucleic Acid Purification Maxi Kit (Slurry Format) + RNA Clean-Up and Concentration Micro-Elute Kit	Initial plasma volume: 1 mL	Yes
	Initial plasma volume: 1.5 mL	Yes
	Initial plasma volume: 2 mL	Yes
	Final Elution volume: 15 µL	Yes
	Final Elution volume: 2x10 µL	Yes
MagMAX™ Viral RNA Isolation Kit + RNA Clean-Up and Concentration Micro-Elute Kit		No

Circulating Nucleic Acid Purification Maxi Kit (Slurry Format) and the *RNA Clean-Up and Concentration Micro-Elute Kit*, with initial plasma volume of 2 mL and the second with the *MagMAX™ Viral RNA Isolation Kit*, with a final elution volume of 20 µL. The qPCR experiment for two of the genes of interest and the chosen housekeeping gene was performed for each of the extraction protocols.

4.1.7.2. Housekeeping gene

Four genes were tested to be used as the housekeeping gene, *β-Actin*, *GAPDH*, *GUSB* and *HPRT1*. However, none of these genes were suitable for this project, as the CT-values for all of these genes were too high, which indicates low expression in the cell-line. As such, there are no results of the qPCR experiment to be presented, since the values obtained from the genes of interest have to be compared with the results from the housekeeping gene. New housekeeping genes need to be tested, in order to study the changes in the expression in the genes of interest. Nevertheless, it was possible to perform the qPCR experiment and obtain amplification of the genes of interest, a novelty in these kind of samples.

4.1.8. Correlation analysis of all variables at baseline state

At baseline, associations between the variables were tested by Pearson Correlation in order to evaluate the influence of physical activity and inflammation on body composition and quality of life. The correlations were made between all variables, however only the correlations statistically significant and with clinical importance are represented and discussed here. Each of the following tables indicate the p-value (p) of the correlation and the Pearson correlation value (r), which ranges from 1 to -1. Negative Pearson correlation values specifies a negative association, where the value of one variable increases and the other one decreases; a positive Pearson correlation value indicates a positive association, meaning that the value of both variables increases.

4.1.8.1. C – reactive protein (CRP) and TNF-like weak inducer of apoptosis (TWEAK)

From all the protein on plasma at baseline, only the levels of CRP and TWEAK had clinically statistically significant correlations.

CRP is a positive acute phase protein, with higher levels associated to inflammatory states (Deans and Wigmore, 2005). In this study we found associations between CRP levels

and alterations in body composition and QoL (**Table 12**). Higher levels of CRP are associated to lower weight (r = -0.424, p = 0.049), lower FFMI (r = -0.481, p = 0.027), lower SMM (r = -0.485, p = 0.030) and lower BMI (r = -0.443, p = 0.044). Higher levels of CRP were also associated with lower Global health status (r = -0.435, p = 0.049).

Table 12 CRP correlation with weight, free-fat mass index (FFMI), skeletal muscle mass (SMM), global health status and body mass index (BMI).

	r	p	N
Weight	-0.424	0.049	22
FFMI	-0.481	0.027	21
SMM	-0.485	0.030	20
BMI	-0.443	0.044	21
Global health status	-0.435	0.049	21

TNF-like weak inducer of apoptosis (TWEAK) is a cytokine that can induce multiple apoptosis pathways and induce the degradation of muscle proteins (Mittal [et al.], 2010). It has shown association with titin, the third most abundant protein present in skeletal muscle and QoL (**Table 13**). Higher levels of TWEAK are associated to higher levels of titin (r = 0.498, p = 0.016).

In the other hand, higher levels of TWEAK are associated with lower QoL (r = -0.452, p = 0.035).

Table 13 TWEAK correlations with titin levels and QoL.

	r	p	N
Titin	0.498	0.016	23
QLQ-H&N35	-0.452	0.035	22

Weight loss in HNC patients has long been associated either with location of the tumor or the side effects of the treatment. However, some types of cancer that do not involve directly the gastrointestinal tract or affect food consumption have also shown deliberate weight loss (Deans [et al.], 2009). In these cases the major correlation found is the presence of systemic inflammation. High circulating levels of positive acute phase proteins, such as CRP, have been shown to increase weight loss in various cancer studies (Simons [et al.], 1999). There are several mechanisms that were proposed to explain the influence of systemic inflammation on the involuntary weight loss observed in cancer

patients. It has also been postulated that the pro-inflammatory cytokines may induce a decrease of food intake (Moldawer [et al.], 1988). This weight loss have also been explained as a direct result of a high intake of amino acid usage, due to hypermetabolism, enhanced gluconeogenesis and acute phase protein production, all cancer-related mechanisms (Andus [et al.], 1988). The most accepted theory is of the pro-inflammatory cytokine-induced activation of the UPS pathway, which will activate the degradation of muscle proteins (Battaglini [et al.], 2012). This theory is confirmed in this study, as TWEAK, a cytokine that induces the UPS pathway is associated with an increase in titin levels on plasma, a result of muscle protein degradation. TWEAK is a long range cytokine, associated with the activation of a variety of cellular pathways. One of those pathways is the activation of NF-κB, a major pro inflammatory cytokine involved in muscle wasting in a variety of disorders. Moreover, inactivation NF-κB has been shown to decrease and even reverse skeletal muscle wasting (Cai [et al.], 2004). In cachexia situations, an over activated NF-κB will induce the MuRF1, an E3 ubiquitin ligase, an important component of the UPS pathway, which function involves the breakdown of myosin heavy chains for degradation. Furthermore, MuRF1 will activate other E3 ubiquitin ligase, Atrogin-1, inducing the whole degradation pathway (Mittal [et al.], 2010). MuRF1 has been shown to bind to titin, the third major component of skeletal muscle cells, and induce its degradation (Glass, 2005). This increase in muscle mass degradation due to cachexia will eventually lead to a decline of physical function. Functional disability is a direct effect of the loss of weight and muscle mass and reduced food intake, all of these related a cancer-induced inflammatory status (Deans [et al.], 2009). More importantly, the loss of muscle mass is proposed to generate overall fatigue, which will lead to weakness, immobility and eventually to loss of respiratory function. All of these factors may contribute to the degradation of the QoL and health status of cachectic patients (Couch [et al.], 2007).

4.1.8.2. Physical activity (PA) variables and overall quality of life (QoL)

Of all PA variables analyzed, sitting time and walking time were the only variables te presented correlations. There was a correlation between the time spent walking and body composition. That is, patients that spent more time walking were associated a higher FFMI ($r = 0.458$, $p = 0.042$).

The time patients spent sitting has shown associations with alterations in body composition and QoL (**Table 14**). More time spent sitting is associated with higher FM% ($r = 0.448$, $p = 0.048$), higher FMI ($r = 0.522$, $p = 0.018$) and higher BMI ($r = 0.463$, $p = 0.035$). Patients that spent more time sitting also experienced more fatigue ($r = 0.431$, $p = 0.035$).

Of all QoL variables analyzed, only global health status and fatigue variables presented correlations statistically significant. Fatigue presented associations with depression. That is, patients that experienced more fatigue also exhibited higher depression symptoms ($r = 0.458$, $p = 0.032$).

Table 14 Sitting time correlations with fat mass (FM) %, fat mass index (FMI), Fatigue and body mass index (BMI).

	r	p	N
FM%	0.448	0.048	20
FMI	0.522	0.018	20
BMI	0.463	0.035	21
Fatigue	0.431	0.045	22

Global health status presented associations with alterations in body composition and depression (**Table 15**). A higher global health status was associated to higher skeletal mass muscle (SMM) ($r = 0.555$, $p = 0.011$). On the other hand, patients with higher global health status exhibited lower depression symptoms ($r = -0.591$, $p = 0.004$).

Table 15 Global health status correlations with skeletal muscle mass (SMM) and depression (HADS-D).

	r	p	N
SMM	0.555	0.011	20
HADS D	-0.591	0.004	22

The effects of PA are well documented. The influence of PA in the prevention of HNC has been mostly inconclusive, however it has been vastly assessed in more common types of cancer, such as breast cancer, where it was shown that both overweight and physical inactivity are factor risks for the development of this type of cancer (Bianchini [et al.], 2002). PA is known to limit weight gain and BMI values. More importantly it is widely associated with a decrease in the risk of development of a variety of disorders, mostly heart-related, but also diabetes and some types of cancer (Kyle [et al.], 2001). It has other health related benefits, such as the decrease of fat mass and an increase of free-fat mass (Donnelly [et al.], 2009). It is believed that even a small amount of PA, such as walking for at least 30 minutes/day may ensure a number long-term health benefits (Kushi [et al.], 2012).

With far more reaching effects than the practice of PA is the lack of it. Overweight and physical inactivity have been associated with several disorders, one of them cancer (Bianchini [et al.], 2002). Additionally, a sedentary lifestyle is associated with an increase of

fat mass, an increase in the risk of cardiovascular and respiratory disorders, and an overall increase in morbidity and mortality rates (Kyle [et al.], 2001). These side effects of physical inactivity are more aggravated with the increase of the subjects' ages, as aging is widely associated with an increase of comorbidities and an increase of weight and fat mass (Donnelly [et al.], 2009, Kyle [et al.], 2001). In cachexia situations, the regular practice of PA is even more imperative. Not only does regular PA reduce the inflammatory status responsible for the degradation of muscle mass, but also compensates the loss of muscle protein with an increase of fat free mass. In obese patients, this situation is even more critical as the sedentary lifestyle and the high levels of fat mass are associated with low muscle mass. Many studies showed that obese patients suffer from sarcopenia, previously to the development of cachexia, and are consequently the group with lower survival rates (Fearon, 2011).

Fatigue has been vastly studied as one of the major influence on the overall QoL in cancer patients, with greater impact in daily activities, as opposed to more common distresses, such as pain and nausea (Stone, 2002). One of the mechanisms associated with an increase in fatigue in cancer patients is physical deconditioning, induced through malnutrition and muscle wasting. Fatigue leads then to an increase in sedentary activities and a decrease of PA, with an eventual increase in body weight and fat mass (Visser and Smets, 1998). In these cases, the sensation of fatigue may become a vicious cycle: patients fell fatigued, therefore they adopt a state of inactivity, which increases the physical debilitation caused by cachexia; when they try to remove themselves from this inactive state, the needed effort will be higher, which will propel them into maintaining their sedentary behavior, thus worsening their overall condition (Stone, 2002, Visser and Smets, 1998). The causes of cancer-related fatigue are yet unknown. Fatigue can be easily confused with early signs of depression and cancer patients can suffer from fatigue because of the cancer itself, but also due to the same reasons the general population become fatigued (Stone, 2002). However, there are studies that contemplate fatigue and depression as two independent factors that may influence the QoL of cancer patients (Visser and Smets, 1998).

The QoL of HNC patients can be affected by many factors. Considering the location of the tumor, patients will often have difficulty speaking and swallowing, which will greatly affect, not only their everyday routine, but also their involvement in social functions. Eventually, their own perception of their health and physical decline will prompt reclusive behaviors, which will lead to depression states (Couch [et al.], 2007). When combined with the muscle wasting effects of cachexia, and the physical disability that accompanies, the probability of developing depression is higher (Davies [et al.], 1986). Despite depression

being most likely related to psychological and social stress, many studies suggest that the action of some pro-inflammatory cytokines, like IL-6 may promote the development of depression (Brown and Paraskevas, 1982, Musselman [et al.], 2001).

4.2. Analysis of alterations throughout the treatment

As it was already mentioned, the patients were subjected to one of the following treatments: Group A) chemotherapy (CT), followed by chemoradiotherapy (CRT); Group B) surgery (SX), followed by radiotherapy (RT); Group C) CT, followed by SX, followed by RT. From the 23 patients of this study, it was only possible to obtain the results throughout the treatment for nine of those patients, eight from the treatment Group B and one from the treatment Group A. As the number of subjects for this study is low, the analysis will englobe the patients of the Group B treatment, and the comparison will be solely on the effect of the treatment in the different variables, as opposed to the comparison of different treatment groups. As such, all the results from this chapter should be regarded as preliminary results.

The treatment regimen studied with these patients is CT, followed by CRT. The 1st time point stands for the patients' analysis before any treatment and the beginning of the CT therapy; the 2nd time point was made at the end of the CT regimen and the beginning of the CRT; the 3rd time point was made at the end of CRT treatment; and the 4th time point are two months follow-ups (**Figure 6**). In this chapter, the 4th time point was discarded since there was not enough data for the analysis, due to the time for the development of the present thesis.

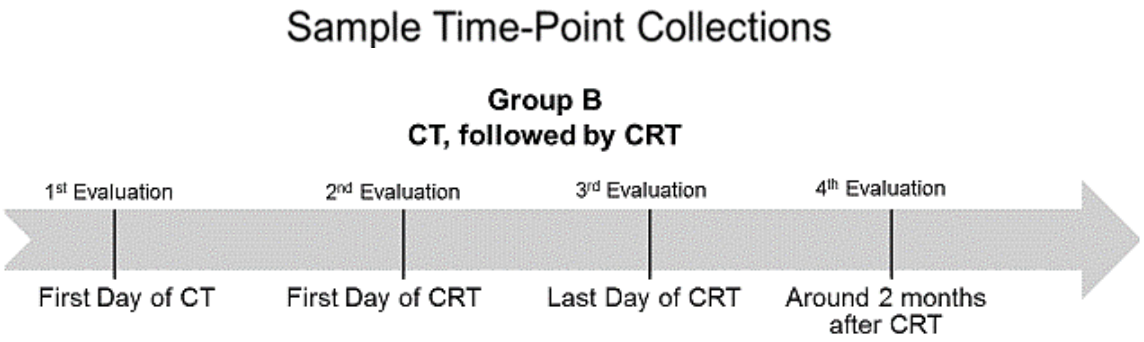

Figure 6 Sample time point collections for the Group B treatment modality.

4.2.1. Body Composition

Body composition variables are indicators of the health state of patients as some side effects of treatment are associated with weight loss, due to the decrease in food intake

(Lees, 1999). TBW can be associated to kidney damage and the physiology of FFM and SMM are indicators of muscle wasting in these patients (Ries and Klastersky, 1986). Body composition variables at the three time-points are present in Table 16. All variables were analyzed for all 8 patients. The average BMI value in all evaluations (1st = 23.94±6.51kg/m^2; 2nd = 23.97±5.63 kg/m^2; 3rd = 21.71±5.20 kg/m^2) are within the normal weight range (20 – 25 kg/m^2), which indicates that the majority of the patients maintained a normal BMI throughout the treatment.

Table 16 Body composition variables for the 1st, 2nd and 3rd evaluations.

Variables	Evaluation	mean ± SD	Range
Weight (kg)	1st	67.44±14.29	46.90 - 88.15
	2nd	67.34±11.55	54.30 - 82.50
	3rd	61.15±11.26	46.00 - 76.90
BMI (kg/m^2)	1st	23.94±6.51	17.47 - 34.01
	2nd	23.97±5.63	17.50 - 32.30
	3rd	21.71±5.20	15.00 - 29.60
FM %	1st	18.73±14.58	2.00 - 42.00
	2nd	20.63±12.05	7.00 - 41.00
	3rd	18.00±13.01	4.00 - 40.00
FFM%	1st	81.27±14.58	58.00 - 98.00
	2nd	79.38±12.05	59.00 - 93.00
	3rd	82.00±13.01	60.00 - 96.00
FMI (kg/m^2)	1st	5.24±5.05	0.40 - 14.20
	2nd	5.54±4.28	1.20 - 13.20
	3rd	4.43±3.94	0.60 - 11.80
FFMI (kg/m^2)	1st	18.70±2.02	16.62 - 22.30
	2nd	18.45±2.04	16.30 - 22.30
	3rd	17.33±2.09	14.10 - 20.40
SMM (kg)	1st	25.45±3.13	21.30 - 30.20
	2nd	25.16±2.59	22.20 - 29.60
	3rd	23.64±3.21	18.50 - 27.80
TBW (L)	1st	57.89±11.82	37.70 - 69.72
	2nd	54.45±13.08	34.90 - 67.67
	3rd	35.70±3.90	31.00 - 42.20

BMI – Body Mass Index; FM – Fat Mass; FFM – Free-fat Mass; FMI – Fat Mass Index; FFMI – Free-fat mass index; SMM – Skeletal Muscle Mass; TBW – Total Body Water.

4.2.2. Physical Activity

PA variables at the three time-points are present in Table 17. The type of PA variables assessed by this form were walking, moderate-intensity activities and vigorous-

intensity activities and were calculated using the predefined MET values (multiples of the rest metabolic rate – see page 25). Data missing for one patient in the 3rd time-point. All active PA values presented ranges starting at 0 MET-min/week, which indicates that this sample size exhibits patients with high levels of PA (15840.00) and patients with almost no PA (0.00). As this is a whole treatment overview of the effect on these variables, it is possible that the patients with no PA were hospitalized during the treatment.

Table 17 Physical activity (PA) variables for the 1st, 2nd and 3rd evaluations.

Variables	Evaluation	mean ± SD	Range
Total PA (MET-min/week)	1st	4823.50±6086.30	33.00 - 15840.00
	2nd	3476.81±4539.88	0.00 - 11544.00
	3rd	2313.43±5060.13	0.00 - 13728.00
Walking (MET-min/week)	1st	478.50±617.63	0.00 - 1485.00
	2nd	1016.81±1871.59	0.00 - 5544.00
	3rd	480.86±153.95	300.00 - 693.00
Moderate PA (MET-min/week)	1st	2125.00±3949.36	0.00 - 11760.00
	2nd	1710.00±2561.50	0.00 - 7560.00
	3rd	154.29±358.09	0.00 - 960.00
Vigorous PA (MET-min/week)	1st	2220.00±4959.77	0.00 - 14400.00
	2nd	750.00±1342.02	0.00 - 3840.00
	3rd	1508.57±3585.88	0.00 - 9600.00
Time sitting (s)	1st	187.50±141.40	60.00 - 480.00
	2nd	311.25±128.22	150.00 - 480.00
	3rd	420.00±158.75	240.00 - 600.00

MET – multiples of the rest metabolic rate.

4.2.3. Quality of Life

QoL variables at the 1st and 3rd time-points are represented in Table 18. All variables were analyzed for all patients. The QoL was solely evaluated on the 1st and 3rd time points, therefore only the effect of all treatment can be analyzed on these variables. This questionnaire evaluates the global health status, the functional scales and the symptom scales of the patients. Additionally, fatigue was also added since it is considered one of the most important side effects associated with cancer and may have some influence in the amount of PA practiced by this set of patients. The values for these parameters can oscillate between 0 and 100, where 0 indicates no outcome in the corresponding item and 100 express the highest level of that item. Since it is a comparative questionnaire, results for this evaluation

are dependent of multiples analysis of the same patient, as only alterations within the same patient can be considered reliable. For the global health status and the functional scales items, an improvement is verified with an increase in the items value from the 1st to the 3rd time point. For the symptom scales and the fatigue items, an improvement is visualized with a decrease of the values throughout the treatment.

Table 18 QoL variables for the 1st and 3rd evaluations.

Variables	Evaluation	mean ± SD	Range
Global Health Status	1st	44.80±16.03	17.00 - 67.00
	3rd	65.64±27.61	16.70 - 100.00
Functional Scales	1st	79.38±7.41	70.3 - 93.70
	3rd	76.99±23.25	28.30 - 95.30
Symptom Scales	1st	19.60±12.04	3.70 - 35.20
	3rd	26.89±16.05	8.60 - 48.80
Fatigue	1st	22.21±19.69	0.00 - 44.00
	3rd	36.06±33.99	11.10 - 100.00

4.2.4. Hospital Anxiety and Depression Scales (HADS)

HADS variables at the 1st and 3rd time-points are presented in Table 19. As with the QoL, HADS was solely evaluated on the 1st and 3rd time points, therefore only the effect of all treatment can be analyzed on these variables. A score of 7 or less is considered normal, from 8 to 10 is a borderline case and with more than 11 is considered an abnormal case. The highest anxiety values (HADS-A) in these patients are reached in the 1st evaluation (2.00 – 17.00), whereas the highest depression values (HADS-D) are reached at the end of the treatment (3.00 – 16.00). It is possible that some of the patients start experiencing anxiety problems, which could then develop into depression symptoms.

Table 19 HADS variables for the 1st and 3rd evaluations.

Variables	Evaluation	mean ± SD	Range
HADS-A	1st	8.75±4.50	2.00 - 17.00
	3rd	7.14±3.02	4.00 - 12.00
HADS-D	1st	6.75±3.37	0.00 - 11.00
	3rd	9.29±4.07	3.00 - 16.00

HADS-A – Anxiety; HADS-D – Depression.

4.2.5. Plasma Levels of Proteins

Protein levels on plasma at the three time-points are presented in Table 20. All variables were analyzed for all patients and the results were obtained from the hospital records. However total billirubun (T.Bil) levels for one patient in the 2nd time-point were impossible to obtain due to hemolyzed plasma and there is data missing for one patient at the 3rd time-point. Titin and TWEAK levels were obtained through ELISA and all data was

Table 20 Protein levels on plasma for the 1st, 2nd and 3rd evaluations.

Variables (normal levels range)	Evaluation	mean ± SD	Range
CRP (mg/L) (1.00 – 3.00)	1st	2.46±3.80	0.14 - 10.20
	2nd	1.81±2.42	0.02 - 6.83
	3rd	3.60±2.50	0.18 - 7.00
ALP (U/L) (44.00 – 147.00)	1st	101.00±47.04	66.00 - 210.00
	2nd	93.38±17.12	73.00 - 118.00
	3rd	102.14±18.17	79.00 - 127.00
GGT (U/L) (0.00 – 45.00)	1st	501.88±1323.92	21.00 - 3778.00
	2nd	91.25±175.14	18.00 - 523.00
	3rd	110.71±142.55	13.00 - 410.00
LDH (U/L) (140.00 – 280.00)	1st	285.25±107.10	135.00 - 423.00
	2nd	195.88±38.42	150.00 - 278.00
	3rd	155.29±40.33	105.00 - 224.00
AST (U/L) (10.00 – 40.00)	1st	47.88±63.58	15.00 - 204.00
	2nd	21.04±14.62	2.30 - 54.00
	3rd	26.57±25.89	14.00 - 85.00
ALT (U/L) (7.00 – 56.00)	1st	27.38±29.35	9.00 - 99.00
	2nd	13.63±7.91	4.00 - 29.00
	3rd	15.86±11.88	9.00 - 41.00
T.BIL (mg/dL) (0.20 – 1.50)	1st	0.40±0.23	0.10 - 0.70
	2nd	0.46±0.14	0.30 - 0.70
	3rd	0.40±0.08	0.30 - 0.50
Titin (ng/mL)	1st	0.99±0.79	0.29 - 2.83
	2nd	0.84±0.68	0.24 - 2.41
	3rd	0.95±0.84	0.32 - 2.90
TWEAK (pg/mL)	1st	872.92±317.06	333.06 - 1242.36
	2nd	889.33±281.93	348.03 - 1317.34
	3rd	830.19±273.55	383.07 - 1292.30

CRP – C-reactive protein; ALP – Alkaline Phosphatase; GGT – Gamma-glutamyl transpeptidase; LDH – Lactate Dehydrogenase; AST – Aspartate transaminase; ALT – Alanine transaminase; TWEAK – TNF-like weak inducer of apoptosis.

available. CRP values for the 3rd time point were above the normal range, which may suggest that the majority of the patients presented a cancer-associated inflammatory state. CRP is a biomarker for the inflammatory response, with higher levels in inflammatory states (Black [et al.], 2004, Don and Kaysen, 2004). High levels of LDH are usually associated to diseased states, such as cancer, inflammation and tissue damage (Miao [et al.], 2013). In these patients, only in the 1st time point were the levels of LDH slightly higher than the normal levels. GGT levels were higher than the normal range in all time points, and AST levels were higher than the normal values in the 1st time point. Abnormal levels of liver proteins (ALP, GGT, AST, ALT and T.BIL) are usually associated to disorders, which in this analysis may be associated with side-effects of the treatment (Korver [et al.], 1995, Vroon and Israili, 1990a, Vroon and Israili, 1990b). From this evaluation, the results obtained from these patients showed that the cancer-associated inflammatory state was set in this group of patients at the 3rd time point, probably due to side effects of the treatment. GGT and AST high levels indicate liver disorders, in part due to the high percentage of patients in this study with a history of alcohol consumption (94%) (**Table 6)** and the toxicity caused by the treatment.

4.2.6. Effects of the intervention

To evaluate the effects of the intervention in the assessed variables the Wilcoxon signed-rank tests was performed for within-group comparisons. This test is used to compare one variable from the same patient in different time points or when subjected to different conditions. Each of the following tables specify the mean ± SD of the variables in both time points analyzed, the p – value (p) and the Z statistics value (Z). The sign of Z indicates if the value of the variable increased (positive sign) or decreased (negative sign) between the two time points. The analysis were made for all variables between all time-points, however only statistically significant alterations and with clinical importance are represented and discussed here

4.2.6.1. Between 1st and 2nd time points

The analysis between the 1st and 2nd time points evaluates the global effect of the CT treatment. The majority of the alterations were of plasma biomarkers (**Table 21**). LDH (Z = -1.970, p = 0.049), AST (Z = -2.380, p = 0.017), ALT (Z = -2.100, p = 0.036) and titin (Z = -2.240, p = 0.025) presented a decrease in plasma levels from the 1st to the 2nd time point. TBW (Z = -2.240, p = 0.025) also displayed a decrease between these time points. The only

noted PA alteration was the increase in the time that patients spent in sitting activities (Z = 2.003, p = 0.045).

Table 21 Variables with alterations between the 1st and 2nd treatment time points.

Variables	1st Evaluation	2nd Evaluation	Z	p	N
TBW (L)	57.89±11.82	54.45±13.08	-2.240	0.025	8
Time Sitting (s)	187.50±141.40	311.25±128.22	2.003	0.045	8
LDH (U/L)	285.25±107.10	195.88±38.42	-1.970	0.049	8
AST (U/L)	47.88±63.58	21.04±14.62	-2.380	0.017	8
ALT (U/L)	27.38±29.35	13.63±7.91	-2.100	0.036	8
Titin (ng/mL)	0.99±0.79	0.84±0.68	-2.240	0.025	8

TBW – Total body water; LDH – Lactate dehydrogenase; AST – Aspartate transaminase; ALT – Alanine transaminase.

CT treatment has already been discussed as causing a large variety of side-effects, such as fatigue, pain and nausea, mostly related to the toxicity of the chemical agent. The fatigue associated with the CT treatment combined with the side-effects that may arise will affect the patients' physical status, which will lead to higher sedentary behavior. High levels of LDH are usually associated to diseased states and is usually used as a prognostic factor (Haas [et al.], 2013, Miao [et al.], 2013). AST and ALT are known indicators of hepatic disorders when their plasma levels are higher than average (Vroon and Israili, 1990b). The fact that all of these biomarkers have decreased during the CT treatment may indicate a higher health status or at least a health improvement, compared to their pre-treatment status. The effects of the more known side-effects of CT are not evidenced in these results, probably because they are of a more physiological nature (Wall [et al.], 2013). The cellular effects of the treatment may become more obvious in a long-term evaluation, as the cytotoxicity of CT may take some time to develop (Cersosimo, 1993). However, one of the more important side-effects of CT is kidney damage, as all the by-products of the CT agents action will undoubtedly end up at the kidneys. These by-products may damage the nephrocytes, as well as the bladder and the urinary tract. One of the signs of nephrotoxicity is the decrease of body water levels in patients (Ries and Klastersky, 1986).

The decrease of titin levels may indicate a reduction in skeletal muscle degradation. As there is no significant alterations in the inflammatory status, through the levels of CRP (Z = -0.280, p = 0.779), it is not possible to confirm if it is a result of a decrease in muscle wasting. However, albeit not statistically significant, there was a decrease in the CRP levels from the 1st (CRP = 2.46±3.80 mg/L) to the 2nd (CRP = 1.81±2.42 mg/L) time point, which can indicate a tendency for a decrease of the inflammatory status. The number of patients

evaluated in this section (N = 8) is too low, and it may contribute for the lack of statistical significance, when discussing the CRP levels. In general, and according to the results obtained in this study, the first stage of treatment may have lightly improved the overall health status of the patients.

4.2.6.2. Between 2nd and 3rd time point

The 2nd to the 3rd time points pertain the second stage of the treatment, where patients are subjected to concurrent CRT. The alterations will allow the study of the effect of CRT in the overall state of the patients. In this analysis, body composition variables, PA variables and biomarkers levels were altered (**Table 22**).

All the body composition variables assessed were affected. The majority of these variables, such as Weight (Z = -2.521, p = 0.012), BMI (Z = -2.521, p = 0.012), FM (Z = -2.546, p = 0.011), FMI (Z = -2.533, p =0.011), FFMI (Z = -2.521, p = 0.012), SMM (Z = -2.524, p = 0.012) and TBW (Z = -2.521, p = 0.012) presented a decrease from the 2nd to the 3rd time points. On the other hand, FFM (Z = 2.546, p = 0.011) was the only body composition variable to display an increase between these time points. Similarly to what occurred in the 1st and 2nd time point analysis, the only noted PA alteration was the increase in the time that patients spent in sitting activities (Z = 2.214, p = 0.027). As for the levels of plasma biomarkers, both CRP and LDH were affected. Between these two time points, the levels of CRP (Z = 2.366, p = 0.036) have increased. On the other hand, the levels of LDH (Z = -2.028, p = 0.043) have decreased.

Table 22 Variables with alterations between the 2nd and 3rd treatment time points.

Variables	2nd Evaluation	3rd Evaluation	Z	p	N
Weight (kg)	67.34±11.55	61.15±11.26	-2.521	0.012	8
BMI (kg/m^2)	23.97±5.63	21.71±5.20	-2.521	0.012	8
FM %	20.63±12.05	18.00±13.01	-2.546	0.011	8
FFM %	79.38±12.05	82.00±13.01	2.546	0.011	8
FMI (kg/m^2)	5.54±4.28	4.43±3.94	-2.533	0.011	8
FFMI (kg/m^2)	18.45±2.04	17.33±2.09	-2.521	0.012	8
SMM (kg)	25.16±2.59	23.64±3.21	-2.524	0.012	8
TBW (L)	54.45±13.08	35.70±3.90	-2.521	0.012	8
Time sitting (s)	311.25±128.22	420.00±158.75	2.214	0.027	7
CRP (mg/L)	1.81±2.42	3.60±2.50	2.366	0.036	7
LDH (U/L)	195.88±38.42	155.29±40.33	-2.028	0.043	7

BMI – Body mass index; FM – Fat mass; FFM – Free-fat mass; FFMI – Free-fat mass index; SMM – Skeletal muscle mass; TBW – Total body water; CRP – C-reactive protein; LDH – Lactate dehydrogenase.

From the results obtained from these time points, it appears that CRT has a deteriorating effect on the patients. Most of the CT effects persist, mostly pertaining its cytotoxicity. The decrease of body water levels in patients are indicative of the continuous nephrotoxicity caused by the by-products of CT and their damage to the kidneys, bladder and urinary tract (Ries and Klastersky, 1986). Once again, there is an increased in the time the patients spent sitting, probably due to fatigue. However, the usual side effects of sedentary behavior, such as increased weight and fat mass, were not observed. In fact, the complete opposed situation is detected. It was verified a decreased weight, BMI, FMI and FFMI, which indicates that the treatment has contributed to a loss of both fat and free-fat mass. The fact that the free-fat mass (FFM%) has actually increased, is not an indication of an increase of free-fat mass, because both free-fat mass index (FFMI) and skeletal muscle mass (SMM) are lower. As FFM% and fat mass (FM%) are comparative measures, the increase in FFM% may indicate that the overall loss of FM is much higher than the loss of FFM. RT on HNC patients affects the whole tissues in and around the oral cavity and the digestive tract, which greatly compromises the nutritional intake of the patients (Chencharick and Mossman, 1983). Additionally, RT is known to cause stomatitis, xerostomia and loss of taste, which also compromises food intake (Lees, 1999).

The RT side effects are related with weight loss as the cause for decrease in food intake, affecting the physiology of fat-mass (Lees, 1999). However, c – reactive protein (CRP) levels also increased with the chemoradiotherapy (CRT) regimen, and have been shown to increase weight loss in various cancer studies (Simons [et al.], 1999). But in this case, the weight loss related with high levels of CRP concerns the loss of FFM. The presence of cancer-related systemic inflammation will induce the degradation of skeletal muscle cells through the activation of UPS pathway. Additionally, both FFMI and SMM decreased during the CRT treatment, which furthers validates the cachectic state of these patients.

However, contrary to what is expected from the results obtained, LDH levels continue to decrease. High LDH levels are usually associated to diseased states as LDH levels in plasma in normal situations are relatively low. They tend to increase in cancer situations due to the high energetic needs of cancer cells. Inflammation and tissue damage are also related to higher levels of circulating LDH (Miao [et al.], 2013). As it is used more as a prognostic factor then an inflammatory biomarker, these low levels of LDH might predict an overall good outcome of the cancer treatment for this group of patients.

4.2.6.3. Between 1st and 3rd time point

This last analysis, between the 1st and 3rd time points, indicates the effect of all treatment in the overall status of the patients. Some body composition variables, PA variables, QoL variables and biomarkers levels were altered (**Table 23**).

Weight (Z = -1.960, p = 0.05), BMI (Z = -1.960, p = 0.05), FFMI (Z = -2.201, p = 0.028) and TBW (Z = -2.521, p = 0.012) were the variables that shown decreased values throughout the treatment. As for PA variables, Total PA (Z = -2.366, p = 0.018) and Moderate PA (Z = -2.201, p = 0.028) have shown a decrease throughout the treatment. On the other hand, the time patients spent in sitting activities (Z = 1.973, p = 0.024) has increased. Global health status (Z = 1.973, p = 0.049) was the only variable that displayed alterations, with an increase of value throughout the treatment, which translates to an improvement in the overall health status of the patients. As for the plasma biomarkers, LDH (Z = -2.201, p = 0.028) and AST (Z = -1.992, p = 0.046), there was also a decrease between these two time points. The decrease of LDH levels may indicate a decrease in the inflammatory state of the patients while the decrease of AST levels could be related with a decrease in liver toxicity.

Table 23 Variables with alterations between the 1st and 3rd treatment time points.

Variables	1st Evaluation	3rd Evaluation	Z	p	N
Weight (kg)	67.44±14.29	61.15±11.26	-1.960	0.05	8
BMI (kg/m^2)	23.94±6.51	21.71±5.20	-1.960	0.05	8
FFMI (kg/m^2)	18.70±2.02	17.33±2.09	-2.201	0.028	8
TBW (L)	57.89±11.82	35.70±3.90	-2.521	0.012	8
Total PA (MET-min/week)	4823.50±6086.30	2313.43±5060.13	-2.366	0.018	7
Moderate PA (MET-min/week)	2125.00±3949.36	154.29±358.09	-2.201	0.028	7
Time sitting (s)	187.50±141.40	420.00±158.75	2.264	0.024	7
Global Health Status	44.80±16.03	65.64±27.61	1.973	0.049	8
LDH (U/L)	285.25±107.10	155.29±40.33	-2.201	0.028	7
AST (U/L)	47.88±63.58	26.57±25.89	-1.992	0.046	7

BMI – Body mass index; FFMI – Free-fat mass index; TBW – Total body water; PA – Physical activity; LDH – Lactate dehydrogenase; AST – Aspartate transaminase.

The side-effects associated with CRT treatment have already been discussed. The lack of food intake cause by damage in the gastrointestinal tract is most probable responsible for the weight loss observed throughout the treatment (Lees, 1999). Additionally, the low levels of TBW was one more indicator of the toxicity associated with CT, in this case associated with kidney damage (Ries and Klastersky, 1986). However AST levels are significantly low, which indicates that there is possibly no liver damage, also associated with this type of therapy (Vroon and Israili, 1990b). LDH's low levels, which is considered a good prognostic factor and the increase in the Global Health Status values might indicate a good outcome for this patients (Miao [et al.], 2013).

In this analysis it was possible to observe significant changes in PA, as opposed to the other time-point study. Both Total PA and Moderate PA have shown a decrease from the pre-treatment status to the end of treatment status of the patients. Combined with the increase of time spent sitting, it is a clear indicator to the physical deconditioning caused by the treatment. Fatigue is the most probable reason for this reduced PA, however throughout the treatment, patients with severe side-effects were most likely hospitalized, and confined to bed rest until the end of the treatment. As such, the significant loss of FFM can be both muscle weakness and sarcopenia caused by physical inactivity and muscle wasting caused by cachexia.

As recent studies suggest that PA can induce protein synthesis in the muscle through the production of anti-inflammatory, the low levels of PA performed in these patients may explain the loss of FFM. Battaglini *et al* (2012) suggested that the anti-inflammatory cytokines will work in two ways: 1) Induce pathways for the synthesis of muscle proteins; and 2) counteract the muscle degradation through the inhibition of pro-inflammatory cytokines. Throughout this study, it was possible to confirm that higher levels of PA were associated to lower muscle wasting and lower inflammation. As no anti-inflammatory cytokine was analyzed in this study, it is not possible to confirm if these beneficial effects were associated to them or to other response to PA, however it was possible to confirm the beneficial effect of PA in the overall state of these patients.

5.Conclusion

The overall aim of this study was to characterize changes in total body composition for patients undergoing radiotherapy or concurrent chemoradiation therapy for HNSCC. For that purpose, correlations between all the variables at the baseline state and changes in each variable throughout the treatment regimen of each patient were analyzed. The main conclusion withdrawn from this project were:

- A cancer-related inflammatory status was associated with lower muscle mass, fatigue and worst quality of life;
- Both TWEAK and Titin were good biomarkers for muscle wasting in cachectic patients;
- The lack of physical activity was associated with higher levels of fat mass, lower levels of free-fat mass and higher levels of fatigue;
- Lower quality of life in HNC patients was associated with more depression symptoms and less muscle mass;
- Cachexia was associated with higher inflammation states, worst quality of life and higher sedentary lifestyles;
- The chemotherapy treatment showed a decrease in muscle degradation;
- The chemoradiotherapy treatment was associated with a worsening of almost all variables and an increased in the inflammatory status;
- The whole treatment was associated with a decrease in physical activity, loss of muscle mass and an increase in the quality of life.

These were preliminary results, however it can be concluded that the cachexia syndrome is a metabolic disorder associated with poor quality of life, increased morbidity and significant lower survival rate in HNC patients. Physical activity can modulate the inflammatory response in cancer, preventing the muscle degradation observed in cachectic patients. However, because of the cancer status, the syndrome is only recognized when it is in its most advanced stages. It is necessary to identify early changes, as to intervene in a precocious manner.

Physical activity can modulate the inflammatory response and has many benefits in cancer patients, so it is important to add this information to the whole population, to healthy individuals. They must be encouraged to practice physical activity in their everyday lifestyles.

6.Future Perspectives

This project comprises a pilot study with the purpose of comparing changes in lean body mass, quality of life and cachexia biomarkers to the pattern of daily physical activity throughout the various treatment modalities. However, at the time of this projects conclusion, the number of patients with data for all time-points was too low and the analysis was only performed for one treatment modality. Therefore, the aim is to reach 20 patients, from all three of the treatment groups, with data for all the treatment time-points.

The optimization of the qPCR experiment is also to be continued, so the study of changes in expression of genes associated to atrophy and hypertrophy pathways in skeletal muscle (*FBXO32*, *TRIM63*, *TNFSF12*, *NFKB1*) for all patients can be conducted. In addition to these biomarkers, the study of some anti-inflammatory cytokines produced during PA and involved in the management of muscle wasting, such as IL-4, IL-10 and sTNFR, could be useful to explore the mechanisms behind the PA modulation process.

In the beginning of this study, the diet of each patient was also added in the analysis, however the questionnaires utilized were too generic and it was not possible to generate any conclusions of the patients' nutritional status. New questionnaires should be tested so that this variable can be incorporated in this project.

During this project, whole blood RNA and DNA was extracted and stored for further use. In the future, the RNA may be used to study the expression profile of HNC patients and eventually identify new RNA biomarkers associated with cachexia or other pathological conditions. The DNA may be used for genetic analysis, such as of DNA methylation studies and analyze and associate epigenetic factors with the development of cachexia in HNC patients.

In the future, and if these results continue promising, a new approach can be tested with a larger study in cachectic HNC patients submitted to a specific regular exercise training program in order to verify if it can also revert the metabolic imbalance caused by cachexia.

7.References

ANDUS, T; GEIGER, T; HIRANO, T; KISHIMOTO, T; HEINRICH, P C - Action of recombinant human interleukin 6, interleukin 1 beta and tumor necrosis factor alpha on the mRNA induction of acute-phase proteins. *Eur J Immunol*. Vol. 18. n.º 5 (1988). pp. 739-46.

ARGIRIS, A; KARAMOUZIS, M V; RABEN, D; FERRIS, R L - Head and neck cancer. *Lancet*. Vol. 371. n.º 9625 (2008). pp. 1695-709.

BARBER, M D; ROSS, J A; FEARON, K C - Changes in nutritional, functional, and inflammatory markers in advanced pancreatic cancer. *Nutr Cancer*. Vol. 35. n.º 2 (1999). pp. 106-10.

BARNARD, N A; SCULLY, C; EVESON, J W; CUNNINGHAM, S; PORTER, S R - Oral cancer development in patients with oral lichen planus. *J Oral Pathol Med*. Vol. 22. n.º 9 (1993). pp. 421-4.

BASKAR, R; DAI, J; WENLONG, N; YEO, R; YEOH, K W - Biological response of cancer cells to radiation treatment. *Front Mol Biosci*. Vol. 1. (2014). p. 24.

BASSUK, S S; RIFAI, N; RIDKER, P M - High-sensitivity C-reactive protein: clinical importance. *Curr Probl Cardiol*. Vol. 29. n.º 8 (2004). pp. 439-93.

BATTAGLINI, C L; HACKNEY, A C; GOODWIN, M L - Cancer cachexia: muscle physiology and exercise training. *Cancers (Basel)*. Vol. 4. n.º 4 (2012). pp. 1247-51.

BENTZEN, S M; TROTTI, A - Evaluation of early and late toxicities in chemoradiation trials. *J Clin Oncol*. Vol. 25. n.º 26 (2007). pp. 4096-103.

BIANCHINI, F; KAAKS, R; VAINIO, H - Weight control and physical activity in cancer prevention. *Obes Rev*. Vol. 3. n.º 1 (2002). pp. 5-8.

BLACK, S; KUSHNER, I; SAMOLS, D - C-reactive Protein. *J Biol Chem*. Vol. 279. n.º 47 (2004). pp. 48487-90.

BOEDEKER, C C; HENSEN, E F; NEUMANN, H P; MAIER, W; VAN NEDERVEEN, F H; SUAREZ, C; KUNST, H P; RODRIGO, J P; TAKES, R P; PELLITTERI, P K; RINALDO, A; FERLITO, A - Genetics of hereditary head and neck paragangliomas. *Head Neck*. Vol. 36. n.º 6 (2014). pp. 907-16.

BONALDO, P; SANDRI, M - Cellular and molecular mechanisms of muscle atrophy. *Dis Model Mech*. Vol. 6. n.º 1 (2013). pp. 25-39.

BRIZEL, D M; VOKES, E E - Induction chemotherapy: to use or not to use? That is the question. *Semin Radiat Oncol*. Vol. 19. n.º 1 (2009). pp. 11-6.

BROWN, J H; PARASKEVAS, F - Cancer and depression: cancer presenting with depressive illness: an autoimmune disease? *Br J Psychiatry*. Vol. 141. (1982). pp. 227-32.

BYERS, R M - Squamous cell carcinoma of the oral tongue in patients less than thirty years of age. *Am J Surg*. Vol. 130. n.º 4 (1975). pp. 475-8.

CABANILLAS, R; ASTUDILLO, A; VALLE, M; DE LA ROSA, J; ALVAREZ, R; DURAN, N S; CADINANOS, J - Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. *Head Neck*. Vol. 35. n.º 3 (2013). pp. E80-4.

CAI, D; FRANTZ, J D; TAWA, N E, Jr.; MELENDEZ, P A; OH, B C; LIDOV, H G; HASSELGREN, P O; FRONTERA, W R; LEE, J; GLASS, D J; SHOELSON, S E - IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell*. Vol. 119. n.º 2 (2004). pp. 285-98.

CAMPISI, G; GIOVANNELLI, L - Controversies surrounding human papilloma virus infection, head & neck vs oral cancer, implications for prophylaxis and treatment. *Head Neck Oncol*. Vol. 1. (2009). p. 8.

CASTELLSAGUE, X; QUINTANA, M J; MARTINEZ, M C; NIETO, A; SANCHEZ, M J; JUAN, A; MONNER, A; CARRERA, M; AGUDO, A; QUER, M; MUNOZ, N; HERRERO, R; FRANCESCHI, S; BOSCH, F X - The role of type of tobacco and type of alcoholic beverage in oral carcinogenesis. *Int J Cancer*. Vol. 108. n.º 5 (2004). pp. 741-9.

CERSOSIMO, R J - Hepatotoxicity associated with cisplatin chemotherapy. *Ann Pharmacother*. Vol. 27. n.º 4 (1993). pp. 438-41.

CHENCHARICK, J D; MOSSMAN, K L - Nutritional consequences of the radiotherapy of head and neck cancer. *Cancer*. Vol. 51. n.º 5 (1983). pp. 811-5.

CHOONG, N; VOKES, E - Expanding role of the medical oncologist in the management of head and neck cancer. *CA Cancer J Clin*. Vol. 58. n.º 1 (2008). pp. 32-53.

COLLEDGE, L - Treatment of Cancer of Pharynx and Larynx. *Br Med J*. Vol. 2. n.º 4046 (1938). pp. 167-8.

COUCH, M; LAI, V; CANNON, T; GUTTRIDGE, D; ZANATION, A; GEORGE, J; HAYES, D N; ZEISEL, S; SHORES, C - Cancer cachexia syndrome in head and neck cancer

patients: part I. Diagnosis, impact on quality of life and survival, and treatment. *Head Neck*. Vol. 29. n.º 4 (2007). pp. 401-11.

COUCH, M E; DITTUS, K; TOTH, M J; WILLIS, M S; GUTTRIDGE, D C; GEORGE, J R; CHANG, E Y; GOURIN, C G; DER-TOROSSIAN, H - Cancer cachexia update in head and neck cancer: Pathophysiology and treatment. *Head Neck*. Vol. 37. n.º 7 (2015). pp. 1057-72.

D'SOUZA, G; CAREY, T E; WILLIAM, W N, Jr.; NGUYEN, M L; KO, E C; RIDDELL, J t; PAI, S I; GUPTA, V; WALLINE, H M; LEE, J J; WOLF, G T; SHIN, D M; GRANDIS, J R; FERRIS, R L - Epidemiology of head and neck squamous cell cancer among HIV-infected patients. *J Acquir Immune Defic Syndr*. Vol. 65. n.º 5 (2014). pp. 603-10.

DANAN, D; SHONKA, D C, Jr.; SELMAN, Y; CHOW, Z; SMOLKIN, M E; JAMESON, M J - Prognostic value of albumin in patients with head and neck cancer. *Laryngoscope*. Vol. 126. n.º 7 (2016). pp. 1567-71.

DAVIES, A D; DAVIES, C; DELPO, M C - Depression and anxiety in patients undergoing diagnostic investigations for head and neck cancers. *Br J Psychiatry*. Vol. 149. (1986). pp. 491-3.

DEANS, C; WIGMORE, S J - Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr Opin Clin Nutr Metab Care*. Vol. 8. n.º 3 (2005). pp. 265-9.

DEANS, D A; TAN, B H; WIGMORE, S J; ROSS, J A; DE BEAUX, A C; PATERSON-BROWN, S; FEARON, K C - The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer. *Br J Cancer*. Vol. 100. n.º 1 (2009). pp. 63-9.

DENARO, N; MERLANO, M C; RUSSI, E G - Dysphagia in Head and Neck Cancer Patients: Pretreatment Evaluation, Predictive Factors, and Assessment during Radio-Chemotherapy, Recommendations. *Clin Exp Otorhinolaryngol*. Vol. 6. n.º 3 (2013). pp. 117-26.

DIVARIS, K; OLSHAN, A F; SMITH, J; BELL, M E; WEISSLER, M C; FUNKHOUSER, W K; BRADSHAW, P T - Oral health and risk for head and neck squamous cell carcinoma: the Carolina Head and Neck Cancer Study. *Cancer Causes Control*. Vol. 21. n.º 4 (2010). pp. 567-75.

DODSON, S; BARACOS, V E; JATOI, A; EVANS, W J; CELLA, D; DALTON, J T; STEINER, M S - Muscle wasting in cancer cachexia: clinical implications, diagnosis, and emerging treatment strategies. *Annu Rev Med*. Vol. 62. (2011). pp. 265-79.

DON, B R; KAYSEN, G - Serum albumin: relationship to inflammation and nutrition. *Semin Dial*. Vol. 17. n.° 6 (2004). pp. 432-7.

DONNELLY, J E; BLAIR, S N; JAKICIC, J M; MANORE, M M; RANKIN, J W; SMITH, B K; AMERICAN COLLEGE OF SPORTS, M - American College of Sports Medicine Position Stand. Appropriate physical activity intervention strategies for weight loss and prevention of weight regain for adults. *Med Sci Sports Exerc*. Vol. 41. n.° 2 (2009). pp. 459-71.

DOUMAS, B T; PETERS, T, Jr. - Serum and urine albumin: a progress report on their measurement and clinical significance. *Clin Chim Acta*. Vol. 258. n.° 1 (1997). pp. 3-20.

DUFFY, S A; TEKNOS, T; TAYLOR, J M; FOWLER, K E; ISLAM, M; WOLF, G T; MCLEAN, S; GHANEM, T A; TERRELL, J E - Health behaviors predict higher interleukin-6 levels among patients newly diagnosed with head and neck squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*. Vol. 22. n.° 3 (2013). pp. 374-81.

EVANS, W J; MORLEY, J E; ARGILES, J; BALES, C; BARACOS, V; GUTTRIDGE, D; JATOI, A; KALANTAR-ZADEH, K; LOCHS, H; MANTOVANI, G; MARKS, D; MITCH, W E; MUSCARITOLI, M; NAJAND, A; PONIKOWSKI, P; ROSSI FANELLI, F; SCHAMBELAN, M; SCHOLS, A; SCHUSTER, M; THOMAS, D; WOLFE, R; ANKER, S D - Cachexia: a new definition. *Clin Nutr*. Vol. 27. n.° 6 (2008). pp. 793-9.

EXARCHOS, K P; GOLETSIS, Y; FOTIADIS, D I - Multiparametric decision support system for the prediction of oral cancer reoccurrence. *IEEE Trans Inf Technol Biomed*. Vol. 16. n.° 6 (2012). pp. 1127-34.

FAKHRY, C; WESTRA, W H; LI, S; CMELAK, A; RIDGE, J A; PINTO, H; FORASTIERE, A; GILLISON, M L - Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst*. Vol. 100. n.° 4 (2008). pp. 261-9.

FEARON, K C - Cancer cachexia and fat-muscle physiology. *N Engl J Med*. Vol. 365. n.° 6 (2011). pp. 565-7.

FEARON, K C; GLASS, D J; GUTTRIDGE, D C - Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell Metab*. Vol. 16. n.° 2 (2012). pp. 153-66.

FUNG, C; GRANDIS, J R - Emerging drugs to treat squamous cell carcinomas of the head and neck. *Expert Opin Emerg Drugs*. Vol. 15. n.º 3 (2010). pp. 355-73.

GARAVELLO, W; BERTUCCIO, P; LEVI, F; LUCCHINI, F; BOSETTI, C; MALVEZZI, M; NEGRI, E; LA VECCHIA, C - The oral cancer epidemic in central and eastern Europe. *Int J Cancer*. Vol. 127. n.º 1 (2010). pp. 160-71.

GARG, P; KARJODKAR, F - "Catch Them before it Becomes Too Late"-Oral Cancer Detection. Report of Two Cases and Review of Diagnostic AIDS in Cancer Detection. *Int J Prev Med*. Vol. 3. n.º 10 (2012). pp. 737-41.

GELBARD, A; GARNETT, C T; ABRAMS, S I; PATEL, V; GUTKIND, J S; PALENA, C; TSANG, K Y; SCHLOM, J; HODGE, J W - Combination Chemotherapy and Radiation of Human Squamous Cell Carcinoma of the Head and Neck Augments CTL-Mediated Lysis. *Clin Cancer Res*. Vol. 12. n.º 6 (2006). pp. 1897-905.

GELIN, J; MOLDAWER, L L; LONNROTH, C; SHERRY, B; CHIZZONITE, R; LUNDHOLM, K - Role of endogenous tumor necrosis factor alpha and interleukin 1 for experimental tumor growth and the development of cancer cachexia. *Cancer Res*. Vol. 51. n.º 1 (1991). pp. 415-21.

GEORGE, J; CANNON, T; LAI, V; RICHEY, L; ZANATION, A; HAYES, D N; SHORES, C; GUTTRIDGE, D; COUCH, M - Cancer cachexia syndrome in head and neck cancer patients: Part II. Pathophysiology. *Head Neck*. Vol. 29. n.º 5 (2007). pp. 497-507.

GLASS, D J - Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol*. Vol. 37. n.º 10 (2005). pp. 1974-84.

GLASTONBURY, C M; PARKER, E E; HOANG, J K - The postradiation neck: evaluating response to treatment and recognizing complications. *AJR Am J Roentgenol*. Vol. 195. n.º 2 (2010). pp. W164-71.

GLEICH, L L; COLLINS, C M; GARTSIDE, P S; GLUCKMAN, J L; BARRETT, W L; WILSON, K M; BIDDINGER, P W; REDMOND, K P - Therapeutic decision making in stages III and IV head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg*. Vol. 129. n.º 1 (2003). pp. 26-35.

GOURIN, C G; BOYCE, B J; VAUGHT, C C; BURKHEAD, L M; PODOLSKY, R H - Effect of comorbidity on post-treatment quality of life scores in patients with head and neck squamous cell carcinoma. *Laryngoscope*. Vol. 119. n.º 5 (2009). pp. 907-14.

GOURIN, C G; MCAFEE, W J; NEYMAN, K M; HOWINGTON, J W; PODOLSKY, R H; TERRIS, D J - Effect of comorbidity on quality of life and treatment selection in patients with squamous cell carcinoma of the head and neck. *Laryngoscope*. Vol. 115. n.º 8 (2005). pp. 1371-5.

GROBBELAAR, E J; OWEN, S; TORRANCE, A D; WILSON, J A - Nutritional challenges in head and neck cancer. *Clin Otolaryngol Allied Sci*. Vol. 29. n.º 4 (2004). pp. 307-13.

GUHA, N; BOFFETTA, P; WUNSCH FILHO, V; ELUF NETO, J; SHANGINA, O; ZARIDZE, D; CURADO, M P; KOIFMAN, S; MATOS, E; MENEZES, A; SZESZENIA-DABROWSKA, N; FERNANDEZ, L; MATES, D; DAUDT, A W; LISSOWSKA, J; DIKSHIT, R; BRENNAN, P - Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. *Am J Epidemiol*. Vol. 166. n.º 10 (2007). pp. 1159-73.

GUO, W; ZHANG, M; SHEN, S; GUO, Y; KUANG, G; YANG, Z; DONG, Z - Aberrant methylation and decreased expression of the TGF-beta/Smad target gene FBXO32 in esophageal squamous cell carcinoma. *Cancer*. Vol. 120. n.º 16 (2014). pp. 2412-23.

HAAS, M; HEINEMANN, V; KULLMANN, F; LAUBENDER, R P; KLOSE, C; BRUNS, C J; HOLDENRIEDER, S; MODEST, D P; SCHULZ, C; BOECK, S - Prognostic value of CA 19-9, CEA, CRP, LDH and bilirubin levels in locally advanced and metastatic pancreatic cancer: results from a multicenter, pooled analysis of patients receiving palliative chemotherapy. *J Cancer Res Clin Oncol*. Vol. 139. n.º 4 (2013). pp. 681-9.

HANASONO, M M - Reconstructive Surgery for Head and Neck Cancer Patients. *Adv Med*. Vol. 2014. (2014). p. 795483.

HASHIBE, M; BRENNAN, P; BENHAMOU, S; CASTELLSAGUE, X; CHEN, C; CURADO, M P; DAL MASO, L; DAUDT, A W; FABIANOVA, E; FERNANDEZ, L; WUNSCH-FILHO, V; FRANCESCHI, S; HAYES, R B; HERRERO, R; KOIFMAN, S; LA VECCHIA, C; LAZARUS, P; LEVI, F; MATES, D; MATOS, E; MENEZES, A; MUSCAT, J; ELUF-NETO, J; OLSHAN, A F; RUDNAI, P; SCHWARTZ, S M; SMITH, E; STURGIS, E M; SZESZENIA-DABROWSKA, N; TALAMINI, R; WEI, Q; WINN, D M; ZARIDZE, D; ZATONSKI, W; ZHANG, Z F; BERTHILLER, J; BOFFETTA, P - Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *J Natl Cancer Inst*. Vol. 99. n.º 10 (2007). pp. 777-89.

HASHIBE, M; BRENNAN, P; CHUANG, S C; BOCCIA, S; CASTELLSAGUE, X; CHEN, C; CURADO, M P; DAL MASO, L; DAUDT, A W; FABIANOVA, E; FERNANDEZ, L; WUNSCH-FILHO, V; FRANCESCHI, S; HAYES, R B; HERRERO, R; KELSEY, K; KOIFMAN, S; LA VECCHIA, C; LAZARUS, P; LEVI, F; LENCE, J J; MATES, D; MATOS, E; MENEZES, A; MCCLEAN, M D; MUSCAT, J; ELUF-NETO, J; OLSHAN, A F; PURDUE, M; RUDNAI, P; SCHWARTZ, S M; SMITH, E; STURGIS, E M; SZESZENIA-DABROWSKA, N; TALAMINI, R; WEI, Q; WINN, D M; SHANGINA, O; PILARSKA, A; ZHANG, Z F; FERRO, G; BERTHILLER, J; BOFFETTA, P - Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiol Biomarkers Prev*. Vol. 18. n.º 2 (2009). pp. 541-50.

HOMANN, N; TILLONEN, J; MEURMAN, J H; RINTAMAKI, H; LINDQVIST, C; RAUTIO, M; JOUSIMIES-SOMER, H; SALASPURO, M - Increased salivary acetaldehyde levels in heavy drinkers and smokers: a microbiological approach to oral cavity cancer. *Carcinogenesis*. Vol. 21. n.º 4 (2000). pp. 663-8.

JHAM, B C; DA SILVA FREIRE, A R - Oral complications of radiotherapy in the head and neck. *Braz J Otorhinolaryngol*. Vol. 72. n.º 5 (2006). pp. 704-8.

JIN, Y; YE, X; SHAO, L; LIN, B C; HE, C X; ZHANG, B B; ZHANG, Y P - Serum lactic dehydrogenase strongly predicts survival in metastatic nasopharyngeal carcinoma treated with palliative chemotherapy. *Eur J Cancer*. Vol. 49. n.º 7 (2013). pp. 1619-26.

JOHNSON, N - Tobacco use and oral cancer: a global perspective. *J Dent Educ*. Vol. 65. n.º 4 (2001). pp. 328-39.

KANG, H; KIESS, A; CHUNG, C H - Emerging biomarkers in head and neck cancer in the era of genomics. *Nat Rev Clin Oncol*. Vol. 12. n.º 1 (2015). pp. 11-26.

KASAPIS, C; THOMPSON, P D - The effects of physical activity on serum C-reactive protein and inflammatory markers: a systematic review. *J Am Coll Cardiol*. Vol. 45. n.º 10 (2005). pp. 1563-9.

KERAWALA, C J; HELIOTOS, M - Prevention of complications in neck dissection. *Head Neck Oncol*. Vol. 1. (2009). p. 35.

KORVER, K D; GRAHAM, S M; HOFFMAN, H T; MCCULLOCH, T; FUNK, G F - Liver function studies in the assessment of head and neck cancer patients. *Head Neck*. Vol. 17. n.º 6 (1995). pp. 531-4.

KREIMER, A R; CLIFFORD, G M; BOYLE, P; FRANCESCHI, S - Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev*. Vol. 14. n.º 2 (2005). pp. 467-75.

KUMAR, A; BHATNAGAR, S; PAUL, P K - TWEAK and TRAF6 regulate skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care*. Vol. 15. n.º 3 (2012). pp. 233-9.

KUNG, T; SZABO, T; SPRINGER, J; DOEHNER, W; ANKER, S D; VON HAEHLING, S - Cachexia in heart disease: highlights from the ESC 2010. *J Cachexia Sarcopenia Muscle*. Vol. 2. n.º 1 (2011). pp. 63-69.

KUSHI, L H; DOYLE, C; MCCULLOUGH, M; ROCK, C L; DEMARK-WAHNEFRIED, W; BANDERA, E V; GAPSTUR, S; PATEL, A V; ANDREWS, K; GANSLER, T - American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. *CA Cancer J Clin*. Vol. 62. n.º 1 (2012). pp. 30-67.

KYLE, U G; GREMION, G; GENTON, L; SLOSMAN, D O; GOLAY, A; PICHARD, C - Physical activity and fat-free and fat mass by bioelectrical impedance in 3853 adults. *Med Sci Sports Exerc*. Vol. 33. n.º 4 (2001). pp. 576-84.

LANGEVIN, S M; MICHAUD, D S; MARSIT, C J; NELSON, H H; BIRNBAUM, A E; ELIOT, M; CHRISTENSEN, B C; MCCLEAN, M D; KELSEY, K T - Gastric reflux is an independent risk factor for laryngopharyngeal carcinoma. *Cancer Epidemiol Biomarkers Prev*. Vol. 22. n.º 6 (2013). pp. 1061-8.

LEES, J - Incidence of weight loss in head and neck cancer patients on commencing radiotherapy treatment at a regional oncology centre. *Eur J Cancer Care (Engl)*. Vol. 8. n.º 3 (1999). pp. 133-6.

LI, W Q; PARK, Y; WU, J W; GOLDSTEIN, A M; TAYLOR, P R; HOLLENBECK, A R; FREEDMAN, N D; ABNET, C C - Index-based dietary patterns and risk of head and neck cancer in a large prospective study. *Am J Clin Nutr*. Vol. 99. n.º 3 (2014). pp. 559-66.

LOPEZ, F; RODRIGO, J P; CARDESA, A; TRIANTAFYLLOU, A; DEVANEY, K O; MENDENHALL, W M; HAIGENTZ, M, Jr.; STROJAN, P; PELLITTERI, P K; BRADFORD, C R; SHAHA, A R; HUNT, J L; DE BREE, R; TAKES, R P; RINALDO, A; FERLITO, A - Update on primary head and neck mucosal melanoma. *Head Neck*. (2014).

LUCENTEFORTE, E; GARAVELLO, W; BOSETTI, C; LA VECCHIA, C - Dietary factors and oral and pharyngeal cancer risk. *Oral Oncol*. Vol. 45. n.° 6 (2009). pp. 461-7.

MACDONALD, N; EASSON, A M; MAZURAK, V C; DUNN, G P; BARACOS, V E - Understanding and managing cancer cachexia. *J Am Coll Surg*. Vol. 197. n.° 1 (2003). pp. 143-61.

MALONE, J; ROBBINS, K T - Neck dissection after chemoradiation for carcinoma of the upper aerodigestive tract: indications and complications. *Curr Opin Otolaryngol Head Neck Surg*. Vol. 18. n.° 2 (2010). pp. 89-94.

MARRON, M; BOFFETTA, P; ZHANG, Z F; ZARIDZE, D; WUNSCH-FILHO, V; WINN, D M; WEI, Q; TALAMINI, R; SZESZENIA-DABROWSKA, N; STURGIS, E M; SMITH, E; SCHWARTZ, S M; RUDNAI, P; PURDUE, M P; OLSHAN, A F; ELUF-NETO, J; MUSCAT, J; MORGENSTERN, H; MENEZES, A; MCCLEAN, M; MATOS, E; MATES, I N; LISSOWSKA, J; LEVI, F; LAZARUS, P; LA VECCHIA, C; KOIFMAN, S; KELSEY, K; HERRERO, R; HAYES, R B; FRANCESCHI, S; FERNANDEZ, L; FABIANOVA, E; DAUDT, A W; DAL MASO, L; CURADO, M P; CADONI, G; CHEN, C; CASTELLSAGUE, X; BOCCIA, S; BENHAMOU, S; FERRO, G; BERTHILLER, J; BRENNAN, P; MOLLER, H; HASHIBE, M - Cessation of alcohol drinking, tobacco smoking and the reversal of head and neck cancer risk. *Int J Epidemiol*. Vol. 39. n.° 1 (2010). pp. 182-96.

MARUR, S; FORASTIERE, A A - Head and neck cancer: changing epidemiology, diagnosis, and treatment. *Mayo Clin Proc*. Vol. 83. n.° 4 (2008). pp. 489-501.

MIAO, P; SHENG, S; SUN, X; LIU, J; HUANG, G - Lactate dehydrogenase A in cancer: a promising target for diagnosis and therapy. *IUBMB Life*. Vol. 65. n.° 11 (2013). pp. 904-10.

MITTAL, A; BHATNAGAR, S; KUMAR, A; LACH-TRIFILIEFF, E; WAUTERS, S; LI, H; MAKONCHUK, D Y; GLASS, D J; KUMAR, A - The TWEAK-Fn14 system is a critical regulator of denervation-induced skeletal muscle atrophy in mice. *J Cell Biol*. Vol. 188. n.° 6 (2010). pp. 833-49.

MOLDAWER, L L; ANDERSSON, C; GELIN, J; LUNDHOLM, K G - Regulation of food intake and hepatic protein synthesis by recombinant-derived cytokines. *Am J Physiol*. Vol. 254. n.° 3 Pt 1 (1988). pp. G450-6.

MOORE-CARRASCO, R; BUSQUETS, S; ALMENDRO, V; PALANKI, M; LOPEZ-SORIANO, F J; ARGILES, J M - The AP-1/NF-kappaB double inhibitor SP100030 can revert muscle wasting during experimental cancer cachexia. *Int J Oncol*. Vol. 30. n.° 5 (2007). pp. 1239-45.

MUSSELMAN, D L; MILLER, A H; PORTER, M R; MANATUNGA, A; GAO, F; PENNA, S; PEARCE, B D; LANDRY, J; GLOVER, S; MCDANIEL, J S; NEMEROFF, C B - Higher than normal plasma interleukin-6 concentrations in cancer patients with depression: preliminary findings. *Am J Psychiatry*. Vol. 158. n.° 8 (2001). pp. 1252-7.

NCCN, National Compreensive Cancer Network - Head and Neck cancer guidelines. *Practice Guidelines in Oncology*. (2009). Available at WWW: <http://oralcancerfoundation.org/treatment/pdf/head-and-neck.pdf>.

NEGRI, E; BOFFETTA, P; BERTHILLER, J; CASTELLSAGUE, X; CURADO, M P; DAL MASO, L; DAUDT, A W; FABIANOVA, E; FERNANDEZ, L; WUNSCH-FILHO, V; FRANCESCHI, S; HAYES, R B; HERRERO, R; KOIFMAN, S; LAZARUS, P; LENCE, J J; LEVI, F; MATES, D; MATOS, E; MENEZES, A; MUSCAT, J; ELUF-NETO, J; OLSHAN, A F; RUDNAI, P; SHANGINA, O; STURGIS, E M; SZESZENIA-DABROWSKA, N; TALAMINI, R; WEI, Q; WINN, D M; ZARIDZE, D; LISSOWSKA, J; ZHANG, Z F; FERRO, G; BRENNAN, P; LA VECCHIA, C; HASHIBE, M - Family history of cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Int J Cancer*. Vol. 124. n.° 2 (2009). pp. 394-401.

NICOLOTTI, N; CHUANG, S C; CADONI, G; ARZANI, D; PETRELLI, L; BOSETTI, C; BRENNER, H; HOSONO, S; LA VECCHIA, C; TALAMINI, R; MATSUO, K; MULLER, H; MUSCAT, J; PALUDETTI, G; RICCIARDI, G; BOFFETTA, P; HASHIBE, M; BOCCIA, S - Recreational physical activity and risk of head and neck cancer: a pooled analysis within the international head and neck cancer epidemiology (INHANCE) Consortium. *Eur J Epidemiol*. Vol. 26. n.° 8 (2011). pp. 619-28.

NIH, National Cancer Institute- - Oral Complications of Chemotherapy and Head/Neck Radiation (PDQ®)–Health Professional Version. (2016). Available at WWW: <http://www.cancer.gov/about-cancer/treatment/side-effects/mouth-throat/oral-complications-hp-pdq#section/_151>.

NOVAKOVIC, D; PATEL, R S; GOLDSTEIN, D P; GULLANE, P J - Salvage of failed free flaps used in head and neck reconstruction. *Head Neck Oncol*. Vol. 1. (2009). p. 33.

O'SULLIVAN, K E; PHELAN, J J; O'HANLON, C; LYSAGHT, J; O'SULLIVAN, J N; REYNOLDS, J V - The role of inflammation in cancer of the esophagus. *Expert Rev Gastroenterol Hepatol*. Vol. 8. n.º 7 (2014). pp. 749-60.

OMURA, G; ANDO, M; SAITO, Y; KOBAYASHI, K; YAMASOBA, T; ASAKAGE, T - Comorbidity as predictor poor prognosis for patients with advanced head and neck cancer treated with major surgery. *Head Neck*. Vol. 38. n.º 3 (2016). pp. 364-9.

PARKIN, D M; BRAY, F - Chapter 2: The burden of HPV-related cancers. *Vaccine*. Vol. 24 Suppl 3. (2006). pp. S3/11-25.

PENG, J; RADDATZ, K; LABEIT, S; GRANZIER, H; GOTTHARDT, M - Muscle atrophy in titin M-line deficient mice. *J Muscle Res Cell Motil*. Vol. 26. n.º 6-8 (2005). pp. 381-8.

PROCTOR, E; ROBBINS, K T; VIEIRA, F; HANCHETT, C; SOMMES, G - Postoperative complications after chemoradiation for advanced head and neck cancer. *Head Neck*. Vol. 26. n.º 3 (2004). pp. 272-7.

RAGIN, C C; MODUGNO, F; GOLLIN, S M - The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. *J Dent Res*. Vol. 86. n.º 2 (2007). pp. 104-14.

RAJAN, V R; MITCH, W E - Muscle wasting in chronic kidney disease: the role of the ubiquitin proteasome system and its clinical impact. *Pediatr Nephrol*. Vol. 23. n.º 4 (2008). pp. 527-35.

REID, B C; ALBERG, A J; KLASSEN, A C; SAMET, J M; ROZIER, R G; GARCIA, I; WINN, D M - Comorbidity and survival of elderly head and neck carcinoma patients. *Cancer*. Vol. 92. n.º 8 (2001). pp. 2109-16.

REIDY, J; MCHUGH, E; STASSEN, L F - A review of the relationship between alcohol and oral cancer. *Surgeon*. Vol. 9. n.º 5 (2011). pp. 278-83.

RICHEY, L M; GEORGE, J R; COUCH, M E; KANAPKEY, B K; YIN, X; CANNON, T; STEWART, P W; WEISSLER, M C; SHORES, C G - Defining cancer cachexia in head and neck squamous cell carcinoma. *Clin Cancer Res*. Vol. 13. n.º 22 Pt 1 (2007). pp. 6561-7.

RIES, F; KLASTERSKY, J - Nephrotoxicity induced by cancer chemotherapy with special emphasis on cisplatin toxicity. *Am J Kidney Dis*. Vol. 8. n.º 5 (1986). pp. 368-79.

ROWLAND, J H; YANCIK, R - Cancer survivorship: the interface of aging, comorbidity, and quality care. *J Natl Cancer Inst*. Vol. 98. n.º 8 (2006). pp. 504-5.

RUSSO, G; HADDAD, R; POSNER, M; MACHTAY, M - Radiation treatment breaks and ulcerative mucositis in head and neck cancer. *Oncologist*. Vol. 13. n.º 8 (2008). pp. 886-98.

SAFDARI, Y; KHALILI, M; FARAJNIA, S; ASGHARZADEH, M; YAZDANI, Y; SADEGHI, M - Recent advances in head and neck squamous cell carcinoma--a review. *Clin Biochem*. Vol. 47. n.º 13-14 (2014). pp. 1195-202.

SAMMUT, L; WARD, M; PATEL, N - Physical activity and quality of life in head and neck cancer survivors: a literature review. *Int J Sports Med*. Vol. 35. n.º 9 (2014). pp. 794-9.

SANABRIA, A; CARVALHO, A L; MELO, R L; MAGRIN, J; IKEDA, M K; VARTANIAN, J G; KOWALSKI, L P - Predictive factors for complications in elderly patients who underwent head and neck oncologic surgery. *Head Neck*. Vol. 30. n.º 2 (2008). pp. 170-7.

SASSOON, C; CAIOZZO, V J - Bench-to-bedside review: Diaphragm muscle function in disuse and acute high-dose corticosteroid treatment. *Crit Care*. Vol. 13. n.º 5 (2009). p. 221.

SAVVIDES, P P - The role of chemotherapy in the management of patients with head and neck cancer. *Semin Plast Surg*. Vol. 24. n.º 2 (2010). pp. 137-47.

SCHANTZ, S P; YU, G P - Head and neck cancer incidence trends in young Americans, 1973-1997, with a special analysis for tongue cancer. *Arch Otolaryngol Head Neck Surg*. Vol. 128. n.º 3 (2002). pp. 268-74.

SEELAENDER, M; LAVIANO, A; BUSQUETS, S; PUSCHEL, G P; MARGARIA, T; BATISTA, M L, Jr. - Inflammation in Cachexia. *Mediators Inflamm*. Vol. 2015. (2015). p. 536954.

SERUGA, B; ZHANG, H; BERNSTEIN, L J; TANNOCK, I F - Cytokines and their relationship to the symptoms and outcome of cancer. *Nat Rev Cancer*. Vol. 8. n.º 11 (2008). pp. 887-99.

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*. Vol. 29. n.º 10 (2007). pp. 893-900.

SIMONS, J P; SCHOLS, A M; BUURMAN, W A; WOUTERS, E F - Weight loss and low body cell mass in males with lung cancer: relationship with systemic inflammation, acute-

phase response, resting energy expenditure, and catabolic and anabolic hormones. *Clin Sci (Lond)*. Vol. 97. n.º 2 (1999). pp. 215-23.

SONI, N; MARGARSON, M - Albumin. Where are we now? *Current Anaesthesia & Critical Care*. Vol. 15. n.º 1 (2004). pp. 61-68.

STONE, P - The measurement, causes and effective management of cancer-related fatigue. *Int J Palliat Nurs*. Vol. 8. n.º 3 (2002). pp. 120-8.

SU, M; WANG, J; KANG, L; WANG, Y; ZOU, Y; FENG, X; WANG, D; AHMAD, F; ZHOU, X; HUI, R; SONG, L - Rare variants in genes encoding MuRF1 and MuRF2 are modifiers of hypertrophic cardiomyopathy. *Int J Mol Sci*. Vol. 15. n.º 6 (2014). pp. 9302-13.

TSOLI, M; ROBERTSON, G - Cancer cachexia: malignant inflammation, tumorkines, and metabolic mayhem. *Trends Endocrinol Metab*. Vol. 24. n.º 4 (2013). pp. 174-83.

VAN WILGEN, C P; DIJKSTRA, P U; VAN DER LAAN, B F; PLUKKER, J T; ROODENBURG, J L - Morbidity of the neck after head and neck cancer therapy. *Head Neck*. Vol. 26. n.º 9 (2004). pp. 785-91.

VAUGHAN, V C; MARTIN, P; LEWANDOWSKI, P A - Cancer cachexia: impact, mechanisms and emerging treatments. *J Cachexia Sarcopenia Muscle*. Vol. 4. n.º 2 (2013). pp. 95-109.

VINCENT, J L - Relevance of albumin in modern critical care medicine. *Best Pract Res Clin Anaesthesiol*. Vol. 23. n.º 2 (2009). pp. 183-91.

VISSER, M R; SMETS, E M - Fatigue, depression and quality of life in cancer patients: how are they related? *Support Care Cancer*. Vol. 6. n.º 2 (1998). pp. 101-8.

VON HAEHLING, S; ANKER, S D - Cachexia as a major underestimated and unmet medical need: facts and numbers. *J Cachexia Sarcopenia Muscle*. Vol. 1. n.º 1 (2010). pp. 1-5.

VROON, D H; ISRAILI, Z - Alkaline Phosphatase and Gamma Glutamyltransferase. Em (WALKER, H. K., HALL, W. D. & HURST, J. W.) Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd. Boston: 1990a.

VROON, D H; ISRAILI, Z - Aminotransferases. Em (WALKER, H. K., HALL, W. D. & HURST, J. W.) Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd. Boston: 1990b.

WALL, L R; WARD, E C; CARTMILL, B; HILL, A J - Physiological changes to the swallowing mechanism following (chemo)radiotherapy for head and neck cancer: a systematic review. *Dysphagia*. Vol. 28. n.° 4 (2013). pp. 481-493.

WARNAKULASURIYA, S - Causes of oral cancer -an appraisal of controversies. *Br Dent J*. Vol. 207. n.° 10 (2009). pp. 471-5.

WEN, Y; GRANDIS, J R - Emerging drugs for head and neck cancer. *Expert Opin Emerg Drugs*. Vol. 20. n.° 2 (2015). pp. 313-29.