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PhD THESIS

**A PARALLEL SCREENING IN VIVO AND IN VITRO
FOR PHARYNGEAL CANCER- CLINICAL OBSERVATIONS
CORRELATED WITH EXPERIMENTAL EVALUATIONS**

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SUMMARY OF THE PhD THESIS

1. INTRODUCTION

Head and neck cancer is a life changing disease, it can affect a variety of anatomical areas. This paper will discuss the pharyngeal cancer with the following subsites: nasopharyngeal, oropharyngeal and hypopharyngeal cancer. The most common form of malignant cell proliferation in this areas, is the development of squamous cell carcinoma. Diagnosed promptly has high life expectation, however during my training as a fellow resident doctor, I noticed that patients with pharyngeal cancer came in a late stage disease which needed a radical therapeutic strategy, especially for those with hypopharyngeal cancer and even sometimes oropharyngeal. The late diagnosis included ganglionic metastases which also was associated with poor prognosis and lowering the quality of life, in some cases the laryngectomy with radiotherapeutic treatment.

The challenge of the specialists in the field is to early diagnose and treatment initiation for pharyngeal cancer patients without delay. The mainstream treatment for pharyngeal cancer consists in radiotherapy mainly with cisplatin. The toxicities associated with mucositis, soreness of throat and mouth, pain, xerostomia due to the affecting of salivary glands, laryngeal edema and nutritional problems. In search of more tolerable and efficient treatment it was also tried with cetuximab but finally not with results as good as cisplatin. Recent research in the field of antitumor therapy is more and more highlighted on the priority of early diagnosis and prompt treatment for preventing tumoral growth and metastasis. Treatment related toxicities are associated with high dosage of medicine, usually for late stages of disease. This type of patients are also more subjected to hospitalisation and failure

finishing treatment with a usually requery of palliative and pain management drugs.

2. CURRENT STATE OF KNOWLEDGE

Head and neck cancer can affect a variety of anatomical areas. This paper will discuss the pharyngeal cancer with the following subsite: nasopharyngeal, oropharyngeal and hypopharyngeal cancer. The most common form of malignant cell proliferation in this areas, is the development of squamous cell carcinoma(SCC). Because of the epithelial origin, it is influenced mostly by the consumption of alcohol and tobacco, but also by other infectious and food factors. In the review of literature men are much more often affected than women with a ratio between 2:1 and 4:1, therefore men are diagnosed at 3.8 per 100,000 and women at 1.3 per 100,000.

Pharyngeal cancer incidence has increased in the last decade by an average of 0.6% per year, of which tonsillar cancer most frequently with 2.9% per year, this is due to the association of HPV infection, which affects the oropharynx site. The Epidemiological Surveillance and End Results Program highlighted the fact that the incidence of cancer linked to HPV increased by 3%, 2.3% and 1.7% annually between 2005 and 2014. While cancer associated with alcohol and tobacco use decreased 3% for oral floor cancer and 2.5% for hypopharyngeal cancer, 0.1% for gingival and oral cavity cancer.

NFC is described according to the extenct of tumor invasion. Most cases, the first sign is the appearance of cervical adenophaty (superior laterocervical). The symptomathology is detemined by the possible routes of spread:

- anteriorly: in the nasal cavity, pterigoid fossa and maxillary sinuses with the following simtpoms: repeted anterior epistaxis, nasal obstruction, hyposmia anosmia.

- lateral extension beyond of the pharyngo- basilar fascia in the pharyngo- temporal and infrateporal spaces with the following symptoms: serous otitis media with transmsion hearing loss.
- supero- posterior extension with the infiltration of the skull base with damage to the cranial nerves: oculomotor(III), abducens(VI), trigeminal(V).

In the last period of time there has been observed a influential rise in oropharyngeal site compared to hypopharingx and laringx, most of them involving the tonsills, with a proportion of 2.9% per year, due to the decrease in smoking and the increase in exposure to the Human Papilloma virus(HPV). Depending on the HPV status, there are two distinct types: molecular and epidemiological. Thus, non-HPV oropharyngeal cancer is epidemiologically similar to the traditional type of squamous cell carcinoma, including the long-term exposure to alcohol and tobacco that induces malignant cell proliferation, and oropharyngeal carcinoma with HPV exposure, most often to the HPV type 16, mostly because sexual behaviour which can develop regardless of alcohol and tobacco consumption.

- Positive HPV SCC are usually middle aged caucasians, with no smoking history, with a superior socio-economical state and a large number of exposure of different sexual partners.
- Negative HPV SCC are older- aged adults with a history of smoking and alcohol consupntion.

Another differentiation in the literature study between the two form of HPV status is that HPV positive occurs less in stage T1-T2 because of the asymptomatic sighs and more in N stage involving one or more latero-cervical adenopathies. Also the incidence of distant metastases was lower in HPV+ patients than in HPV- patients, therefore HPV+ oropharyngeal cancer had a 28% lower risk of death with a 48% risk of disease recurrence.

Therefore, the incidence of distant metastases was much lower in patients with positive HPV compared to patients with negative HPV, as well as

the development of secondary tumors, establish the fact that patients with positive oropharyngeal carcinoma have a much better survival rate than patients with HPV negative oropharyngeal cancer.

Due to the anatomy of the hypopharynx, which can indicate the natural progression of the disease, primarily through its unique lymphatic and vascular anatomy. This allows the tumors to metastasize in the regional cervical nodes as well as in more distant places. The lymphatic network in the region drains mainly to the cervical jugular nodes (II-IV) and the retropharyngeal nodes. The hypopharynx is closely connected to the nearby structures (oropharynx, larynx and esophagus) and due to the configuration of the pharynx through the lateral and posterior pharyngeal walls, the retrocricoid space and the piriform sinus, the tumor formation can have a substantial growth before causing invasion and damage to these structures

The treatment management is made taking into account the following criteria:

1. Tumor factors - which can be divided into histological (referring to tumor type, stage, tumor cell differentiation, level of invasion, extracapsular spread, and the presence of nearby or distant metastases) and molecular features (presence or absence of HPV or HBV, the expression of programmed death ligands (PD-L1 and PD-L 2), mutational burden and the involvement of specific genetic mutations, genomic deletions or amplifications, CMV).
2. Patient characteristics including : age, sex, smoking, comorbidities, treatment compliation.

Nevertheless the major factors that determine the choice of treatment remain: tumor location, size, histological type, lymph node invasion or involvement, extracapsular extension and neurovascular invasion.

The main cytotoxic drug used in the treatment of pharyngeal cancer is platinum-based chemotherapy (predominantly Cisplatin). It is administered

concurrently with radiation for radiosensitization or in a primary, adjuvant or palliative. The therapeutic changes usually are trying to make improvements in treatment modalities such as modifications in radiation delivery (intensity-modulated radiation therapy [IMRT] and fractionation delivery), the timing of chemotherapy administration (induction, concurrent, and/or adjuvant), and minimally invasive surgery; nevertheless, a slight improvement in survival for advanced cancers has been achieved. The newest version of guidelines for the diagnosis, therapy and follow-up of patients with HNC SCCs are provided by the European Society for Medical Oncology (ESMO)

Modern therapeutic techniques used in HNC are: de-escalation radio-chemio RT doses or volumes- the idea of reducing the usual treatment dose came due to the knowledge that an increased dose of radiation affects the neighboring structures (pharyngeal constrictor muscles, the base of the tongue and the supraglottic floor of the larynx and the major salivary glands) having side effects that decrease the patient's quality of life: xerostomia and difficulty swallowing , transoral robotic surgery- has the advantage of guiding the postoperative treatment, thus allowing in low-risk patients, the application of de-intensification of adjuvant therapy. This type of robotic head and neck surgery is performed using a da Vinci Surgical System of transoral otolaryngology, intensity-modulated radiation therapy [IMRT] and fractionation delivery- the goal is to increase tumor local control without affecting the nearby tissues due to the sensitive nearby structures that involve a HNC and a personalized treatment approach. The advantage of IMRT compared with 2- dimensional or 3- dimensional radiotherapy is that it can project a dose distribution of the tumor configuration(dependable of the shape or size) in the selected area, with the benefits of disease control rate and survival at patients with NPC. This type of treatment has better effect on nasopharynx and oropharynx with the benefit of avoiding important anatomical structures such as parotid salivary glands, optic stem, brain stem, brain, optic emerging a better quality of life after and in the

period of treatment decreasing xerostomia, trismus, pain, sticky saliva, mucositis, fatigue

Cancer still remain a huge health concern worldwide, with an incidence, at the present time, over 4.9 million of cases in Europe, from which over a hundred thousand only in Romania. The incidence from cancer in Romania, are expected to rise with 5.4% by 2030. According to global cancer observatory data, till now, deaths from cancer in Europe are over 2.2 million, from which, over fifty thousand in Romania, being expected to rise with 7.6% by 2030. For this purpose, in the last decade, considerable measures has been developed for the diagnosis, monitoring and therapy of cancer. The diagnostic stage is primordial because the discovery of cancer in an early phase is determined by the choice of the most suitable and effective cancer therapy. Although it presents many disadvantages and side effects, the viable conventional therapies used for cancer treatment are radiation therapy, chemotherapy and surgery.

The prevalence of malnutrition in patients with pharyngeal cancer varies from 42-77% and worsens throughout treatment. Nutritional support is an essential part of multidisciplinary care from diagnosis to institution of oncology treatment. Weight loss is one of the independent negative factors in the prognosis and development of complications. In recent years, several studies have demonstrated the importance, not only of weight, but also of body composition changes throughout cancer treatment. Thus, muscle loss may result in a much more limited administration dose of antineoplastic drugs due to a high volume of distribution of adipose tissue (patients with more adipose tissue have much slower elimination of the drug).

The state of hipovitaminosis in patients diagnosed with cancer has several possible causes: unbalanced food intake, altered metabolism, adverse effects of treatment and inflammation. Deficiencies in certain vitamins correlate with increased inflammation and an increase in C-reactive protein. Currently there is a low level of evidence regarding the condition of hypovitaminosis.

Adherence to nutritional treatment is complicated by the symptoms of acute toxicity: nausea and vomiting, thick and abundant saliva,odynophagia.

The mechanisms of carcinogenesis extended in the context of the function of vitamins, i.e. regulation and participation in metabolic processes in the cell, suggest a substantial impact of these compounds in the main stages of carcinogenesis. Among others, due to their antioxidant properties, i.e. by releasing carcinogenic reactive oxygen, and due to their participation in carbonic metabolism DNA methylation process basic epigenetic process for carcinogenesis involving: vitamins B6, B3, folic acid, but also vitamin A, C and D.

Therefore, the present thesis describe , the newest data from the specialized literature are described with reference to: (a) Head and neck cancer. Epidemiology. Rhynopharingx Cancer. Oropharingx Cancer. Hypopharynx cancer (b) Treatment strategies used in the field of ENT, current observations regarding the screening and therapy of pharyngeal cancer (c) The role of vitamins in the prevention of pharyngeal cancer (d) Pharyngeal cancer at an experimental level: types of cell lines used, applied experimental methods. The special part is structured in three chapters, as follows: clinical retrospective study focusing on incidence of pharyngeal cancer in western Romania, comparing the diagnosis strategies employed by practitioners. The relevance in the relapse and survival rate based on the initial lymphadenopathy stage (N0/N1) of the patients at admission in the hospital; the effects of time-delay treatment and treatment package time upon patients with pharyngeal cancer; assessment of the biological effects of cetuximab and methotrexath on keratinocytes and HET-CAM Test – on eggs. The paper also presents a part of general conclusions and particular contributions and ends with bibliographic references that support the information presented and the original results obtained.

3. AIM AND OUTLINE

Unfortunately, head and neck cancer is still diagnosed in late stages of disease, mostly with nodal involvement. Initiation of therapy due to the delay of diagnosis may affect tumor local control and the survival of the patient. Concerning the tumor sites, it seems that oropharynx and hypopharynx cancers may be associated with an increased mortality as compared to nasopharynx cancer. In order to secure a more accurate evaluation, larger studies concerning time delays and outcomes are necessary in prospective multicenter studies. Recurrences are expected to develop in 30–40% of treated patients if they do not follow a healthy lifestyle with improved personal care modalities, related to physical, mental, emotional, and social functioning, which contributes to preserving or increasing the life quality and overall satisfaction. Unfortunately, even the treatment strategies for patients with head and neck cancer play a crucial role in the alteration in quality of life.

The doctoral thesis makes an important contribution in this direction, due to the fact that is important to identify risk factors associated with malignancy and toxicity in patients with head and neck cancer. Early diagnosis and access to multidisciplinary teams are crucial for improving survival rates and HRQoL. Financial toxicity remains a major concern for HNC patients

Study highlights the evidence regarding the consequences of treatment delay on the prognosis of pharyngeal cancer-diagnosed patients undergoing therapy, offering a comprehensive overview of the cases registered in the western region of Romania between 2014 and 2018. This study focused particularly on the evaluation of Methotrexate and Cetuximab in keratinocytes, since there are a few reports of evidence of the modification that can generate on this cell type. Additionally, there is little information on Methotrexate and

Cetuximab effects in the wound remodeling process that allow a comprehensive evaluation of the impact that this drugs may have on the quality of the healing process and cell proliferation. Experimental research carried out was based on a series of relevant methods for preclinical studies, and the results obtained led to the successful fulfilment of the objectives.

4. PERSONAL CONTRIBUTIONS

4.1. CHALLANGES OF PHARYNGEAL CANCER SCREENING IN LOWER-INCOME COUNTRIES DURING ECONOMIC AND SOCIAL TRANSITIONS: A POPULATION BASED ANALYSIS

The results of this study are presented in the PhD thesis in **Chapter 5** and refers to the survival rate for patients that live in countries with limited access to tertiary health care opportunities are very low, showing a rate around 30-40%. 38 different symptoms were reported in patients with benign and malignant tumors. Ten symptoms were common to both groups but with different frequencies. In the malignant tumor group, the most common symptoms were dysphagia (40%), sore throat (39%), dysphonia (15%), and reflex otalgia (12%) (Figure 2a).

In the benign tumor group, the most common symptoms were nasal obstruction (15%), oral respiration (11%), and dysphagia (7%) (Figure 2b).

Since benign and malignant symptoms overlap, the clinical investigation must

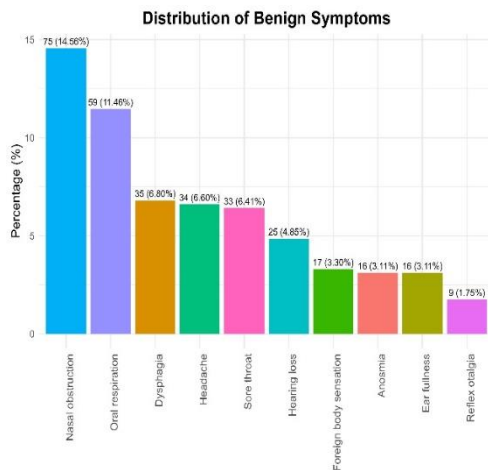


Figure 2a. Distribution of the Top 10 Non-Malignant Symptoms

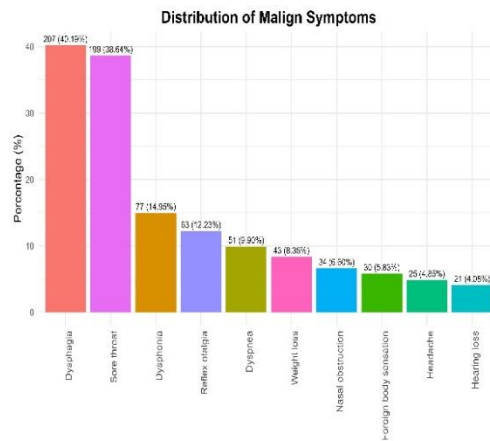
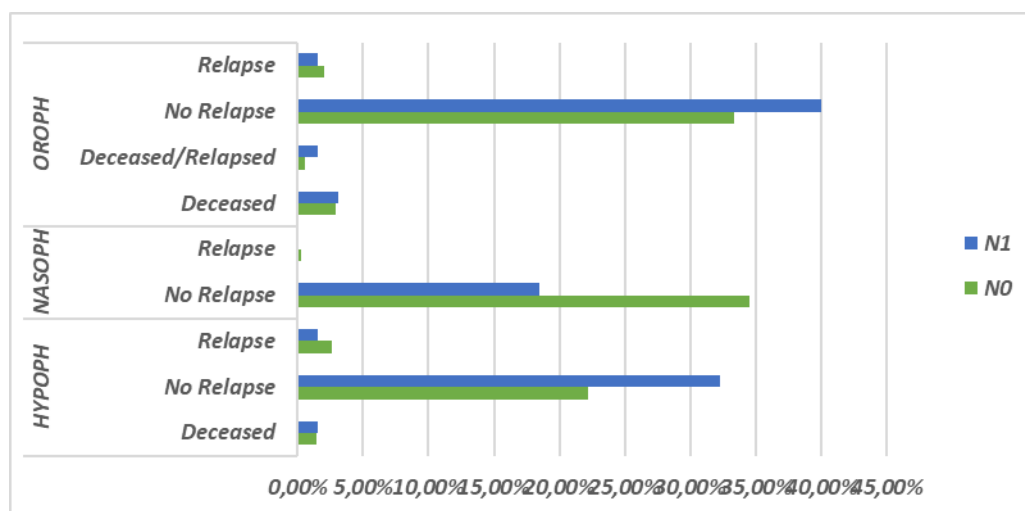


Figure 2b. Distribution of the Top 10 malignant Symptoms

be performed completely and the follow-up employed all the time, to accurately exclude a possible malignant diagnosis.

For Figure 3, survival time was calculated from initiation of therapy until disease remission. Patients with oropharyngeal tumors had the longest median survival of over 3 years, while median survival could not be estimated for nasopharyngeal and hypopharyngeal tumors due to insufficient events. However, the survival curve for hypopharyngeal tumors was below that of oropharyngeal tumors, indicating worse survival.



4.2. TIME-TO-TREATMENT DELAYS AND THEIR PROGNOSTIC IMPLICATIONS IN PHARYNGEAL CANCER- AN EXPLORATORY ANALYSIS IN WESTERN ROMANIA

In **Chapter 6** was studied effects on time-delay-treatment. The present study was conducted to investigate the effects of time delay treatment upon the prognosis of patients diagnosed with pharyngeal cancer in the western region of Romania. The major findings indicated that a time delayed treatment prolongs the treatment package time and therefore it has an unfavorable impact on the fast growing of tumors. The nasopharynx group had the most delayed TPT but with a better outcome than the other two sites. We had a more than 50% delayed DTI especially for oropharynx which increased mortality (HR=6.21, 95%CI=2.1-18.3).

Our retrospective study group showed that survival analysis in the risk of death stratified by the therapy regimen, the p-value of the treatment with chemotherapy alone group presented a higher mortality rate compared to radiotherapy alone. In this chapter, MIONPs via green chemistry approach were obtained for the first time, starting from two aqueous extracts of wormwood, based on leaves and stems. However, most head and neck cancer cases

were found in the oropharynx and hypopharynx. Pharyngeal cancer treatment aims to eradicate tumors while minimizing adverse effects and preserving the quality of life. Treatment choice is multifaceted, factoring in tumor characteristics and patient profiles. Platinum-based chemotherapy, especially Cisplatin, remains the mainstay.

The present study was meant to evidence the consequences of treatment delay on the prognosis of pharyngeal cancer-diagnosed patients undergoing treatment, offering a comprehensive overview of the cases registered in the western region of Romania between 2014 and 2018. The novel findings revealed in the current research showed a significant correlation between time delay and the evolution of pharyngeal cancer (especially, oropharynx and hypopharynx cancers), standing as a starting point for further investigations in this regard.

4.3. METHOTREXATE AND CETUXIMAB- BIOLOGICAL IMPACT ON NON-TUMORIGENIC MODELS: IN VITRO AND IN OVO ASSESSMENTS

Chapter 7 present the evaluation in vitro and in ovo the effects of methotrexate and cetuximab to determine their toxic potential in healthy cells. Thereby, the present study focuses on the evaluation of different concentrations (5, 15, 20, 30, 60, 90, 120, 150 g/mL) of Methotrexate and Cetuximab on HaCaT cell line, in terms of cell viability and anti-proliferative/anti-migratory capacity, when a stimulation time interval of 24 h was employed. To evaluate the mechanism associated with cell death, it was decided to apply the RT-PCR method by which the effect of Methotrexate and Cetuximab was evaluated in concentration of 150 g/mL in the expression of pro-apoptotic genes (Bax and Bad) and anti-apoptotic (Bcl-xL and Bcl-2). In addition, the potential irritant effect of the two cytostatics on the vascular plexus was also examined in ovo, by employing the chick chorioallantoic membrane (HET-CAM) assay.

The results showed that the wound healing rate of human immortalized keratinocytes (HaCaT) after exposure to different concentrations (5, 15, 20, 30, 60 µg/mL) of Cetuximab. The migratory capacity of HaCaT was monitored by taking pictures of the scratched area initially and 24 h post stimulation. Graph bars are calculated as the percentage of the scratched surface at 24 h, compared to the initial scratched surface (0 h). Scale bars denote 100 µm. The results represent the mean values \pm standard deviation (SD) of three independent experiments. One-way ANOVA was employed to determine the statistical differences, followed by a Tukey post-test (** $p < 0.01$; *** $p < 0.001$).

In summary, the results obtained in the current study revealed that human immortalized keratinocytes viability was more affected after exposure to Methotrexate, when compared to the impact induced by Cetuximab, under the same experimental parameters (concentrations and stimulation time interval of 24 h).

Based on the cell viability data, the expression of the main genes involved in the apoptosis process was analyzed. As seen, Methotrexate elicited an up-regulation of mRNA expression for the pro-apoptotic markers (Bax and Bad), while Cetuximab did not induce significant changes in the expression of these genes. Finally, by using the *in ovo* method, it was determined that Methotrexate has a stronger irritant effect compared to Cetuximab.

5. CONCLUSIONS

The present PhD thesis highlights the evidence regarding the consequences of treatment delay on the prognosis of pharyngeal cancer-diagnosed patients undergoing therapy, offering a comprehensive overview of the cases registered in the western region of Romania between 2014 and 2018. The novel findings revealed a significant correlation between time delay and pharyngeal cancer evolution (especially, oropharynx and hypopharynx cancers). As regards the treatment strategy, the results obtained from the survival analysis on the risk of death stratified by the therapy regimen reveal

that applying chemotherapy as treatment led to a higher mortality rate as compared with radiotherapy. In addition, concerning the tumor sites, it seems that oropharynx and hypopharynx cancers may be associated with an increased mortality as compared to nasopharynx cancer. In order to secure a more accurate evaluation, larger studies concerning time delays and outcomes are necessary in prospective multicenter studies. Recurrences are expected to develop in 30–40% of treated patients if they do not follow a healthy lifestyle with improved personal care modalities, related to physical, mental, emotional, and social functioning, which contributes to preserving or increasing the life quality and overall satisfaction. Unfortunately, even the treatment strategies for patients with head and neck cancer play a crucial role in the alteration in quality of life. Therefore, the treatments and protocol interventions should be focused both on survival and on ensuring quality of life. The particularities that should be considered refer to the management of pain, emotional and psychosocial instability, as well as rehabilitation support to overcome the debilitating physical barriers regarding the change in physical appearance, and the loss or modification of certain organs/functions.

In summary, treatment delay is associated with a bad prognosis in patients with head and neck cancer. It is suggested that a direct collaboration with or patient transition to a specialized hospital with improved healthcare capacity available to provide the most innovative treatment approaches represents a good strategy. However, further research should be conducted to verify if this strategy increases survival rate.

Met and Cet are two basic chemotherapies used successfully in cancer therapy. However, the toxic effects associated with them have not yet been fully elucidated. The results obtained in the current study showed that Met causes a dose-dependent cytotoxic effect in human keratinocytes. Cell death has been associated with up-regulation of pro-apoptotic markers (Bax and Bad). In addition, in the vascular plexus, Met caused irritant effects translated into hemorrhage, lysis, and vascular stasis. In comparison, Cet has a better

safety profile, which does not lead to significant changes in cell viability, apoptotic gene expression, and vascular irritant effect. In conclusion, the use of Met raises toxicity issues, but nevertheless, additional in vivo studies are needed to fully elucidate the mechanisms leading to the toxic side-effects associated with the administration of these drugs.