

**“VICTOR BABEȘ” UNIVERSITY OF MEDICINE
AND PHARMACY FROM TIMIȘOARA
FACULTY OF MEDICINE
DEPARTMENT IX: SURGERY I
ENT**

SITARU ADRIAN MIHAIL



PHD THESIS

**ASPECTS RELATED TO NEOPLASMS OF THE
UPPER RESPIRATORY TRACT AND THEIR
ASSOCIATION WITH NICOTINE**

– R E S U M E –

Scientific Coordinator
PROF. MARIOARA POENARU

**Timișoara
2022**

CONTENTS

List of scientific published articles	VI
List of abbreviations and symbols	VII
List of figures	VIII
List of tables	XII
Acknowledgement	XIII
INTRODUCTION	XIV
GENERAL PART	1
CHAPTER I. PATHOLOGIES OF THE UPPER RESPIRATORY TRACT	1
I.1 Introduction.....	1
I.2 Risk factors.....	5
I.2.1 Nicotinic acetylcholine receptors and upper respiratory tract health	6
CHAPTER II. MANAGEMENT OF MALIGNANT DISEASES OF THE UPPER RESPIRATORY TRACT	10
II.1 Introduction.....	10
II.2 Diagnostic.....	11
II.3 Treatment	15
II.3.1 Surgery	16
II.3.2 Radiation therapy	17
II.3.3 Drug therapies	19
CHAPTER III. IMPORTANCE OF PRECLINICAL RESEARCH TO SUCCESSFUL CLINICAL MANAGEMENT	22
III.1 Introduction.....	22
III.2 <i>In vitro</i> tests in the study of tumor pathologies	29
SPECIFIC PART	33
CHAPTER IV. CONTRIBUTIONS RELATED TO CYTOTOXIC EFFECTS OF NICOTINE ON HEALTHY CELLS AND SPECIFIC OBSERVATIONS RELATED TO IRRITANT POTENTIAL	34
IV.1 Introduction	34
IV.2 Materials and methods	36
IV.2.1 Reagents.....	36
IV.2.2 Cell cultures.....	36
IV.2.3 Assessment of cell viability	37
IV.2.4 Cell morphology.....	37

IV.2.5 Nuclear staining.....	38
IV.2.6 Chorioallantoid membrane test (CAM)	38
IV.2.7 Hen's egg test on the chorioallantoic membrane (HET-CAM)	39
IV.2.8 Statistical analysis	40
IV.3 Results	40
IV.3.1 Cell viability	40
IV.3.2 Cell morphology.....	43
IV.3.3 Nuclear labeling.....	48
IV.3. HET-CAM test	52
IV.4 Discussions	56
IV.5 Conclusions	61
CHAPTER V. CONTRIBUTIONS TO PRECLINICAL STUDIES CONCERNING NICOTINE AND ITS ROLE IN ENT MALIGNANT DISEASES	62
V.1 Introduction	62
V.2 Materials and methods	64
V.2.1 Reagents and cells	64
V.2.2 Evaluation of cytotoxicity	64
V.2.3 Statistical analysis	65
V.3 Results and Discussions	65
V.4 Conclusions	71
CHAPTER VI. INCIDENCE OF LARYNX CANCER DIAGNOSED IN THE WESTERN AREA OF ROMANIA	72
VI.1 Introduction	72
VI.2 Materials and methods	74
VI.2.1 Data source	74
VI.2.2 Histopathological evaluation	75
VI.2.3 Ethical considerations.....	75
VI.2.4 Statistical analysis	75
VI.3 Results	76
VI.3.1 Epidemiological data, special features and interventions	76
VI.3.2 Histopathological evaluation	81
VI.4 Discussions	88
VI.5 Conclusions	91
GENERAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS	92
REFERENCES.....	96
ANNEXES	I

RESUME

Laryngeal neoplasms are one of the most common malignancies of the head and neck, accounting for one third of all head and neck cancers, being a significant source of morbidity and mortality. In 2020, in our country, laryngeal neoplasms were diagnosed in more than 1,900 and caused more than 1,100 deaths. Several risk factors are involved in the malignant pathology of the larynx, the most important of which is represented by smoking.

The latest reports place Romania first among the EU-27 countries in terms of mortality due to laryngeal neoplasms, with a relative rate of change in ASR of over 133%. In these statistics, Romania was followed by Poland, Bulgaria, Lithuania, and Hungary. According to the same report, countries that in the past had an increased incidence of mortality (such as France, Spain, and Italy) now showed significantly reduced values.

The main risk factor for the appearance and development of laryngeal neoplasms is tobacco consumption (associated with more than 90% of cases, identified especially in the glottic area).

Cancer cell lines are valuable in vitro model systems that are widely used in cancer research and the discovery/development of new therapeutic protocols. Their use is primarily related to the ability to provide an undefined source of biological material for experimental purposes.

The current thesis was written according to specific rules and is structured in two parts, the general part, and the special part. The general part includes three chapters in which the following are addressed according to the latest guidelines and research: the types of malignant pathologies of the respiratory tract, the management of these pathologies and the relevance of preclinical studies for the success of clinical management. The special part, in turn, also includes three chapters, namely: contributions related to the cytotoxic effects of nicotine on some healthy cells and specific observations related to

the irritating potential; contributions to preclinical studies aimed at nicotine and its role in malignant pathologies in the ENT sphere and the incidence of laryngeal cancers diagnosed in the western area of Romania.

Early diagnosis plays an important role as it contributes to a high survival rate compared to late diagnosed counterparts. In recent years, although a slight decrease in the incidence of laryngeal neoplasms has been observed, the mortality rate has surprisingly increased. Until the early 1990s, the standard treatment for laryngeal neoplasms was total laryngectomy in combination with radiotherapy. Early-stage tumors are treated with transoral carbon dioxide laser microsurgery, while more advanced stages may involve partial laryngectomies and combination therapies. The treatment protocol for diagnosed stage I and II laryngeal neoplasms shows a lack of prospective data, as there is no common approach in clinical practice.

Despite the various reports currently available, which provide more epidemiological data associated with laryngeal neoplasms, the local epidemiology is poor and the global perspective is incomplete. The costs associated with the treatment of laryngeal neoplasms are high, and the patient's quality of life is greatly affected. Accurate epidemiological information is needed to develop effective prevention strategies and to contribute to their early diagnosis.

Regarding the scientific objectives, they were three in number, namely: (a) the determination through preclinical studies, in vitro, of the effects exerted by nicotine (in terms of viability, morphology and structure of the nucleus) on healthy cells (keratinocytes, hepatocytes and cardiomyocytes) and the effect related to irritant potential by means of the embryonated egg chorioallantoic membrane test, (b) selection of two cell lines (human gingival keratinocytes and pharyngeal carcinoma) to evaluate their behavior (using cytotoxicity and cell proliferation tests) in the presence of nicotine and (c) analysis of epidemiological data and histopathological features in patients with laryngeal neoplasm diagnosed in western Romania.

The methods approached to carry out the activities necessary to achieve the objectives are up-to-date methods that lead to reproducible results. For the in vitro preclinical studies, specific tests were used to analyze cell morphology, to quantify cell viability, to analyze changes at the nucleus level, while for the in ovo studies, the embryonated egg chorioallantoic membrane test was used, which allowed visualization of the effects at the level of blood vessels. Regarding the analysis of the incidence of laryngeal neoplasms diagnosed in the western part of Romania, the data source was presented and the histopathological analyzes were analyzed in a retrospective study.

The general part comprises three chapters addressing: (a) pathologies of the upper respiratory tract, (b) management of malignancies of the upper respiratory tract, and (c) the importance of preclinical research for successful clinical management.

The experimental part is structured on three chapters that describe the main directions of research and which are interconnected. The first chapter/study is focused on the cytotoxic effects of nicotine on healthy cells and specific observations related to irritant potential. Nicotine is a psychoactive substance known for the significant dependence it is capable of inducing. In addition, it has a high toxicological profile, belonging to the category of the most harmful substances. However, about the effect of nicotine on smokers, it is more difficult to establish a direct causal relationship between it and systemic toxic effects, due to the fact that both regular cigarettes and e-cigarettes contain a multitude of other potentially toxic substances. Several diseases associated with tobacco use have become more prevalent in recent years because of increased tobacco use. There are many known consequences of smoking in adults, including the development of cancerous processes in the organs exposed to tobacco, as well as several chronic conditions, such as eye disorders and cardiovascular, pulmonary, and periodontal diseases. Smoking has a toxic effect on the body, affecting a variety of cells in the body. Transdermal nicotine patches and the toxic effects

of smoking can adversely affect keratinocytes. Nicotine is primarily metabolized by hepatocytes, which is why tobacco consumption leads to various toxic changes at this level. Therefore, the current study was designed to assess the effects of nicotine on these cells at the cellular level. In addition, to obtain a more comprehensive understanding of the toxic potential of nicotine, the HET-CAM method was applied to determine the irritation potential of the chorioallantoic membrane of hen eggs. By calculating the milligram equivalent based on the nicotine density, the following concentrations were obtained: 0.10, 10.1, 25.2, 50.4 and 101 mg. The concentrations tested in this study were chosen primarily based on the nicotine content of e-cigarettes. It has been shown that there is a significant problem with the reporting of nicotine content. Additionally, the average nicotine content of e-cigarettes has been found to range from 6 to 36 mg/mL and can reach up to 60 mg/mL. Furthermore, the nicotine content of a cigarette was also considered, which varies according to the type and size of the product, at a concentration of 6 to 28 mg/g of tobacco. However, the amount of nicotine absorbed after smoking a cigarette is about 1.1 to 1.5 mg of nicotine per cigarette. Given that a person may smoke an average of 20 cigarettes per day, the amount of nicotine they may be exposed to on any given day varies between 22 and 30 mg. During the study, it was observed that nicotine can induce a marked reduction in cell viability, especially at the highest concentrations tested (50 and 100 μ L/mL), resulting in an average viability of only 2% for all cell lines tested. In addition, a significant step in this study was to evaluate the effect of nicotine on the blood vessels of the hen's egg chorioallantoic membrane. According to our findings, the lowest concentration tested, 0.1 μ L/mL, did not significantly contribute to changes in vascular architecture. In contrast, exposure to concentrations of 50 and 100 μ L/mL induced widespread irritant effects, including hemorrhage, coagulation, and lysis of blood vessels. In this case, the irritation score calculated for the two concentrations was analogous to that of the positive control, suggesting that nicotine is a highly irritating substance. To the best of

our knowledge, the irritant effect on the chorioallantoic membrane has not yet been explored.

The second chapter focused on preclinical studies through the lens of analyzing the effect of nicotine on some types of healthy and tumor cells closely related to malignant diseases of the upper respiratory tract. Nicotine uptake can occur in the oral cavity, nasal cavity, pharynx (nasopharynx, oropharynx, and hypopharynx), laryngeal lining, lungs, urinary bladder and gastrointestinal tract, and the degree of absorption is dependent on pH. In the ionized state in acidic environments, nicotine slowly crosses biological membranes. Absorption of nicotine in the lungs is rapid (occurs at a rate similar to that after intravenous administration) due to the large surface area of the lung alveoli and the pH of about 7.4, while in the stomach, nicotine absorption is reduced due to the acidic pH of the gastric juice but is well absorbed in the intestine due to the alkaline pH and the large absorption surface.

The third chapter is a retrospective study that addresses the incidence in the histopathological particularities of laryngeal neoplasms in the western area of Romania. Several risk factors are involved in the malignant pathology of the larynx, which are most often diagnosed in patients with a significant history of smoking and can involve different locations of the larynx. Data showed a high risk of developing laryngeal cancer in smokers (10-15 times higher compared to non-smokers) and an extremely high risk in heavy smokers (30 times higher compared to non-smokers). Early diagnosis plays an important role as it contributes to a high survival rate compared to late diagnosed counterparts. In terms of staging, carcinoma in situ is classified as stage 0 (the earliest stage) with the best response rates. The other stages are from I to IV, divided into substages, with stage IVc being the most advanced with the lowest response rates. Despite the various reports currently available, which provide more epidemiological data associated with laryngeal neoplasms, the local epidemiology is poor, and the global perspective is incomplete. The costs associated with the treatment of laryngeal neoplasms are high, and the

patient's quality of life is greatly affected. Accurate epidemiological information is needed to develop effective prevention strategies and contribute to the early diagnosis of laryngeal neoplasms.

The main conclusions that can be drawn are reproduced in the following.

Study 1. Contributions related to the cytotoxic effects of nicotine on healthy cells and specific observations related to irritant potential. It involved evaluating the toxicological profile of nicotine both in vitro, at the level of three healthy cell lines, and in ovo, at the level of the chorioallantoic membrane.

- the results obtained in the present study contribute to the understanding of the activity of nicotine on oral cells, but also on tumor cells of the pharynx.
- at certain concentrations, nicotine exerts harmful effects on cells, destroys healthy cells, which results in cell injury and the appearance of precancerous lesions.
- in the case of a malignant disease, nicotine can intensify cellular manifestations, and the results are negative for the patient's recovery.
- five different concentrations of nicotine were used in keratinocytes, cardiomyocytes, and hepatocytes in order to evaluate cell viability, cell morphology and its impact on nuclei.
- the hen egg chorioallantoic membrane test method (HET-CAM) was used to analyze biocompatibility and irritant potential.
- in all cell lines studied, nicotine was found to significantly impair cell viability, with the highest concentration tested resulting in less than 2% viable cells. Moreover, the morphology of the cells changed dramatically, with changes in their shape and confluence. Nicotine-induced cell death appears to be apoptotic based on its impact on the nucleus.

- it has been shown that nicotine has a very strong irritating effect on the chorioallantoic membrane.
- nicotine has an extremely strong toxicological profile, as demonstrated by the drastic reduction of cell viability and the induction of morphological changes and nuclear alterations associated with cell apoptosis.

Study 2. Contributions to preclinical studies targeting nicotine and its role in ENT malignancies. It involved investigating the effect of nicotine on primary gingival keratinocytes but also on pharyngeal tumor cells, by evaluating viability and apoptotic processes.

- several studies highlight the harmful effects of smoking and the extremely serious consequences of most of the compounds found in cigarettes and cigarette smoke.
- the obtained results contribute to the understanding of the activity of nicotine on oral cells but also on pharyngeal tumor cells.
- at certain concentrations, nicotine exerts harmful effects on cells, destroys healthy cells, which results in cell damage and the appearance of precancerous lesions.
- in the case of a malignant disease, nicotine contributes to the intensification of cellular morphological changes, and the results are negative for the patient's recovery.

Study 3. Incidence of laryngeal cancers diagnosed in the western part of Romania. It involved the analysis of epidemiological data on laryngeal cancer and histopathological features in patients diagnosed in western Romania.

- within three years and 10 months (between October 2016 and July 2020) in the ENT Clinic of the Timișoara Municipal Emergency Clinical Hospital, 194 patients with laryngeal neoplasms were diagnosed.

- the majority of patients were men (93.8%), mainly from Timiș county, and from the neighboring counties of Caraș-Severin, Hunedoara, Mehedinți and Arad.
- the average patient age (both sexes) was 62.6 years, with a range from 38 to 84 years and the highest percentages in the 60+ age group (48%).
- regarding smoking status, the main cause of the development of laryngeal neoplasms, 93.9% of patients were active.
- in terms of location [International Classification of Diseases 11th Revision (ICD-11)], approximately 69% were malignant neoplasms of the glottis (C32.0), followed by the supraglottis (C32.1), unspecified larynx (C32.9) and subglottis. (C32.2).
- from a histopathological point of view, several types of tumors were identified, the majority being non-keratinized and keratinized squamous cell carcinomas (about 90%).
- types of verrucous carcinoma, acantholytic carcinoma, squamous papilloma, a possible pleomorphic sarcoma, and pyogenic granuloma were identified.
- the data presented in this study highlight the increasing incidence of laryngeal neoplasms, which mainly affect men, at an increasingly younger age.
- it is imperative to involve specialists in nutrition, general medicine, ENT, dentistry to start awareness programs and develop prevention protocols.

The use of tobacco products is a major public health problem worldwide, as it is the leading preventable cause of death worldwide. Nicotine is a key component of electronic and conventional cigarettes; the addictive potential of nicotine is well known but its health effects are still not fully understood. The connection with the occurrence of laryngeal neoplasms is a

strong one and future studies to deepen and understand the mechanisms involved are necessary. At the same time, different awareness programs involving specialists from different specialties should be designed and implemented in the shortest possible time.