Oral and oropharyngeal squamous cell carcinoma

epidemiology and targeted treatment

Aisha Ahmed Hussein Al-Jamaei

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Dedicated to the memory of my brother in law

Abdul Kader Ali Hilal, who died tragically in 2016

Though you are gone, you are profoundly appreciated

"O my lord advance me in knowledge"

Qur'an, Surah Taha (114)

Contents

CHAPTER 1

General introduction

General introduction

Etiology and epidemiology

Head and neck cancer (HNC) is a broad term used to describe a variety of neoplasms occurring in different anatomical structures including among others the oral cavity, oropharynx, hypopharynx, and the larynx. Worldwide, HNC is one of the common and deadly diseases, with an estimated annual incidence of 650,000 cases for the year 2018, which is expected to increase to 833,000 new cases in 2020 (1,2). Based on the global estimate of the year 2012, the anatomical subsites with the highest prevalence of HNC are cancer of the oral cavity (202,000 cases) followed by oropharyngeal cancer (100,500 cases), and more than 90% of these malignancies are squamous cell carcinoma (SCC) (3). Therefore, this thesis focuses only on SCC of these two anatomical subsites of the head and neck region. Oral and oropharyngeal SCC (OOSCC) usually affects elderly people having a long and significant history of combined tobacco and alcohol use (4). However, in the last two decades, changes in trends for OOSCC have been reported, particularly in the western countries. The first noticeable change is an increase in the number of the patients who are being diagnosed with such diseases at ages younger than 45 years, which have no clear correlation with the classical risk factors smoking, drinking, and/or HPV (5-8). Revealing incidence of OOSCC for this young age group is significantly important to public health in planning services and prevention strategies not only in the western world, but also worldwide.

There are many cancer statistic web-based platforms such as the Surveillance, Epidemiology, and End Results (SEER) database, GLOBOCAN, Cancer Incidence in 5 Continents (CI5) and the European Network of Cancer Registries (EUREG) that essentially provide an estimate for the current incidence burden, mortality and surveillance of all cancers, for all age groups and covering all countries. However, there is an important drawback shared by all these registries, which concerns lack of the anatomical distinction between oral and oropharynx subsites. For example, in the SEER database, cancers from the tonsils are separated from oropharyngeal cancer incidence, while in gross anatomy it is part of it. Similarly, in the GLOBOCAN database, there is an aggregation for the anterior-two third and posterior-one third of the tongue as one subsite of the oral cavity. However, the anterior two-third is related to the oral cavity and the posterior-one third is part of the oropharynx (9-11). Because of that, a knowledge gap about the accurate global incidence rate of these malignancies in young patients is still apparent so far.

Another issue is the categorising of age groups since there is a lack of consensus on the definition of what age should be considered as "young" for patients with an OOSCC. The adolescents and young adults oncology groups (AYAO) agreed on the age range for adolescents (15-19 years) and the lower limit for young adults (early twenties) (12). However, there is no accepted age for the upper limit internationally. In SEER, the definition for AYAO group as a whole are the people with an age range of 15-39 years, while in EUROCARE it refers to individuals aged 15-24 years (13, 14). In the Netherlands, a different age range based on epidemiological studies was accepted for young patients with various oncological diseases; i.e 18-35 years (15). In published Dutch studies on the prevalence of OOSCC, 45 years has been used as the upper limit for young patients, which is not in accordance with the Netherlands AYAO group definition (16,17). Therefore, there is a need to evaluate the accurate incidence of these two neoplasms with emphasis on subdividing the young patients based on the quite commonly used cut-off points (20-34 years vs 35-44 years) to determine whether or not there is a difference between them and which age group needs more attention.

Equally important, gender disparity in incidence, disease prognosis, and mortality is a significant finding in a variety of cancers, including HNC (18-20). In fact, gender incidence disparity has been clearly observed in classical OSCC, showing that behavioural differences in smoking and drinking habits have been identified in men vs. women causing predominance of OSCC in elderly men (21, 22). However, with the emergence of a new trend of this kind of malignancy in people younger than 45 years which shows a slight propensity for women without smoking or drinking history, the need for further gender disparity evaluation, and research extending to potential alternative causes such as genetic and molecular mechanisms underlying oral carcinoma carcinogenesis in males and females, and investigation of gene modulation by sex hormones is deemed necessary. Moreover, the comprehensive understanding of socio-cultural differences associated with gender in this group of young individuals could result in better prevention strategies.

The second trend change in OOSCC is the upsurge in the incidence of oropharyngeal SCC (OPSCC), specifically those related to HPV infection. A recent systematic review evaluated frequencies of HPV-related OPSCC worldwide, and revealed a steadily increase over time in Europe reaching 50%, while a plateau at 65% has been reported in North America (23). For the Netherlands, despite the fact that several studies have been conducted to evaluate prevalence of HPV among OPSCC patients, the reported results were inconsistent, in the range of 30-40%, and somewhat heterogeneous (24-26). This is mostly because these studies were based largely on single

institutional data, which are strongly influenced by referral pattern. Therefore, a nationwide evaluation of the prevalence of HPV in OPSCC is necessary to determine the exact burden of this problem for the Dutch population. This will provide a baseline estimate to evaluate any future preventive measures such as HPV vaccination.

Oral SCC monitoring and current treatment modalities

Oral SCC (OSCC) represents the site with the highest frequency for HNC globally. It is well-documented that OSCC can develop in clinically normal mucosa or is preceded by oral potentially malignant disorders, or synonymously potentially premalignant oral epithelial lesions, such as leukoplakia (Fig.1) or erythroplakia (Fig 2). The main problem with these lesions is that they are relatively silent and progress into an invasive carcinoma without any specific alarming symptoms. This causes delay in cancer diagnosis, eventually leading to a poor prognosis. Of note, the malignant transformation rate of oral leukoplakia into an OSCC varies between 0.13% to 36.4%, depending on the study used definitions, and geographical location, which relates to the etiological factors (27,28). However, there is almost always severe dysplasia or even an invasive SCC on histopathological examination in oral erythroplakia (29). Intriguingly, the risk for malignant transformation becomes higher when these lesions affect the lateral border of the tongue or the floor of the mouth.

The mobile part of the tongue represents the most commonly affected mucosal site involved by SCC (41%) in the oral cavity worldwide (30). Mobile tongue SCC (MTSCC) is characterized by an aggressive clinical behaviour, where 40% of all patients already have cervical lymph node metastasis at initial diagnosis. Importantly, the extent of involvement of cervical lymph nodes is known to be the most important independent prognostic factor in OSCC that significantly affects the survival rate of patients (31). This, indeed, is one of the reasons for failure treatment and unsatisfactory 5-year survival which remains at approximately 50% since the last three decades despite advancements in various treatment modalities (32, 33). Therefore, there is an intensive focus on this specific subset to find alternative strategies to overcome such associated tragic outcomes.

The first possible strategy is to find a reliable and objective measurable biomarker that could detect the carcinoma as early as possible, and identify the high-risk patients. Equally important, biomarkers can also provide information on how the body will respond to any therapeutic intervention; this may help in making treatment decisions, and eventually could substantially reduce the tongue cancer mortality. Generally, biomarkers are categorized into three groups: diagnostic,

prognostic, and predictive. A diagnostic biomarker allows confirmation of the existence of the diseases. A prognostic biomarker is used to predict the expected clinical course, i.e. whether there will be aggressiveness and progression or recurrence of the disease, more or less irrespective of the treatment. Finally, the presence or absence of a predictive biomarker can be used to categorize patients in either more or less likely to respond in a favorable or unfavorable manner to a medication or a treatment (34). Of particular concern is that translation of the potential biomarkers from bench to clinic is a very long and complicated process. In order to facilitate the process of biomarker development and promote it efficiently, a guideline consisting of several stages has been suggested (35). Simply put, this guideline could be divided into 4 stages, starting with preclinical exploratory and ending with prospective validation. Since, indeed, this field was enticing for many researchers, a huge number of biomarkers studies about tongue cancer have been published in the last decade (36). Over 100 biomarkers in saliva were suggested as potential oral cancer biomarkers (37), and maybe more than this number was studied in tissues samples as well. Nonetheless, according to the suggested pipeline of developmental process, we do not know in which phases these biomarkers are, and which of those biomarkers could be considered as promising candidates and need further validation to proceed towards clinical use. Hence, there is an urgent need for a critical evaluation of those studies to advance the research in this field.

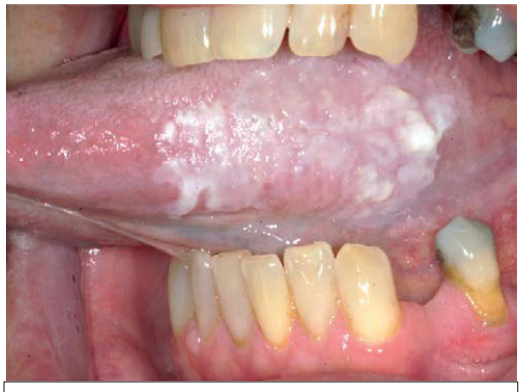

Fig. 1 oral leukoplakia on the lateral border of the tongue

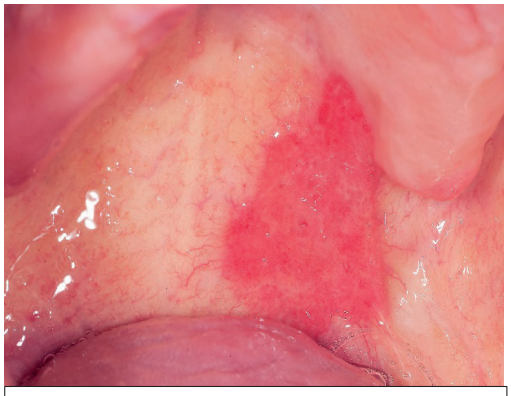

Fig.2 oral erythroplakia on the soft palate

If preventive measures were insufficient and/or monitoring (either or not with the use of biomarkers) shows that premalignant lesions have turned into malignancies, more radical treatments may be necessary. Up till now, surgery is considered as the cornerstone for treatment the small-sized MTSCC lesions. Radiotherapy is also an important modality used for patients with MTSCC as a part of their primary treatment and has shown a success rate similar to surgery when the

disease in stage I and II, though no clinical trial has made a direct comparison between them yet (38,39). However, for patients with locally advanced lesions in stages III and IV, radiation or chemoradiation are usually used as adjuvant treatment modality after surgery, particularly when some risk of relapse such as unclear margin or poorly differentiated disease exists (40, 41).

Development of novel treatment modalities: targeted therapies

Although these multimodality options may improve outcomes, it is frequently associated with disfigurements, high toxicity, and other sequelae that impair quality of life (42, 43). Therefore, in the last two decades great efforts have been put in finding alternative therapeutic options. These include, apart from immunotherapy and gene therapy, also targeted therapy. Targeted therapy is based on the advancement of understanding of the genetic and molecular cancer biology, and aims at specifically targeting and killing the cancer cells, while causing little side effects and maintaining cell viability of normal cells. In this context, some targeted molecules have been successfully developed for advanced head and neck cancer and approved by the US Food and Drug Administration (FDA). For example, a well-established and currently evaluated molecular agent is the epidermal growth factor receptor (EGFR) inhibitor Cetuximab, which demonstrated a significant improved overall survival when used concomitantly with radiation (44). Nonetheless, the rather low response rate and rapidly occurring resistance to Cetuximab warrant further efforts for novel therapeutics options (45, 46).

A recent and promising new therapeutic approach that has lately received great interest is the use of nanoparticle technology and its application in modern cancer treatment modalities. Briefly, nanoparticles are biocompatible and biodegradable delivery systems that are characterized by their ability of transporting several therapeutic agents such as gene therapy (siRNA) and conventional chemotherapeutic drugs simultaneously(47, 48). Moreover, these nanoparticles can be prepared with molecules on their outer surface to target surface markers that are upregulated on cancer cells when compared to healthy tissue cells. This can ensure selective accumulation and superior cytotoxic effects of the medications specifically at the cancer site, while leaving healthy tissues alone (49). To the best of our knowledge, targeted nanoparticle treatment has not been explored for MTSCC so far.

We envision a treatment approach in which we create nanoparticles that are coated with moieties specifically targeted against MTSCC cells (i.e., extracellular targeting), and which contain agents that interfere with aberrant intracellular processes specific for cancer cells (i.e., intracellular

targeting). These nanoparticles preferably are used as an adjuvant therapy in order to enhance or restore efficacy of conventional treatments such as chemo- or radiation therapy. Within this PhD study, we will perform initial steps to develop these dual-targeted nanoparticles for therapeutic purposes.

As an extracellular target, we will consider tyrosine kinase receptors (RTKs), which have been shown to be frequently upregulated in cancer cells (50). RTKs are transmembrane glycoproteins, comprising an outer, trans-membrane, and an inner domain. The RTKs consist of several families, among others epidermal growth factor receptor (EGFR), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), vascular endothelial growth factor receptors (VEGFRs), and Met (hepatocyte growth factor/scatter factor [HGF/SF] receptor). RTKs represent essential components of cellular signaling pathways that are activated upon binding of their ligands (50, 51). Moreover, it has been widely reported that RTKs show increased density of RTKs after radiation in many cancers (52, 53) and once radiation-activated, promote events that are implicated in cancer proliferation, invasion, and metastasis. In addition, radiation-activated RTKs have been shown to enhance DNA repair (54) after radiation-induced DNA damage, which suggest a potential role in the often occurring phenomenon of radio- and chemoresistance in many cancers including MTSCC.

Perhaps one of the most promising members of the RTK family is c-Met of which it is reported that a five-fold increase in its expression occurs upon exposure to irradiation in several cancers (52). In tongue carcinoma, c-Met receptor is a potential candidate because it is highly expressed in this type of cancer and was found to enhance the *in vitro* and *in vivo* metastasis, thus resulting in a poor prognosis (55). However, there is limited knowledge about its expression pattern upon exposure to radiation in oral cancer. Hence, studying the intra-and extracellular expression level of this receptor would ultimately confirm whether or not c-Met is a promising candidate for targeted delivery of medications with radiotherapy.
Ultimately, but beyond the scope of this thesis, we will explore the feasibility of a WEE1 inhibitor, MK-1775, as an intracellular therapeutic target. WEE1 is a molecule specifically involved in the temporary halt of the cell cycle in cancer cells to allow DNA repair to be completed prior to commencement of cancer cell proliferation, and thereby imposing the radioresistance to cancer cells. The MK-1775 agent will then be incorporated in the targeted nanoparticle to eradicate the tongue carcinoma cells specifically and effectively by potentiating the radiotherapeutic treatment.

RESEARCH AIMS AND OUTLINES OF THIS THESIS

In the light of the research context and problems addressed above, **one of the major objectives of this thesis is to determine the incidence trends of oral and oropharyngeal SCC both nationally and internationally in all age groups, with special emphasis on patients younger than 45 years. These evaluations should be useful in planning and designing specific and better prevention and treatment strategies to combat these types of HNSCCs. Other objectives are to assess the level of validation of MTSCC biomarkers that are available in the literature, and to perform initial research on identifying a suitable surface receptor that can enhance selective delivery of targeted therapies to the tumor sites of oral and potentially also oropharyngeal cancers.**

In **chapter 2** we conducted a systematic review to summarize and discuss the existing data worldwide regarding the incidence rate of oral and oropharynx cancer, with particular emphasis on patients aged less than 45 years, to determine the burden of this type of malignancy and increase awareness among this age group. Based on the results from chapter 2 we also investigated in **chapter 3 and 4** the incidence trends of oral and oropharyngeal squamous cell carcinoma in the Netherlands in more detail, again with special emphasis on patients younger than 45 years old. The data from the Netherlands Cancer Registry (NCR) were used to analyze changes in trends over the period 1989-2016 in young patients in two age subgroups, i.e. patients with age 20-34 and 35-44 years. These data were compared to those from the older populations. Since the data of the years 2015 and 2016 contained information about the classic risk factors smoking and drinking, and for oropharyngeal cancer also for HPV status, we determined whether correlations existed between these risk factors and the incidence trends within these two types of HNC. In **chapter 5** an evaluation of published MTSCC biomarkers was performed in order to identify as well as classify the biomarkers into validated and exploratory level of evidence. In **chapter 6** we describe the expression of c-Met protein in MTSCC cells upon exposure to ionized radiation at different time points. We selected flow cytometry and western blot approach to give us a complete picture about the intra- and extracellular expression of this receptor. In **chapter 7** the results of the topics covered in this thesis are discussed, and suggestions for future research are given. Finally, the thesis ends with a summary in English (**chapter 8**) of the main findings from the preceding chapters (2-6).

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CHAPTER 2

GLOBAL INCIDENCE OF ORAL AND OROPHARYNX CANCER IN PATIENTS YOUNGER THAN 45 YEARS: A SYSTEMATIC REVIEW

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is typically regarded as a disease of elderly people. However, increasing numbers of patients worldwide with HNSCC at younger age (defined as < 45 years old) have been reported in recent years.

To assess geographical variations and trends worldwide in incidence of oral and oropharyngeal cancer in young patients, a systematic review was conducted in Pubmed and Google scholar databases from 1975 to June 2016. Seventy-eight studies were selected for further study.

Nineteen population-based studies on incidence rate were available from 13 countries, showing a prominent increase over time except for The Netherlands. A notable rise of oral(mobile) tongue cancer among white women and oropharyngeal cancer in white men was observed. Data suggest that cancer in young patients may be a distinct clinical entity and characterized by different etiology and pathogenesis. Additionally, the relative proportion of oral and oropharyngeal cancer in young patients to total incidence revealed a significant difference between estimates from North America (5.5%) and both Africa (17.2%) and Middle East (14.5%).

It is concluded that (i) a rising trend in oral and oropharynx cancers is observed in young patients worldwide; (ii) incidence studies should properly define outcomes in age cohorts and use a consensus cut-off for young patients; (iii) more population-based studies should be performed in non-western regions to get accurate global measures of incidence for these cancers in young subpopulations; and (iv) there is an urge to identify new etiological factors in these young patients.

Key words: Oral cancer; Oropharynx cancer; Young patients; Incidence rate; Relative proportion

Introduction:

Head and neck (HN) cancer is a broad term used to describe a variety of neoplasms occurring in different anatomical structures including oral cavity, oropharynx, hypopharynx, and the larynx. More than 90% of these malignancies are squamous cell carcinoma (SCC) [1]. HNSCC is a serious global health problem, with estimated more than 550,000 new cases and 300,000 deaths annually [2]. 2015 data revealed 45,780newly diagnosed cases with HNSCCs and 8,650 expected related deaths in the USA alone [3]. In Europe, the situation is not different. 99,630 new cases of lip, oral cavity and pharynx cancer were reported in 2012, and 43,704 of deaths were reported in the same year[4].

These types of tumors are typically regarded as diseases of the elderly and predominantly seen in men in their sixth and seventh decades after many years of tobacco and alcohol abuse [5-8]. However, over the past 30 years, increasing numbers of patients worldwide are being diagnosed with HNSCC at a younger age (<40–45years old) [9]. From this point on, we will refer to these patients as "young patients". This new trend of high incidence among young patients was primarily observed in oropharyngeal (base of tongue, tonsil, and oropharynx) and oral tongue cancer [10,11].It is noteworthy that the demographic pattern of this disease among young patients is different with regard to the etiological factors and gender. Whereas human papillomavirus (HPV)- related oropharyngeal cancers are more likely to occur in men who are non-smokers, non-drinkers and have a good socio-economic status, oral (freely mobile portion) tongue cancer mostly affects young white women with unknown causes at the moment [12-16].

Little is known about the true incidence of oral and oropharyngeal SCC in young patients. The main difficulties arise from deficient cancer registries in non-western countries and the notable heterogeneity in the studies. Different age thresholds have been used in several studies to define "young" (below an age between 30 and 45 years), and/or no subtype

specifications for HN structures were performed. Even though several published papers have estimated the incidence rate in specific countries or regions, there is no study, to our knowledge, that has evaluated the global incidence of SCC in young patients. Therefore, this systematic review aimed to summarize and discuss the existing data regarding the incidence rate of oral and oropharynx cancer in patients aged less than 45 years, to highlight similarities and differences by geographic region, and to examine trends over time.

MATERIALS AND METHODS

Data Sources

For the review, search strategies involved the following four steps (Figure 1).

1- Query online database of Pubmed and Google scholar2- Review of all reference lists3- Contacting the authors4- Review data from International agency for research on cancer/cancer incidence in five continents **Database searches.** PubMed and Google scholar were searched for articles from 1975till June 2016, only in English with MESH terms "incidence rate", "epidemiology ","trend of incidence", "demographics", "oral cancer", "oral squamous cell carcinoma", "oropharyngeal cancer", "head and neck cancer", "young patients", "patients under 40years".

Step 1: Inclusion and exclusion criteria

a) Abstracts that discussed epidemiological issues such as incidence, trend, prognosis, and demography were identified.

b) Full-text articles of abstracts selected in Step 1 were retrieved and reviewed.

c) Articles were excluded for the following reasons:

1- Being case reports,

2- Age cut-off values for young patients > 45 years,

3- No specific analysis for oral or oropharynx sites (i.e. only HN cancer in general)

4- Incidence estimates based on fewer than 10 cases.

d) Studies were included if they met the following inclusion criteria:

a) Reporting incidence data in young age group < 45

b) Reporting incidence rates clearly in a population-based study or giving sufficient data to allow calculation of relative proportion

c) Specific cancer subsets and duration of case ascertainment

Step 2: Review of all reference lists Reference lists of all articles selected in Step 1 were subsequently examined for eligibility criteria.

Step 3: Contact authors for further information Seven authors were asked for full-text papers and/or for specification of results on relevant subgroups.

Step 4: Review data from international agency for research on cancer/cancer incidence in five continents http://ci5.iarc.fr/Default.aspxWe could not use this, because CI5 considers both mobile and posterior part of the tongue as one unit. However, the mobile part is in the oral cavity and base belongs tothe oropharynx. Also, the other parts of oropharynx are separated, like tonsil.

Data extraction

The following data for each study were extracted from full-text articles by the first author and reviewed by two other authors (MNH and HCWdV): author, country and region, study period, age groups, sites (international classification of diseases (ICD)was not included because many studies did not use it, and the rest used different versions of the coding system), patients number of young age in comparison to patients of all ages, gender, pathology, data source, relative proportion, incidence rate per 100,000 of population (either crude rate or age-standardized rate), and trends overtime. Because we could not find an adequate number of population-based studies to estimate the incidence in some areas of the world, we calculated the relative proportions. The relative proportion is defined as the number of cases in young age group divided by the number of cases in all ages. Finally, a pooled analysis was done for seven geographical locations and genders by Medcalc version 15.2. From each study, one estimate was chosen. In case of presence of multiple estimates, the most appropriate one was selected. For example, one study mentioned an estimate for people <40 years and another estimate for people <45 years. Here, the latter one was selected because it was more comprehensive and would cover the former value as well.

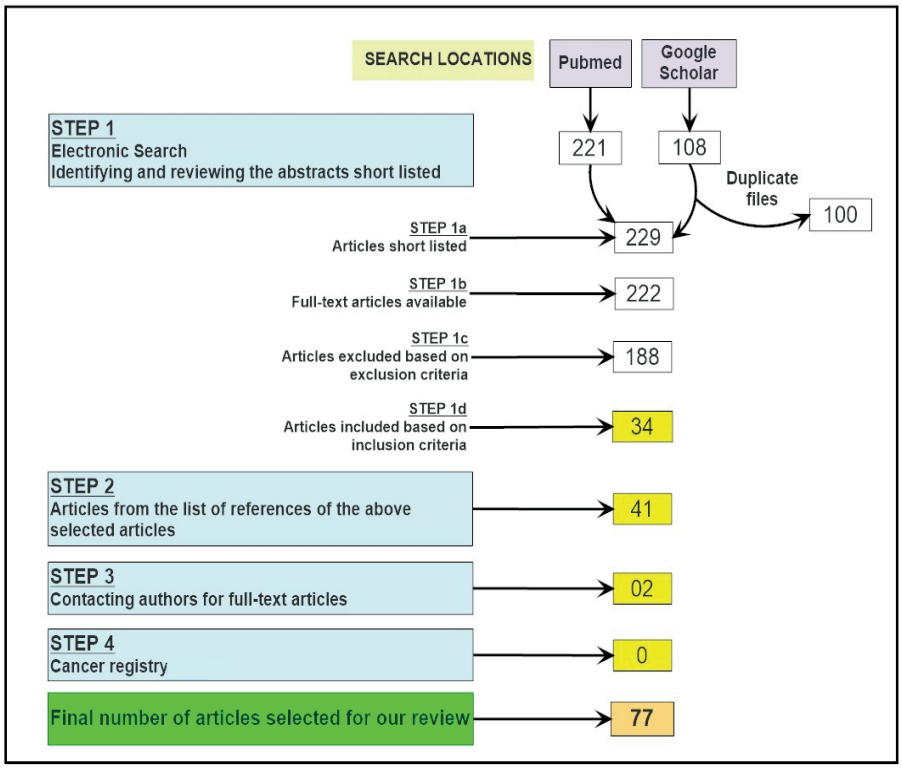


FIGURE. 1. Diagram of study selection

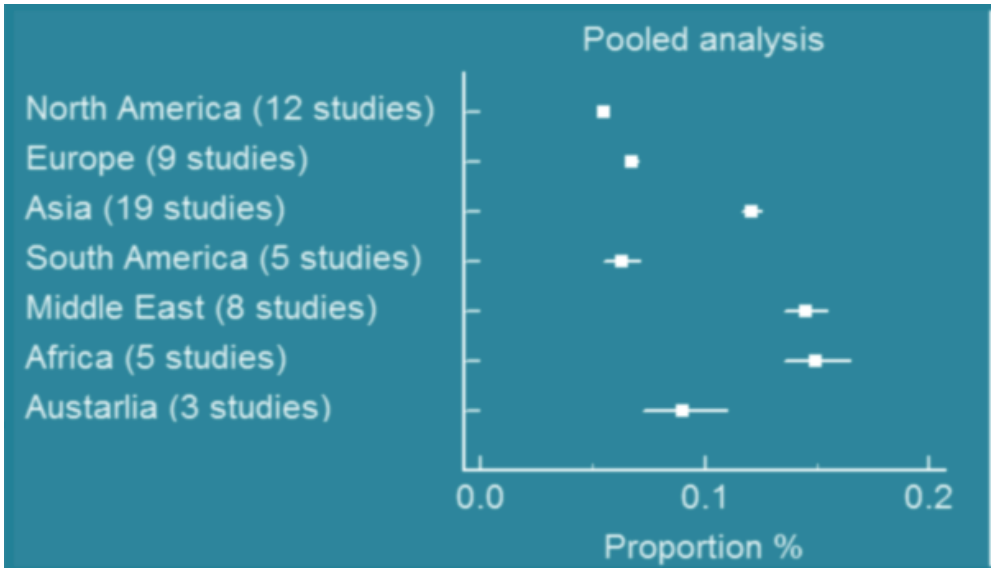
Results

A total of 78 papers were identified from North America (n=17), from Europe (n=18),from South America including Mexico (n=5), from Asia (n=21), from Middle East(n=8), from Africa (n=6), and from Australia (n=3). These studies provided data from48 different countries worldwide over a period of four decades. Nineteen population-based data on incidence rates were available from 13 countries(Table 1). These studies covered mostly the Western population due to accurate registry systems for age-stratified cancer cases and population size. A comparison of incidence data from cohorts diagnosed in 1960s and 1970s with those in later decades showed a doubling or even tripling in incidence in young age groups in several countries. Twelve studies conducted in USA, Canada, and Europe revealed substantial increasing trend of oral and oropharynx cancer among young patients[10,11, 16-20, 22-24, 28, 29]. Additionally, a notable increase in oral tongue cancer was reported in most of these studies, which showed a significant predilection for

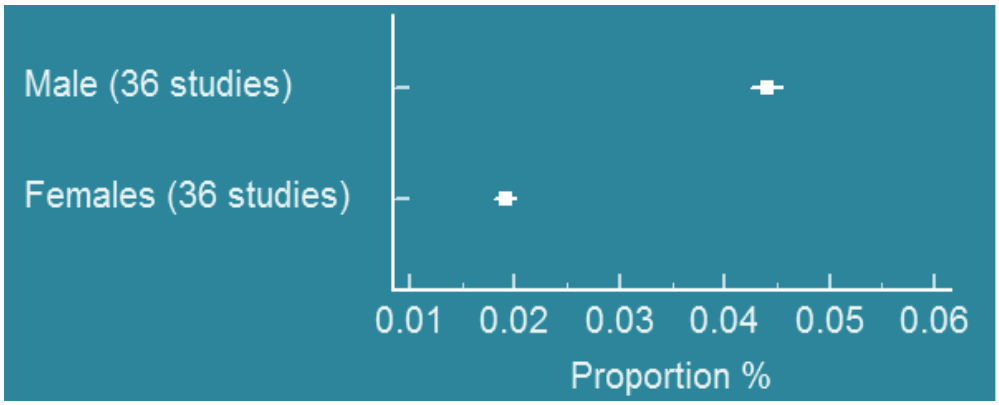
females[11, 16, 23]. In The Netherlands, a different pattern was noticed; there was significant decrease in cancer of oropharynx for both genders and an increase in oral tongue for men only [27]. With regard to race, a noteworthy difference was observed in some studies with higher cancer frequencies in young white people compared to the other races [10, 16, 18].Table 2 shows the relative proportion of oral and oropharyngeal cancer among patients(less than 45 years) in 68 studies. In North America, most of studies were conducted to evaluate tongue cancer and a remarkable increase in its proportions was noticed. It increased from 3.0% in 1975 to more than 11% in 2011 and 2013. In Europe, Scandinavia has shown a stable percentage of tongue cancer within younger age groups, but the trend revealed a persistence increase in females only. Less than 4.0%relative proportion of oral and oropharyngeal cancer in patients less than 40 years of age were reported in The Netherlands, Germany, and Poland. Greater than 6.5% was found in England, Spain, Finland, Portugal and France. Studies from Asia, Middle East and Africa have shown high percentages, particularly in Nigeria and Pakistan, with percentages as high as 29% and 30% respectively. This means that nearly one third of patients in these two countries were within the younger age group. Conversely, a very recent study from Brazil (South America) showed promising result with much higher reduction in relative proportion within youngpatients [71]. Though only 3 studies from Australia were included in this study, also a high relative proportion in young patients was identified [88-90].The overall pooled analysis for oral and oropharynx cancer proportions in youngpatients revealed a significant heterogeneity across the studies ($P < 0.0001$). Large differences in geographic location were noticed: Lowest estimates were from North America (5.4%) and South America including Mexico (5.7%), and highest for both Africa (17.2%) and the Middle East (14.5%) Figure (2). Estimates for Europe (6.8%)and Australia (9.0%) were in between. At the same time, the pooled proportions of gender showed higher percentage of males (4.7%) than females (2.1%).

FIGURE 2. Pooled Proportions of oral and oro- pharyngeal cancer in young adults according to geographic Location &gender

Geographic Location



Gender



Discussion

This systematic review brings together 78 papers on the incidence of oral and oropharynx cancer among patients younger than 45 years over the past four decades. To the best of our knowledge, this is the first study on this subject. It is known that the most reliable data on cancer incidence rate usually come from population-based studies, but unfortunately studies that discussed oral and oropharynx cancer in youngpatients were limited. Based on the available data, the most striking finding was that there is a growing incidence of oral and oropharynx cancer among young patients. Despite the fact that a few studies reported stability[21, 25, 31] or even reduction (the latter in The Netherlands [26, 27]), the general trend reflected an alarming worldwide increase in the rate. The increase, from 1960-1970 to the next decades, was double or triple in most countries. Another remarkable point is that the overall incidence of these cancers was gender-, ethnic-, and site-specific. Additionally, the relative proportion of oral and oropharyngeal cancer in young patients to total incidence revealed a significant difference between estimates from North America and both Africa and Middle East.

The data showed that the incidence rate in young patients has increased over several decades in particular in Western populations. However, this increase of oral and oropharyngeal cancers has been noticed simultaneously with a decline in rate of the classical type - occurring in older patients - which is likely the result of a reduction in smoking and alcohol consumption[91-93]. Thus, considering any correlation between traditional risk factors and cancer in young subgroups would be illogical. Additionally, smoking and alcohol consumption are known to be time- and dose related, so a short exposure in young patients would not be enough to cause any malignant transformation[33, 94]. Nevertheless, some authors examined the association and found many young patients were never smokers and never drinkers[33, 95]. Since then, awareness has been raised to study other etiological factors like the role of HPV[96], familial risk[97, 98], immunodeficiency [9, 99], and predisposition to genetic instability [100-103]. Concerning oral cancer there was not any evidence for a specific carcinogen so far, but a strong association was found between oropharyngeal cancer and HPV-infection [96].

In this study, oral (mobile) tongue has been shown to be the most common site of occurrence of oral SCC among the younger age group. This was in line with Müller et al who reported 62.1% of cases affecting tongue [37]. Slightly lower proportions of cases affecting the tongue were found by other authors (e.g. 38% by Hart et al [104],41% by Son and Kapp [105], and 45% by Mackenzie et al [106]). On the contrary, a study conducted in Taiwan, where betel quid chewing is common among youngpatients, a higher incidence of oral SCC in the buccal area (53.6%) was observed[107]. In Germany [42] and Brazil [70], the floor of the mouth was the most commonly involved site for oral SCC. These controversies are probably due to variations in social lifestyles in different countries.

Combining incidence analysis by ethnicity, gender and sites, we observed that the white females showed predominance of oral tongue cancer and white males for oropharyngeal sites. This was inconsistent with data from The Netherlands that showed tongue cancer more often in males although the exact reasons are not known for this predilection [27]. It is worthy to note that there is high incidence of tongue cancer in women <45 years of age, which was observed in both in the western population as well as people from countries such as India [62], China [51-53], and Korea [30]. Hence, it is necessary to evaluate tongue cancer in females to ascertain the causes for this gender predilection. One should assess whether hormonal changes or differential stress responses may play a role or whether it is purely because of environmental or genetic factors. Another intriguing aspect is why it occurs in particular in the mobile part of the tongue.

The surge in HPV infection (associated with sexual behaviors such as oral sex and multiple sexual partners) could explain the differences in gender- and ethnic specific incidence of oropharynx, particularly in base of tongue and tonsil cancers in white men. It has been reported that white individuals engage in these sexual activities more often than the blacks and that could be the reason for this differential incidence [108-110].

Although relative proportion is not an accurate measure of cancer incidences, it is useful to extract information about age-distributions in studies on case series. In this review, the proportions of oral and oropharyngeal cancer in young patients were considerably different

in various parts of the world. A proportional pooled analysis was done to get an understanding of the extent of the problem in different regions of the world, and to determine in which countries the young population is at high or low risk. According to this analysis, North America has the lowest proportion of 5.4%,consistent with the reported values in literature (1-6%) [37, 111, 112]. The main attributable factor for this could be the awareness among professionals and the population about general risk factors for cancer, and timely treatment and removal of precancerous lesions. South America, including Mexico, was very close to North America, but Europe had a slightly higher percentage than it, and much lower than the other regions. Interestingly, in Europe there was a considerable variation between countries ranging from 3.1% to 11.1%. This variety in incidences may reflect differences in life style and habits amongst different populations. Australia's percentage was in between Europe and Asia (see below), but in general it could also be considered as an area of high risk.

In Asia, Africa and Middle East, the estimated percentages were the highest. One explanation may be that the relative proportion of young patients may be higher due to a lower life expectancy in these countries. In particular, India and Pakistan both reported some of the highest incidence of oral cancer in old [113] as well as in youngpatients [68, 114, 115], which may in turn explain the corresponding high relative proportions in all Asia. A similar situation occurs in Africa, where for example the incidence of oral cancer in young patients was reported to be 3 to 6 times in Nigeria compared to the incidences in USA and Europe. The reasons suggested are poor diet and the habitual use of kola nuts and tobacco [83]. On the other hand, these risk factors appear inadequate to justify the high relative proportion in the Middle East. Perhaps, this reflects an overestimate because of the reluctance of old, and often illiterate, people to attend a hospital. Thus, they might not have been registered in the cancer registry. In contrast, the younger, educated generation, will seek medical help earlier, i.e. as soon as cancer is suspected. However, there may be specific risk factors which may not have been identified yet. Nonetheless, more studies are necessary to investigate the exact extent of the problem and the etiology.

In agreement with many previous studies [16, 50, 42,70, 85, 90], our pooled analysis showed that males outnumbered females. This result is similar to the general trend of classical type of oral SCC where men have always had higher incidence than women[116].

Within this first review of global incidence studies regarding oral and oropharyngeal cancer in young patients, a large number of studies have been included with various incidence measures. Using this approach, we could deduce that even though there is a large range in the soundness of the data sets depending on the region, a rising trend in oral and oropharynx cancers is being observed in young patients worldwide. Their incidence rates appear to be determined at least in part by gender, region, social habits and race. Overall, the cancers may even represent different entities in young vs. older patients, and may have distinct etiology and clinical behavior. However, the data on incidence rates and distinct characteristics of the tumors in young patients need to be substantiated with more and properly conducted incidence studies, in particular in nonwestern countries. Moreover, it is important to realize that other limitations of the current study include possible bias due to the heterogeneity in incidence measures and age cut-off to define the young patients, the retrospective analysis of the presented datasets that could threaten the overall validity and reliability, and the fact that some studies did not use the international coding system to describe the precise cancer sites, potentially affecting our interpretations. These limitations should be acknowledged and where possible avoided in future studies, in order to allow a more accurate measure of true global incidence for, and biological/clinical behavior of these cancers in young subpopulations.

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Table (1): Population-based Incidence rate of oral and oropharyngeal cancer worldwide in patients younger than (40-45 Yrs)

First author	Year	Country	Time-period	Age	Site	Patients No	Pathology	Data source	Incidence rate/100.000 time	Trend over time
Davis S & Severson RK [17]	1987	USA	1973-1984	<40	Oral tongue+ BT[#]	205	------	SEER[§]	0.45[a]	↑ about 3 fold in young male of age 30- 39 years
Shiboski CH [18]	2000	USA	1973-1996	<40	Oral cavity	2760	Mixed	SEER	W.F [d]: 0.6 in 1973, 1.9 19951.2 in 1996[b]	↑ significantly among in white males and females
Schantz SP [19]	2002	USA	1973-1997	<40	Oral tongue+ BT	617	------	SEER	0.13 from 1973-1984 0.21 from 1985-1997	Sharp increase over time
Shiboski CH [10]	2005	USA	1973-2001	20-44	Tongue & Tonsil	1807	SCC	SEER	Oral tongue 0.09 in 1973 & 0.48 in 2001 in white male. 0.61in 1973 &0.13 in 2001 In non-white. Oral tonsil 0.18 in 1973 &0.25 in 2001[b]	↑ significantly in mobile tongue, base of tongue and palatin tonsil in white individuals
Patel SG [16]	2011	USA	1975-2007	18-44	Oral cavity	2223	SCC	SEER	----------	↓ in young male, but ↑ in white females
					Oral tongue	814	SCC		----------	↑ in white individuals, Significantly in white females
Rodu B [20]	2007	USA	1973-2003	<40	Oral cavity	-----	Mixed	SEER	Males: 0.6[b] Females: 0.3 [b]	Non discernable in Male , ↑ in Females
Mehta V [21]	2013	USA	1975-2006	<40	Oral cavity & oropharynx	2309	SCC	SEER	0.5 *	Stable over time
Forte T [22]	2012	Canada	1992-2009	30-39	Oropharyngeal-HPV associated	202	SCC	Cancer registry	0.1 in 1992 & 0.2 in 2009 [b]	↑ significantly in males
Annertz K [11]	2002	Scandinavia	1960-1994	20-39	Oral tongue	276	SCC	Cancer registry	Men: 0.06 in 1960& 0.32 in 1994. Women: 0.03 in 1960 & 0.19 in 1994[b]	↑ 5 fold in males and 6 fold in females
Annertz K [23]	2012	Scandinavia	1960-2008	20-39	Oral tongue +BT	673	SCC	Cancer registry	--------	Persistence ↑ in females, stable trend in males
Conway DI [24]	2006	UK [e]	1990-1999	<45	Oral cavity & Oropharynx	------	Carcinoma	Cancer registry	Men : 0.8 & women: 0.4[b]	↑ by 24.7% in males and 43.2% in females

Doobaree IU [25]	2009	UK	1995-2004	<40	Intraoral	316	Mixed	Cancer registry	0.40 in 1995 &0.41 in 2004[b]	generally stable
Braakhuis BJM [26]	2009	The Nether-lands	1989-2006	<45	Oral cavity & Oropharynx	------	SCC	Cancer registry	------	↓ in both genders for all sites (Not significantly)
Braakhuis BJM [27]	2014	The Nether-lands	1989-2011	<45	Oral cavity &	------	SCC	Cancer registry	------	Stable over time, but tongue Cancer ↑ in males
					Oropharynx	------	SCC		------	↓ in both genders
Levi F [28]	1995	Switzerland	1974-1992	20-44	Oral cavity & pharynx	144	------	Cancer registry	Men: 3.9 in 1974-79& 6.5 in 1986-92 & Women: 0.5 in 74-79& 1.3 in 86-92[b]	↑ in both genders
Monteiro LS [29]	2013	Portugal	1998-2007	<45	Oral cavity & oropharynx	----	Mixed	Cancer registry	1.57 in 1998 &1.98 in 2007[c]	↑ for all sites in both genders
Choi SW [30]	2014	Korea	1999-2010	<40	Oral cavity	929	-----	Cancer registry	0.19[b]	↑ predominantly in oral tongue
Sunny L [31]	2004	India	1986-2000	<40	Oral cavity	1074	Mixed	Cancer registry	Men: 1.0 [b] Women: 0.6	Stable
Arora RS [32]	2012	England India	2001-2003	15-29	Mouth &tongue	60 120	-----	Cancer registry	0.21[b] 0.34[b]	-------

↑ : increase, ↓ : decrease, BT, base of tongue; SEER, Surveillance, Epidemiology, and End Results. a Annual average incidence (The author did not mentioned whether it used crude rate or age-standardised rate), b Age-standardised incidence rate, c Crude rate., d W.F, white female, e UK exclude North Ireland

Table (2): Relative proportion of oral and oropharyngeal cancer in patients of age (≤45 Years) among patients of all ages

First author	Year	Country	Time period	Age	Site	Patients No/All cases	Gender		Pathology	Relative proportion	Trend over Time
							Male	Female			
Byers RM [33]	1975	USA	1956-1973	<30	Oral Tongue	11/407	7	4	SCC	3.0 %	-----
Shemen LJ [34]	1984	USA	1955-1982	<40	Oral Tongue Oral Tongue	68/1590 124/3741	38 78	30 46	SCC Mixed	4.3 % 3.3%	↑ non-linearly
Davis S &Severson RK [17]	1987	USA	1973-1984	<40	Oral Tongue &BT	205/5481	135	70	-----	3.7%	↑
Schantz SB [35]	1988	USA	1944-1984	<40	Oral Tongue Other oral Cavity Sites	79/1349 64/3443	----- -----	----- -----	SCC SCC	6.0% 2.0%	↑ linearly
Myers JN [36]	2000	USA	1973-1995	<40	Oral Tongue	64	-----	-----	SCC	<10% in 1963 15% &25% in 1990s	↑
Shiboski CH [18]	2000	USA	1973-1984 1985-1996	<40	Oral cavity	1026/20224 1734/22499	----- -----	----- -----	Mixed	5.7% 7.7%	↑
Schantz SP [19]	2002	USA	1973-1984 1985-1997	<40	Oral Tongue +BT	204/5302 413/7246	----- -----	---- ----	------ ------	3.8% 5.7%	↑
Shiboski CH [10]	2005	USA	1973-2001	20-44	Oral cavity Pharynx[a]	2262/33,864 1067/23,460	----- -----	---- ----	SCC SCC	6.7% 4.5%	↑↓
Mu¨ ller S [37]	2008	USA	1971-2006	<40	Oral cavity	95/1919	55	40	SCC	5.0%	↑↓
Saba N [2]	2011	USA	1973-2008	<40	Oral Tongue &BT&Tonsil	1,941/51,092	-----	----	SCC	3.8%	-----
Patel SG [16]	2011	USA	1975-2007	18-44	Oral cavity Oral Tongue	2223/32,776 814/6,810	1666 506	557 308	SCC SCC	6.7% 11.9%	↑↓
Li R [38]	2013	USA	1990-2009	<40	Oral Tongue	------	-----	----	Mixed	3.8% black people 11.3% white People	-----
McGregor GI [39]	1983	Canada	1972-1981	<40	Oral cavity	19/518	-----	----	SCC	3.6%	↑
Howell RE [40]	2003	Canada	1983-1997	≤40	Oral cavity	57/1155	-----	----	------	5.0%	-----
Annertz K [11]	2002	Scandinavia	1960-1994	20-39	Oral tongue	276/5024	174	102	SCC	5.5%	↑
Annertz K [23]	2012	Scandinavia	1960-2008	20-39	Oral tongue +BT	673/12280	404	269	SCC	5.5%	↑ Till 2004 then slightly ↓

Sasaki T [41]	2005	UK	1990-1999	<40	Oral cavity	35/529	20	15	SCC	6.6%	-----
Udeabor SE [42]	2012	Germany	1980-1999	<40	Oral cavity & Oropharynx	38/977	30	8	SCC	3.90%	-----
van Monsjou HS [43]	2013	The Nether lands	1977-2008	<40	Oral cavity& oropharynx	54/1762	35	19	SCC	3.1%	↑↓
van Dijk BA [44]	2016	The Nether lands	1991-2010	≤ 45	Oral cavity	1140/13108	-----	----	Carcinoma	8.7%	↑↓
Girod A [45]	2009	France	1989-2002	≤45	Oral cavity& oropharynx	19/171	-----	19	SCC	11.1%	-----
Martin-granizo R [46]	1997	Spain	1979-1994	18-40	Oral cavity& oropharynx	24/294	15	9	SCC	8.2%	↑
Monteiro LS [29]	2012	Portugal	1998-2007	<45	Oral cavity& oropharynx	------	-----	----	Mixed	10.8%	↑↓
Atula S [47]	1996	Finland	1953-1962 1983-1992	<40	Oral Tongue	16/302 42/585	-----	----	SCC	5.3% 7.2%	↑
Pabiszczak MS [48]	2013	Poland	2000-2008	<40	Oral cavity& oropharynx	13/360	-----	----	SCC	3.6%	-----
Gatta G [49]	2015	All Europe	1999-2007	15-44	Oral tongue +BT oral cavity	3345/39592 3138/46206	2290 2226	1055 912	Mixed	8.40% 6.80%	-----
Liao CT [50]	2006	China	1996-2003	≤40	Oral Tongue	76/296	71	5	SCC	25.8%	-----
Park JO [51]	2010	China	1994-2008	<45	Oral Tongue	23/85	11	12	SCC	27.1%	-----
Fang QG [52]	2014	China	2011-2005	<40	Oral Tongue	15/176	6	9	SCC	8.50%	-----
Sun Q [53]	2015	China	2005-2012	<40	Oral cavity	31/430	19	12	SCC	7.2 %	-----
Zhang J [54]	2016	China	1960-2013	< 45	Oral cavity	1094/4097	-----	----	Mixed	26.7%	-----
Yip Connie SP [55]	2010	Singapore	1998-2006	≤40	Oral Tongue	17/123	8	9	SCC	14.0%	-----
Lim AA [56]	2014	Singapore	1991-2001	≤40	Oral cavity	16/173	-----	----	Mixed	9.2%	-----
Yamamoto N [57]	2013	Japan	2006-2011	≤40	Oral cavity	21/205	15	6	Mixed	10.2%	-----
Choi KK [58]	2006	Korea	1984-1996	<35	Oral cavity	59/861	-----	----	SCC	6.9%	-----
Choi SW [30]	2014	Korea	1999-2010	<40	Oral cavity	929/10282	-----	----	-----	9.0%	-----

Iamaroon A [59]	2004	Thailand	1991- 2000	<45	Oral cavity	75/587	43	32	SCC	12.8%	↑↓
Vatanasapt P [60]	2011	Thailand	1985-2001	<45	Oral cavity & Pharynx [b]	95/1038	-----	----	Mixed	9.2%	-----
Komolmala i N [61]	2015	Thailand	2001-2010	<40	Oral cavity	36/874	23	13	SCC	4.12%	-----
Kuriakose M [62]	1992	India	1988-1990	<35	Oral cavity	37/2046	17	20	SCC	1.30%	-----
Iype EM [63]	2001	India	1982-1996	<35	Oral cavity	264	184	80	Mixed	2.8%	-----
Subapriya R [64]	2007	India	1991-2003	<40	Oral cavity	66/388	-----	----	SCC	17.0%	-----
Sherin N [65]	2008	India	2002-2007	<40	Oral cavity	44/606	-----	----	SCC	7.5%	↑
Gupta PC [66]	2014	India	1983-1987 2010	<40	Oral cavity	42/310 88/398	42 88	---- ----	-----	13.5% 22.1%	↑↓
Ranganathan K [67]	2015	India	2000-2013	≤40	Oral cavity	32/151	22	10	SCC	21.2%	-----
Bhurgri Y [68]	2005	Pakistan	1995-2002	<40	Oral cavity	--------	-----	----	Mixed	30.0%	-----
Hirota SK [69]	2008	Brazil	1994-2004	≤ **40**	Oral cavity	13/121	8	5	SCC	10.7%	-----
Ribeiro AC [70]	2009	Brazil	1990-2005	<40	Oral cavity	46/400	38	8	SCC	12.0%	-----
Santos HB [71]	2016	Brazil	2000-2012	<45	Oral cavity	76/2311	62	14	SCC	3.3%	↑↓
Hernandez-Guerrero JC [72]	2013	Mexico	1990-2008	<40	Oral cavity	62/531	-----	----	SCC	11.6%	↑↓
Brandizzi D [73]	2008	Argentina	1992-2000	<45	Oral cavity	9/274	-----	----	Mixed	3.0%	-----
Andisheh- Tadbir A [74]	2008	Iran	1992-2007	<40	Oral cavity	30/200	18	12	SCC	15.0%	-----
Falaki F [75]	2011	Iran	1996-2009	<40	Oral cavity	21/158	12	9	SCC	13.2%	-----
Duzlu M [76]	2014	Turkey	1993-2013	<45	Oral cavity	48/230	-----	----	Mixed	20.8%	-----
Halboub E [77]	2012	Yemen	1994-2008	<40 <45	Oral cavity	62/457 105/457	34 57	28 48	SCC SCC	14.0% 23.0%	-----
Subhashraj K [78]	2009	Libya	1991-2007	≤40	Oral cavity	12/81	10	2	SCC	15.0%	-----
Zini A [79]	2010	Israel	1970-2006	<45	Oral cavity	531/4017	-----	----	SCC	13.2%	-----

Soudry, E [80]	2010	Israel	1992-2007	<30	Oral Tongue	11/85	5	6	SCC	13.0%%	-----
Hilly O [81]	2013	Israel	1996-2012	≤30	Oral Tongue	16/113	9	7	SCC	14.0%	-----
Chidzonga MM [82]	2006	Zimbabwe	1982-1991	≤40	Oral cavity& lip+Maxillay antrum	68/358	39	29	SCC	19%	-----
Otoh EC [83] ------	2005	Nigeria	1987-2002	<40	Oral cavity	11/43	-----	-----	Mixed	25.5%	-----
Oji C [84]	2007	Nigeria	1998-2003	<40	Oral cavity	24/81	14	10	Mixed	29.6%	-----
Khammissa RA [85]	2014	South Africa	1995-2002	≤40	Oral cavity	32/496	21	11	SCC	6.4%	-----
Onyango JF [86]	2004	Kenya	1978-1997	<40	Oral cavity	130/821	-----	----	------	15.8%	------
Idri AM [87]	1995	Sudan	1970-1985	<40	Oral cavity	103/616	-----	----	Carcinoma	16.7%	------
Hyam DM [88]	2003	Australia	1979-2000	<40	Oral Tongue	15/192	9	6	SCC	12.0%	------
Veness MJ [89]	2003	Australia	1980-2000	≤40	Oral Tongue	22/164	13	9	SCC	13.4%	------
Lam L [90]	2006	Australia	1977-2001	≤40	Oral Tongue	50/611	31	19	SCC	8.1%	------

↑ : increase., ↓: decrease , ↑↓ Fluctuated , ------ : not mentioned, a : excluded nasopharynx and hypopharynx , b : excluded nasopharynx

CHAPTER 3

TREND ANALYSIS OF ORAL SQUAMOUS CELL CARCINOMA INCIDENCE AND RISK FACTORS AMONG DUTCH PATIENTS, WITH EMPHASIS ON YOUNG ADULTS

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Submitted

Abstract:

Objectives: Worldwide, oral squamous cell carcinoma (OSCC) incidence is increasing among young adults. However, the definition of "young" varies; the cutoff of < 35 and < 45 years are both commonly used. In our trend of incidence and etiology analysis of Dutch patients with OSCC, we therefore subdivided our young cohort in 20-34, and 35-44 age groups, and compared these to the older patients.

Materials and Methods: Data from the Netherlands cancer registry database (1989-2016) were analyzed using join point regression software for time trends in incidence rates by age, gender, and clinical stage. Smoking and drinking data were available from 2015 onwards.

Results: 17,289 cases of OSCC were reported, with an overall male-to-female ratio of 1.3:1. Annual incidence increased significantly by 2.8 % for patients aged 20-34 years, while it decreased for those aged 35-44 years by -1.1 %. In both groups > 75 % had tongue carcinoma. In adults aged 45-59 years, incidence rates declined slightly from 1992-2010 [annual percentage change (APC) of -0.1%], while steeply in 2010-2016 (APC -4.6%). In patients older than 60 years, incidence rates increased overall, with an APC for women being twice as high as men. Of all patients, 67% were smokers, and the same pattern was observed about prevalence of alcohol consumption (67%).

Conclusions: The striking difference in incidence trends in the two young age groups demonstrates that subcategorization may substantially affect outcomes. Further studies elucidating the underlying reasons for the observed differences are needed.

Key words: Oral squamous cell carcinoma, young adults, incidence rate , join point analysis, risk factors

Abbreviation: Oral squamous cell carcinoma (OSCC); European Standardized Rate (ESR) ; annual percentage change (APC) ; average annual percentage change (AAPC)

Chapter 3

Introduction

Internationally, incidence of oral cancer varies considerably and because of its high mortality rate, it remains a serious problem for global public health. Based on the global estimate of the year 2012, cancer of the oral cavity alone was responsible for 202,000 incident cases [1]. The most recent GLOBOCAN data available (2018) showed that a total of 354,864 new cases of lip and oral cavity cancer were diagnosed worldwide, and highest incidence rates were reported in Melanesia and South Central Asia [2]. In India, for example, cancer of the oral cavity accounts for up 30 % of the total cancer cases, in contrast to only 3 % in the western world [3]. More than 90% of malignant oral tumors are squamous cell carcinoma (SCC) and most patients are men between 50-70 years of age with a history of tobacco and alcohol use [4]. Lately, a trend of increasing numbers of patients younger than 45 years old has been reported in several countries of the world, except for the Netherlands [5].

Interestingly, the subgroup of young patients was different with regard to etiological factors and gender distribution as compared to the older age group, since a remarkable rise of mobile tongue cancer was observed among non-smokers / non-drinkers white women [5]. These findings were inconsistent with results from the Netherlands which showed that tongue cancer more often occurred in young males [6]. Disappointingly, risk factors responsible for this trend in both genders remain ill-defined [6, 7].

Until now, a lack of consensus exists on what age should be considered to characterize "young" patients. The vast majority of published studies arbitrarily used cut-off values of either 35 or 45 years [8], [9], [10]. Unavoidably, incidence rates and their trends will therefore vary, thus making it impossible to compare reported incidences of his malignancy in young patients.

In the Netherlands, the age range for adolescent and young adults (AYA) group has been determined at 18-35 years [11]. Nonetheless, published studies so far have evaluated OSCC incidence using an upper age limit of 45 years for young adults [6, 12, 13]. Hence, in order to provide detailed information on OSCC in young patients, in this study we aimed to first analyze changes of OSCC trends over the period 1989-2016 in young patients in two age subgroups, i.e. patients with age 20-

34 and 35-44 years, and describe these changes for older patients as well. In this way, we intended to provide a more complete picture than is commonly presented in previous studies, and to highlight which age strata may need more awareness. The second research aim of this study was to shed some lights at population-level on differences in smoking, and drinking habits between OSCC age groups. This is critically important for developing targeted and tailored preventive measures for specific age subgroups.

Methods

Data Source and Population

Using the Netherlands Cancer Registry (NCR), all newly diagnosed patients (aged 20 years and older) with oral epithelial carcinoma from 1989 to 2016 were included. Comprehensive evaluation of the data of NCR has shown that the registry database is complete and recording approximately 98% of all cancers [14]. The current analysis was limited to cases diagnosed with SCC (morphology codes M8050–M8084) based on International Classification of Diseases for Oncology, 3rd edition (ICD-O-3), localized at the following subsites: mucosa of lip (C00), mobile tongue (C02), gum (C03), floor of mouth (C04), palate (C05), and other or unspecified parts of the oral cavity (C06). Epithelial carcinoma of the external lip (C00.0-2, C00.6), and salivary gland carcinoma (C07–08) were not considered. Standard clinical TNM staging was used, comprising four stages that were subdivided into 2 groups: local (stage I and II) and advanced disease (stage III and IV). The available data included all variables needed in the current analyses (histopathology, primary site, age at diagnosis, gender and clinical TNM stages). Incidence rates for gender, sites, and clinical TNM stages by age group were expressed as European age-standardized rate per 100,000 person-years (ESR), and data were classified in four age groups: young adults (20-44 years), adults (45-59 years), early elderly (60-74 years) and late elderly (over 75 years old). However, “young adults” as mentioned above were subdivided into two subgroups, patients aged 20-34 and those aged 35-44 years old. In the Netherlands, an institutional review board approval was not required for a descriptive study of this type because the registry data are de-identified and are presented in aggregate numbers. The study was approved by the Privacy Review Board of the Netherlands Cancer Registry.

Information about the classic risk factors such as smoking and drinking habits was available in the registry only for the last two years of the study period (2015 and 2016). Smoking tobacco was

defined in terms of cigarettes/cigars, and was reported as smoking status (current/past smoker, and never). Quantification was calculated in pack-year and 20 pack-year was chosen as cut-off point for subgrouping the patients. Similarly, patients consuming alcohol were defined as ”current drinker/past drinker” and “never”. Regarding alcohol amount, 20 beverages/week was used as cut-off point to dichotomize the patients into two groups. This information was extracted from the patient electronic files. To facilitate understanding in depth characteristics and risk factors for this disease, we additionally analyzed differences between younger and older patients with regard to gender, sites and subsites, clinical stage, smoking, and drinking.

Statistical analysis

Trends in the incidence rates for the five age-groups were assessed by the annual percent change (APC), average annual percent change (AAPC) and the corresponding 95% CIs, with the Join point Regression Analysis program (version 4.6.0.0), obtained from the National Cancer Institute (http://surveillance.cancer.gov/joinpoint) [15, 16]. This analysis program selected the best-fitting log-linear regression model to identify calendar years (i.e. the joinpoints) when APC changed significantly, allowing for the minimum number of joinpoints necessary to fit the data [15]. However, since these tumors are rare, splitting up according to gender and clinical stages led to ESR-values of 0, specifically in the youngest female population aged 20-34 years; therefore in this subgroup the APC was calculated on ESRs clustered on the year of diagnosis in four equally spaced calendar periods (1989-1995, 1996-2002, 2003-2009, 2010-2016).

To investigate differences in patient and tumor characteristics by age category for data of the years 2015 and 2016, we used Kruskal-Wallis for continuous variables (Kolmogorov–Smirnov test, $p < 0.05$) and Pearson ($\chi 2$) or Fisher’s exact tests with the Monte Carlo simulation for categorical variables. For the risk factors with significant results ($p<0.05$), adjusted standardized residuals (roughly comparable to a z-score) were converted to chi-square values and the corresponding p-value was calculated and compared to the Bonferroni-adjusted p-value to assess which observation(s) contributed to this finding. Measured data of continuous variables were presented as a median and p25 and p75 (allows for interquartile range calculation), and count data as N (%). All statistical analysis was performed using SPSS version 22 (IBM Corp. New York, USA, 2012).

Results

In the 28-year period , there were 17,289 cases of oral cavity SCC in The Netherlands. The male-to-female ratio was 1.3:1. Of all patients, 1.2 % were aged 20-34 years and 5.0 % were aged 35-44 years at time of diagnosis (Table 1).

During the study period, the ESR increased significantly from 3.0/100,000 person-year in 1989-1995 to 3.6/100,000 person-year in 2010-2016 (Table 2). Table 2 shows the results of join point analysis on the OSCC trends, in which the corresponding average annual percentage change (AAPC) was 0.5 % per year in males and 1.7% per year in females, overall.
For the youngest age group, join point regression analysis showed a steeper increase over time with an AAPC of almost 4% in males under 35 years old. An upward but non-significant trend was also observed among females in this youngest cohort. Trends toward increase were observed in all age subgroups except in those aged 35-44 years: the annual rate of incidence decreased by 1.1% . The decline in the incidence rate in this group was similar between males and females (AAPC of -1.3 & -1.0, respectively), though only significant in males. In males aged 45-59 years, distinct and significant trends were noted for different time periods, starting with a steep increase (APC: 9.9%;), followed by a period with a modest decline (APC: -0.5) and finally a strong decline (APC: -4.1). The increase in those aged 60 years and older, was stronger in females than males in the entire period (1989-2016), [See figure 1 for graphic presentation].

Stratified by clinical stage, the ESR increased with 1.7% in local, and 0.6% in advanced disease (Table 2). Noteworthy, the elderly groups (60 years and older) showed significant increasing trends for local and advanced diseases in a parallel pattern (Supplementary Figure S1).

Associations between tumor characteristics, patients characteristics and classical risk factors in relation to age groups are presented in Table 3. The most common OSCC site in all age groups was the mobile tongue, ranging from 79% in patients less than 35 years old to 38% in patients aged 75 years and older. Gender differences were noted particularly among patients under 45 years. In those younger than 35 years, 67% of females and 84% of males were diagnosed with mobile tongue carcinoma, whilst in those aged 35-44 years the females slightly outnumbered the males with tongue carcinoma (78% vs. 72%, respectively) (data not shown).

Overall, 67% of the patients with OSCC were either current/past smokers, which was also observed for alcohol consumers (67%). Smoking and drinking status significantly differed over the age groups (both $P<0.001$). We found that the patients aged 45-59 years and those aged 60-74 years

were more likely to be current/former smokers (Z-residual =3.5, P = 0.0004; Z-residual = 7.2 P <0.0001), whereas the opposite was true for patients age 75 years or older (Z-residual = -10.7, P <0.0001). Regarding alcohol consumption, significantly higher rates of current/former drinker were reported by patients aged 60-74 years old (Table 3). Post-hoc chi-square analyses evaluated the interaction between tobacco and alcohol use among all age subgroups, presented in supplementary figure S2. It was found that in the young cohorts (20-34 and 35-44 years), unhealthy lifestyle habits were rather common- i.e ., more than half were tobacco and alcohol co-users.

Discussion

The key finding in this study was a significant increase in annual incidence of OSCC in those aged 20-34 years, and a decline in those aged 35-44 years of age. Interestingly, this finding differs from the previous publications which have concluded an overall downward or stable trend in young Dutch patients [6, 12, 13]. This is mainly because the prior studies collectively categorized the young adults into one cohort aged less than 45 years. When we applied this "common" <45 years interval, our findings were in accordance with those reports (APC= -0.24%). This, indeed, shows how estimation of incidence rates could be quite sensitive to grouping during analysis, and revealed that our sub-classifying the young age group in two cohorts was rather powerful and allowed unraveling important trends in the youngest age group which would otherwise be masked by the much larger number of patients in the 35-44 years subgroup. Increasing incidence in the youngest age group, which was only statistically significant in males, seems to be consistent with other studies from many regions of the world, although age subgroup classification slightly differs. In the US, an analysis for Surveillance, Epidemiology, and End Result data set from 1973-1997 found nearly four-fold increase of OSCC incidence in males aged 30-39 years [17]. Data from Taiwan also showed a progressive increase in oral cancer in males aged 30-39 years, but not in those aged 20-29 years [18]. A German analysis over a 20-year period revealed a significant increase in OSCC incidence among patients aged 30-39 years, with a males-to-females ratio of 3.8:1[19]. However, one study from India reported a preponderance of females cases over males without a clear risk factor in patients younger than 35 years [20].

There is general agreement that the mobile tongue is the most common site for OSCC in young adults [21]. This matches our finding: 78% of people aged 20-34 years and 74% of those aged 35-44 years old, but less than 50% of the older age groups had cancer in this region. Prior studies have found that white females younger than 45 with mobile tongue cancer outnumbered males [5, 7]. We

confirmed this for our population aged 35-44 years, but not in patients under 35 years. Further, we noticed that with aging the proportionate share of mobile tongue cancer became less, reaching lowest percentage in oldest group (patients older than 75 years). However, this may be a reflection of the increasing number of tumors at other sites. Increasing incidence of OSCC in young adults makes it paramount for dentists to consider tongue SCC in this age group as they do now for the adult and elderly populations, and make the necessary investigations or referrals to improve the possibility of early detection.

Interestingly, our study also found an enigmatic change in trend pattern of OSCC among males aged 45-59 years old: incidence rates increased steeply from 1989-1993, then declined slowly during 1993-2010, and dropped dramatically from 2010-2016. It is well documented that the people at their 50s have substantially the highest risk for OSCC [22], but why this particular group showed such a pattern is not clear. Some may assume that to be a depiction of the decline in smoking prevalence in the Netherlands [23], however, since the consumption of cigarettes and other tobacco products was reduced (from 35% in 1995 to 23 % in 2014) quite equally in all age groups, this seems not a likely explanation [24, 25]. We postulate that one possible reason underlying this finding could be the better awareness and higher alertness level among the dentists which may have enhanced opportunities of early detection and treatment at pre-cancer stages.

It is well documented that the OSCC 5-years survival rate is approximately 62 % in developed countries, but hardly reaches 30% in developing countries [26-28]. This is largely because in the latter case, most of the cases are only diagnosed at stages III or IV. In our young and adult cohorts, related to the advances in high- resolution imaging and awareness among patients and clinicians, a shift in the clinical diagnosis from advanced toward early stages is observed. However, the results showed an increasing trend in advanced stages in the elderly groups. We hypothesize that a possible explanation may lie in patient bias, we think that the ignorance or reluctance of elderly patients to seek professional care may be the cause for late presentation. Hence, further efforts to encourage people older than 60 years of both sexes to visit the dentists periodically, but at least in case of suspicious signs or symptoms such as appearance of red or white painless areas or dysphagia are warranted.

Another distinguishing aspect is the male-to-female ratio. The reported ratio of males-to-females in most western countries is 3:1 or 2:1[1, 22]. However, in this analysis the male preponderance was

smaller: about 1.3:1. In addition, we found a more profound increase in the overall annual percentage change of the incidence rate for females compared to males, particularly among those older than 60 years. We speculate that this may be at least partly related to risk factor profiles.

Based on solid observational design studies since the 70's, tobacco smoking and alcohol drinking have been identified as major risk factors for OSCC with a well-defined dose-response relationship [29, 30] . Despite the still inconsistent evidence regarding the independent casual association between these factors and OSCC, evidence for the carcinogenic effect of heavy drinking has been considered sufficient by IARC Monograph 96, regardless of smoking status [31] . Moreover, one study found that the association between ever smoking and the risk of head and neck cancer among females was stronger with an odd ratio (OR) of 2.33 (95% CI=1.56 to 3.49) than for males OR of 1.65, (95% CI=1.14 to 2.39) [32] . Intriguingly, some meta-analysis found that the effects of smoking was more profound on larynx and pharynx than oral cavity [33, 34]. Although numerous authors have suggested that exposure to high dose of tobacco and alcohol for at least 21 years is required to cause malignant transformation [35, 36], other data by Castellsague and colleagues showed that smoking-drinking interaction significantly increases the risk of cancer by 5-fold in a synergic fashion even with moderate consumption level [37] . In this study, they noted a 2-4 fold increase in risk of OSCC with ever smoking or drinking only, while a 13-fold increase in risk was found with simulations exposure to both habits.

How do these findings relate to our results? For the youngest patient group aged 20-34 years, it is always questionable whether the occurrence of OSCC is sporadic or hereditary [38]. Our data demonstrated that more than half of the patients within this strata had a background of tobacco and alcohol co-use. Although data are still conflicting about the etiology of OSCC in young patients, this study appears to support the assumption that these traditional risk factors play an essential role in oral carcinogenesis in the young groups as they do in the elderly ones. This is also in agreement with Iamaroon et al. who suggested that smoking and alcohol consumption at a very young age play a crucial role in development of malignancy [10]. However, it is highly unlikely that this youngest age group will be exposed for at least 21 years to one or both risk factors, the time frame considered to be required for developing malignancies as mentioned above. It is worth noting that some people suggest a causal link between the observed trend and human papillomavirus (HPV) infection, especially because this age group is known to be sexually active. In fact, such an assumption seems possible in a country like Brazil, where 32% of OSCC young patients are HPV positive [39]. However, this is not the case in the Netherlands, in which HPV infection prevalence in OSCC was

found to be very rare (3%) for young patients [40]. Hence, HPV testing is not routinely recommended for Dutch OSCC patients. Other studies have also investigated the association between familial history of cancer and OSCC, but the results were inconclusive [41, 42]. All of these controversies lead some authors to conclude that the etiology of OSCC in the young group of the patients could be multifactorial [43, 44]. Together, these data suggest that the young patient groups are either more susceptible to risk factors, or other factors may play a role as well.

As indicated above, the adult and elderly age groups displayed a more profound increase in the overall annual incidence for females compared to males, in particular in the older than 60 year old groups. One aspect possibly contributing may be that 22% of the Dutch females has been reported to be a heavy drinker, compared to 14% of men, especially those above 55 years [45]. Another aspect is that the WHO reported that Dutch females smoke almost as much as Dutch males [46]. These findings, combined with the odds ratio to develop head and neck cancer apparently being higher for females than for males (see above), may be explanations for the increased APC values for females vs. males in the elderly.

Our findings should be understood in the context of some limitations. First, our risk factor analysis was based on the available data for only two years, consequently, we cannot relate these findings to changes in incidence rates over time. Moreover, because patient and tumor characteristics including lifestyle habit were only available at the time of diagnosis of the tumor, we can't say anything about the causal link; this was just to illustrate the differences by age group. Furthermore, there is quite some missing information regarding smoking and alcohol drinking, so our findings with regard to these factors should be regarded as a first indication only.

To the best of our knowledge, this is the first and largest study that evaluated specifically oral SCC annual incidence across different strata of young patients and compared that to the elderly groups. Additionally, the study is population-based (covering all cancer cases in The Netherlands), thus avoiding any selection bias of clinical series. At the same time, we investigated the relevance of the well-known risk factors for OSCC at a population level which allowed us to explore additional differences between age groups.

We conclude from this study that patients aged 20-34 years may be a unique entity from those aged 35-44 years, as incidence rate increased in the youngest subgroup of the patients, but decreased in those aged 35-44 years. This may suggest focusing on other venues of research, such as potential

genetic differences between these two young strata. This may help in more understanding and delineation of the risk factors, and consequently may guide diverse treatment plans. An estimated overall incidence showed a predilection in particular in older women, which could at least partially be explained by behavioral factors. Finally, still many of the oral cavity cancers were diagnosed at high stage disease levels, so early detection, early treatment intervention, and withdrawal from risk habits remain important factors to reduce the burden of oral cancer.

Supplementary data

Supplementary data includes two figures S1 &S2. S1 represents the graphic presentation of joinpoint analysis of the clinical stages for all age groups. S2 represents smoking and drinking interaction within the patients with OSCC, including values of posthoc chi-square analysis.

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Table 1: General characteristics of 17,289 patients with oral SCC diagnosed in 1989-2016 by age groups							
			Age groups				
Variables		**Total**	**20-34 y**	**35-44 y**	**45-59 y**	**60-74 y**	**+75 y**
Total	N (row%)	17,289 (100)	224 (1.2)	858 (5.0)	5,336 (30.9)	7,018 (40.6)	3,853 (22.3)
Gender	N (column%)						
	Male	9,955 (58)	136 (61)	552 (64)	3,410 (64)	4,315 (62)	1,542 (40)
	Female	7,334 (42)	88 (39)	306 (36)	1,926 (36)	2,703 (38)	2,311 (60)
Clinical stage	N (column%)						
	local disease (stage I & II)	9,131 (53)	148 (66)	499 (58)	2,838 (53)	3,738 (53)	1,908 (50)
	advanced disease (stage III & IV)	7,338 (42)	60 (27)	313 (37)	2,276 (43)	2,983 (43)	1,706 (44)
	Unknown	820 (5)	16 (7)	46 (5)	222 (4)	297 (4)	239 (6)

Table 2: Trend in incidence for OSCC in the Netherlands, 1989-2016

Variables	Overall trend (1989-2016)	Join point analysis						ESR/ (100,000)			
		Trend I		Trend II		Trend III					
	AAPC (95 CI%) (%)	Year	APC (%) 95CI%	Year	APC (%) 95CI%	Year	APC (%) 95CI%	1989**	1996	2003	2010
Overall	1.0* (0.7, 1.3)							3.0	3.2	3.5	3.6
Gender											
Males	0.5* (0.1, 0.8)							4.0	3.9	4.3	4.3
Females	1.7* (1.3, 2.1)							2.1	2.3	2.7	2.9
Age groups											
20-34 years T	2.8* (1.2,4.4)							0.2	0.2	0.3	0.3
M	3.9 * (2.5,5.3)							0.2	0.2	0.3	0.4
F#	2.1 (-5.1,9.7)							0.1	0.2	0.3	0.2
35-44 years T	-1.10* (-2.0, -0.1)							1.5	1.2	1.2	1.2
M	-1.3* (-2.4,-0.1)							1.9	1.4	1.6	1.5
F	-1 (-2.6, 0.6)							1.1	0.9	0.8	0.9
45-59 years T	0.4 (-0.8, 1.7)	1989-1992	14.5* (4.2, 25.8)	1992-2010	-0.1 (-0.7, 0.6)	2010-2016	-4.6* (-7.6, -1.5)	5.8	6.3	6.4	5.5
M	-0.1 (-1.3,1.3)	1989-1993	9.9 * (2.7,17,5)	1993-2010	-0.5 (-1.3,0.3)	2010-2016	-4.9* (-8.2,-1.4)	7.7	8.0	8.0	6.8
F	0.5 (-0.4, 1.4)							3.8	4.6	4.8	4.1
60-74 years T	2.0* (1.6, 2.3)							9.3	10.1	12.1	14.0
M	1.2* (0.8,1.6)							13.4	13.2	15.3	16.7
F	3.0* (2.7, 3.3)							5.9	7.3	9.1	11.0
+75 years T	1.4* (1.0, 1.8)							11.7	12.0	13.6	15.8
M	0.8 * (0.2,1.5)							13.8	12.9	15.1	16.4
F	1.7 * (1.2, 2.3)							10.5	11.23	12.5	15.3
Clinical stage											
local disease (stage I& II)	1.7* (1.3, 2.1)							1.5	1.6	1.9	2.0
advanced disease (stage III & IV)	0.6* (0.2, 1.0)							1.3	1.4	1.5	1.5
Unknown	-3.2 * (-5.8, -0.6)	1989-2002	0.8 (-3.4, 5.2)	2002-2016	-6.9 * (-10.4,-3.2)			0.2	0.2	0.2	0.1

*The APC or AAPC is significantly different from zero (p <0.05), AAPC, average annual percentage change; APC, annual percentage change, T: total, M: males, F: females, # calculation was based on clustering years of diagnosis, ** based on 7 years interval (1989- 1995, 1996-2002, 2003-2009, 2010-2016)

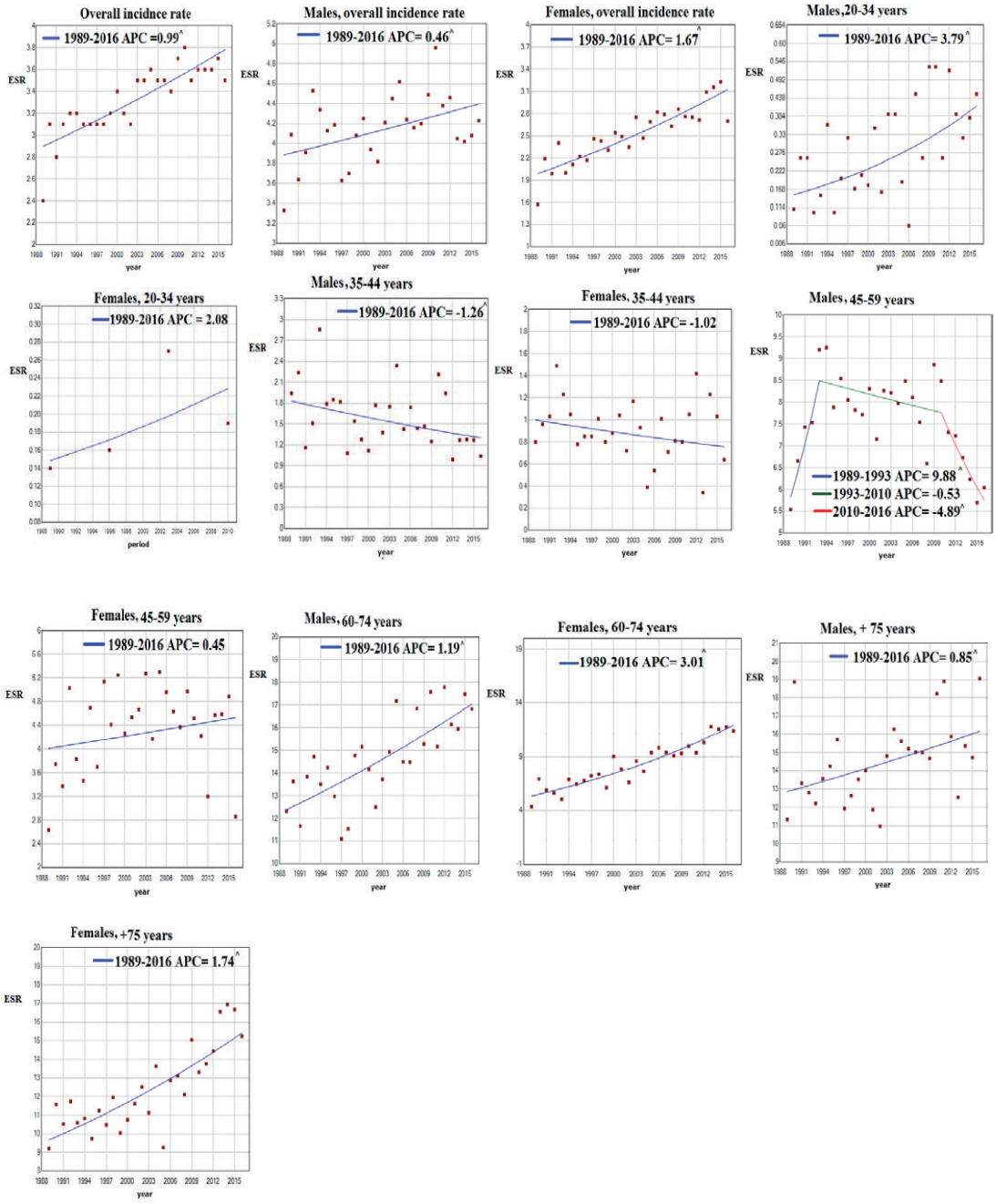
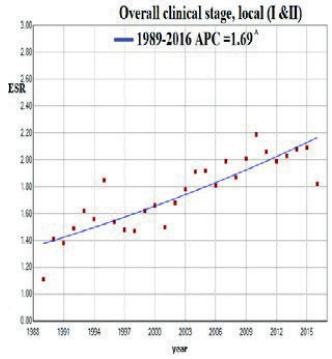


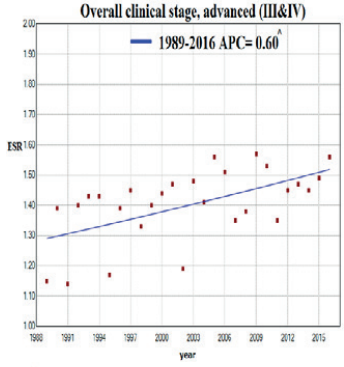
Figure 1: Join point regression analysis shows trend of incidence of oral squamous cell carcinoma (1989-2016), ESR :European age-standardized rate per 100,000 person-years. ^ indicates that the Annual Percent Change (APC) is significantly different from zero at the alpha = 0.05 level. The APC of females aged 20-34 years was calculated in period of four equally spaced calendar (1989-1995, 1996-2002, 2003-2009, 2010-2016).

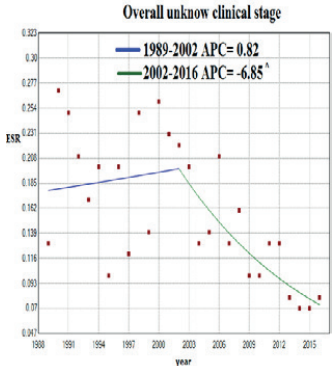
Table 3: Tumor characteristics and differences of individual risk factors in oral SCC patients from different age groups for years 2015-2016

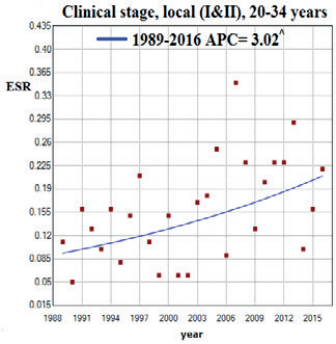
		Age groups					
Variables	**Total**	**20-34y**	**35-44y**	**45-59y**	**60-74y**	**+75y**	**P-value**
Total N(raw%)	1680(100)	19(1)	43(2)	368 (22)	818 (49)	432 (26)	
Gender N (column%)							
Males	922 (55)	13 (68)	25 (58)	222 (60)	482 (59)	180 (42)	**<0.001***
Females	758 (45)	6 (32)	18 (42)	146 (40)	336 (41)	252 (58)	
Anatomical sub-sites N (column%)							
Mobile tongue	700 (42)	15 (79)	32 (74)	178 (48)	315 (39)	160 (37)	
Gingiva	292 (17)	1 (5)	4 (9)	39 (11)	128 (16)	120 (28)	**<0.001****
Floor of mouth	356 (21)	0 (0.0)	2 (5)	88 (24)	211 (26)	55 (12)	
Buccal mucosa	271 (16)	2 (11)	4 (9)	50 (14)	134 (16)	81 (19)	
Hard palate	29 (2)	1 (5)	1 (2)	8 (2)	11 (1)	8 (2)	
Unknown	32 (2)	0 (0.0)	**0** (0.0)	5 (1)	19 (2)	8 (2)	
Clinical stage N (column%)							
local (stage I & II)	905 (54)	12 (63)	29 (67)ll	217 (59)	445 (54)	202 (47)	**0.002***
Advanced (stage III &IV)	738 (44)	5 (26)	13 (30)	146 (40)	355 (43)	219 (51)	
Unknown	37 (2)	2 (11)	1 (2)	5 (1)	18 (2)	11 (2)	
Smoking status N (column%)							
Current or past	1133 (67)	8 (42)	26 (60)	271(73.6)[a]	622 (76)[a]	206 (47.6)[b]	**<0.001***
Never	213 (13)	2 (10.5)	11 (25.5)	39 (10.5)	69 (8)[b]	92 (22.3)[a]	
Unknown	334 (20)	9 (47)	6 (14)	58 (15.7)	127 (15.5)	134 (31)	
Pack-years N (column%)							
1-20 pack-year	157 (14)	8 (100)	9 (35)	48 (18)	70 (11)	22(11)	**<0.001****
≥ 21 pack-year	487 (43)	0 (0.0)	6 (23)	127 (47)	278 (45)	76 (37)	
Unknown	489 (43)	0 (0.0)	11(42)	96 (35)	274 (44)	108 (52)	
Median (P25, P75) #		5.5 (1.75, 13.75)	20 (15, 25)	32 (20, 40)	40 (25, 50)	35 (23, 50)	**<0.001*****
Alcohol status : N (column%)							
Current or past	1126 (67)	11 (58)	28 (65)	260 (71)	602 (73)[a]	225 (52)[b]	**<0.001****
Never	84 (5)	1 (5)	2 (5)	18 (5)	30 (4)	33 (8)	
Unknown	470 (28)	7 (37)	13 (30)	90 (24)	186 (23)	174 (40)	
Number of alcoholic beverages per week : N (column %)							
1-20	443 (39)	7 (64)	11 (39)	87 (33)	226 (37)	112 (50)	**<0.001****
≥ 21	394 (35)	0 (0.0)	6 (21)	111 (43)	239 (40)	8 (17)	
Unknown	289 (25	4 (36)	11 (39)	62 (24)	137 (23)	75 (33)	
Median (P25, P75)#		5 (2, 15)	10 (5.5, 24.5)	21 (8, 42)	21 (7, 30)	14 (5.75,21)	**<0.001*****

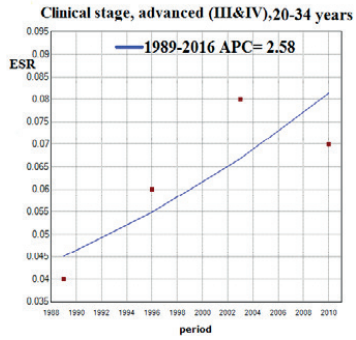
*chi-square, ** Fisher exact, *** Kruskal wallis test, # Median calculated by interquartile range = Percentile 75th- Percentile 25th , a:The observed count of this cell was significantly higher than expected per null hypothesis,
b: The observed value of this cell was significantly lower than expected per null hypothesis. Statistically significant p-values are shown in Bold.

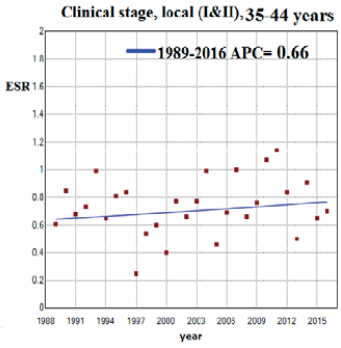

Overall clinical stage, local (I &II)
1989-2016 APC =1.69^
ESR
year

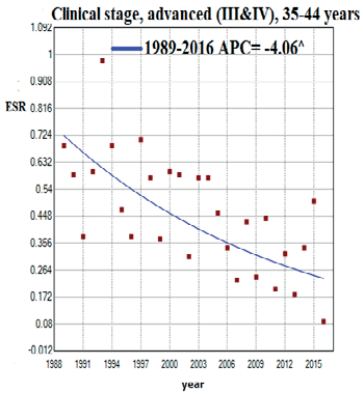

Overall clinical stage, advanced (III&IV)
1989-2016 APC= 0.60^
ESR
year

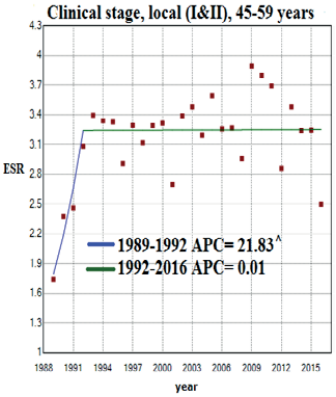

Overall unknow clinical stage
1989-2002 APC= 0.82
2002-2016 APC= -6.85^
ESR
year

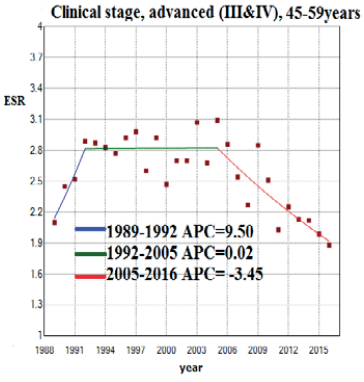

Clinical stage, local (I&II), 20-34 years
1989-2016 APC= 3.02^
ESR
year

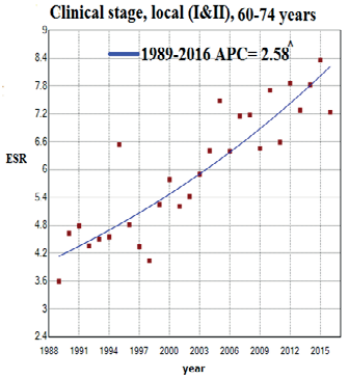

Clinical stage, advanced (III&IV),20-34 years
1989-2016 APC= 2.58
ESR
period

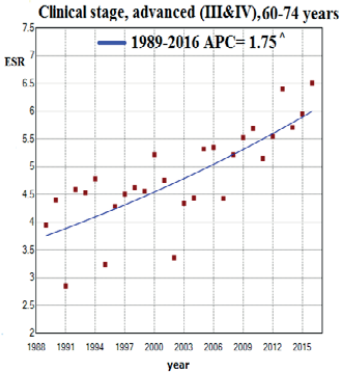

Clinical stage, local (I&II), 35-44 years
1989-2016 APC= 0.66
ESR
year

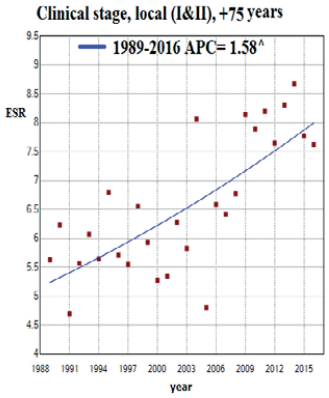

Clinical stage, advanced (III&IV), 35-44 years
1989-2016 APC= -4.06^
ESR
year

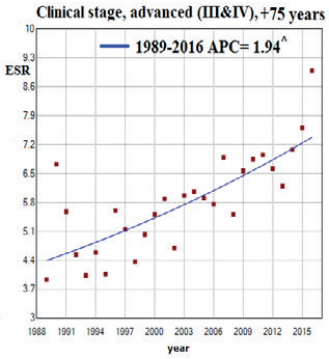

Clinical stage, local (I&II), 45-59 years
1989-1992 APC= 21.83^
1992-2016 APC= 0.01
ESR
year


Clinical stage, advanced (III&IV), 45-59years
1989-1992 APC=9.50
1992-2005 APC=0.02
2005-2016 APC= -3.45
ESR
year


Clinical stage, local (I&II), 60-74 years
1989-2016 APC= 2.58^
ESR
year

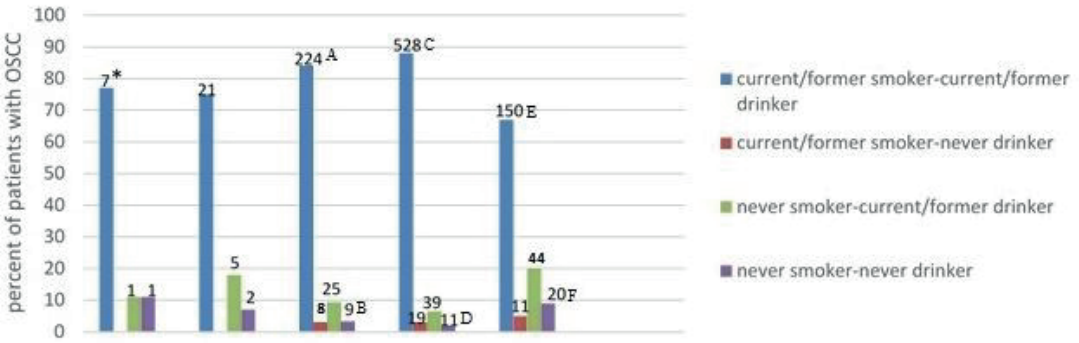

Clinical stage, advanced (III&IV),60-74 years
1989-2016 APC= 1.75^
ESR
year


Clinical stage, local (I&II), +75 years
1989-2016 APC= 1.58^
ESR
year



Supplementary figure S1: Join point regression analysis shows trend of **clinical stages (Local and advanced)** of oral squamous cell carcinoma`(1989-2016), ESR : European age-standardized rate per 100,000 person-years. ^ indicates that the Annual Percent Change (APC) is significantly different from zero at the alpha = 0.05 level. The APC of advanced stage for those aged 20-34 years was calculated in period of four equally spaced calendar (1989-1995, 1996-2002, 2003-2009, 2010-2016).

Note: During the study period of time, several editions of the International Union against Cancer (UICC) TNM classification were used to record tumor stages : 4th edition (1989–1998), 5th edition (1999-2002), 6th edition (2003-2009), and 7th edition (2010-2016).



Supplementary figure S2 : percentage of patients with OSCC within each of the four conditions of smoking and drinking interactions.
* Number of the patients. A (Z-residual= 6.3 , P <0.0001), B (Z-residual= 5.3, P = 0.0001), C (Z-residual = 10.9, P <0.0001), D (Z-residual = 5.6, P < 0.0000), E (Z-residual = 7.5, P <0.000), F (Z-residual = 5.5, P < 0.0000). Missing patients were excluded.

CHAPTER 4

INCIDENCE AND RISK FACTORS OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA IN THE NETHERLANDS: A POPULATION-BASED STUDY

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Submitted

Abstract

Background: Data on incidence of oropharyngeal squamous cell carcinoma (OPSCC) in the Netherlands has been reported before, but not population-based in the HPV era. Our goal was to expand and update epidemiological information on incidence trend and risk factors of OPSCC at the population level.

Methods: Data from the Netherlands Cancer Registry (NCR) from 1989-2016 were analyzed using join point regression software for time trends in incidence rates by age, gender, and clinical stage. Data on HPV status, smoking and drinking habits were available from 2015 onwards.

Results: The overall incidence rate as measured by annual percentage change (APC) increased remarkably during the early 90's (5.3%), but from 1997 slowed to an APC of 1.2 %. Incidence rates showed a significant decline in age group 35-44 years for both males and females with APC of -3.5 and -5.2, respectively. In adults aged 45-59 years, incidence rates increased significantly from 1989 to 2000, and thereafter showed a significant decline. In patients older than 60 years, incidence rates increased overall, with an APC for women being consistently higher than men. The prevalence of HPV infection among patients was about 31%, however, smoking and alcohol consumption were more prevalent, i.e. 79% and 76 % respectively.

Conclusions : We observed significant decreases in incidence of OPSCC in 35-44 year-olds and 45-59 year-olds after 2000, while the incidence increased in all other age groups. Smoking and alcohol consumption are still prevalent, while the role of HPV infection and its interactions with other factors needs further elucidation.

Key words

Oropharyngeal squamous cell carcinoma, incidence trend, annual percent change, HPV status, risk factors

Abbreviation: Oropharyngeal squamous cell carcinoma (OPSCC); European Standardized Rate (ESR) ; annual percentage change (APC) ; average annual percentage change (AAPC); human-papilloma virus (HPV)

INTRODUCTION

Head and neck cancer (HNC) is a major public health problem worldwide nowadays, with an estimated annual incidence of 550,000 cases, which is expected to increase to 833,000 new cases in 2020 (1). Based on the global estimate of the year 2012, the anatomical subsites with the highest prevalence of HNC were cancers of the oral cavity (202,000 cases) followed by oropharyngeal cancer (100,500 cases) (2). However, in 2018, the most recent year for which data is available, the estimated number of oropharyngeal cancer cases were 92,887, and the number of deaths 51,005 (3). In the literature, oral cavity and oropharyngeal carcinomas were often reported in aggregate (4). However, the exclusive association between human-papilloma virus (HPV) and oropharyngeal but not oral squamous cell carcinoma provides clear indications that these two types of cancers should be regarded as separate and distinctive entities (5).

Remarkably, in the last two decades, the oral cancer incidence rate decreased in parallel to tobacco use, but with a dramatic rise in oropharyngeal squamous cell carcinoma (OPSCC) in many developed countries, including the Netherlands (6-9). These shifted trends are mostly attributed to a high prevalence of HPV-positive OPSCC. Interestingly, HPV-positive OPSCC is established as a unique disease with specific biological and epidemiological features distinct from HPV-negative OPSCC. Firstly, HPV-positive OPSCC commonly affects patients at a younger age with less tobacco exposure and has a high propensity to occur at the base of the tongue and tonsils (10). Further, HPV-positive tumors have a good response to chemo-radiation therapy and a better survival rate (11). However, OPSCC profiles in relation to HPV appear to be changing: a very recent study using data from the Surveillance, Epidemiology, and End Results (SEER) database has shown a significant change in the demographics of HPV-positive OPSCC patients, and found that the incidence is not limited anymore to the younger population, but is expanding in the elderly groups as well (12).

In the Netherlands a few studies have reported the annual incidence of OPSCC, but a lot of detailed information on population-based patient demographics is missing. This is because most of the published studies reported the incidence trends of OPSCC as part of comprehensive head and neck cancer analysis, or focused on cohorts form one or two centers. One study (Rietbergen et al.) reported a steady increase in the prevalence of HPV-positive OPSCC among Dutch patients, ranging

from 5.1% in 1990 to 20% in 2004, to as high as 29% in 2010 (13). The data were updated in 2015 and revealed an attributable fraction of 50% in 2015 (14). However, this study made estimates that were largely based on single-institution data, making it difficult to be considered as a national prevalence estimate. So far, it remains unknown whether or not the difference in prevalence of HPV versus non-HPV OPSCC is also changing in the Netherlands.

For the above reasons, we performed a population-based study to update and expand the epidemiological information on OPSCC and determine its burden on the Dutch society. In this study an analysis of the population-based trends in incidence rates of OPSCCC in 5 age-subgroups will be performed, based on data from the Netherlands cancer registry (NCR), covering the period 1989-2016. We intend to provide more detailed information and to highlight which age strata may need more awareness. For 2015-2016, the NCR collected also information about HPV status, smoking and alcohol consumption and the prevalence of these factors in patients with OPSCC will be evaluated. This information may be important in developing tailored preventive and/or treatment measures.

Materials and Methods

Data Source and Population

Using the Netherlands Cancer Registry (NCR), all newly diagnosed patients aged 20 years and older with oropharyngeal squamous cell carcinoma from 1989 to 2016 were included. The completeness of the Netherlands Cancer Registry was estimated to be at least 95% (15). We limited our analysis to cases diagnosed with squamous cell carcinoma SCC based on International Classification of Diseases for Oncology, 3rd edition (ICD-O-3) and histology codes (M8050–M8084), localized at the following subsites: base of the tongue (C01), soft palate (C05.1), uvula (C05.2), tonsil (C024, C09) and other or unspecified parts of the oropharynx (C10, C142) (16). Standard clinical TNM staging, is the main tumor staging system used in the NCR and comprises four stages that were combined into 2 clinical disease stages, i.e. early (stage I and II) and advanced disease (stage III and IV) to obtain a robust clinically relevant classification with sufficient numbers for our main analysis. The collected data included all variables needed in the current analyses (histopathology, primary site, age at diagnosis, gender, and clinical TNM stages). Incidence rates for gender and clinical TNM stage by age group were expressed as the European age-standardized rate (ESR) per 100,000 person-years and the data were classified into four age groups: young adults (20-44), adults (45–59), early elderly (60-74) and late elderly (75 years or older). However, “young adults” is often differentially

defined, with cutoff points < 35, or < 45 years old. In the Netherlands, and based on the adolescent and young adults oncology group (AYAO), the age range of young adults has been determined at 18-35 years and a specific guideline is followed in their treatment (17). Hence, in order to provide detailed and accurate information on OPSCC in young patients, we subdivided this category into two subgroups, patients aged 20-34 and those aged 35-44 years old, even though the number of the patients thereby becomes small.

Information about HPV status, smoking and drinking habits was available since 2015. In the Netherlands, there is a national guideline for the detection of high-risk HPV in OPSCC tumors: performing p16 immunostaining as screening test that is followed by HPV type-specific DNA PCR in case p16 is positive in >70% of the cells; both tests should be positive. Although the majority of Dutch cancer centers follow this guideline, a few centers use only immunostaining of p16 to classify the patients into positive or negative HPV-related OPSCC. In the NCR, patients were considered as positive if p16 immunostaining was positive and not followed by a negative HPV PCR test. Patients with a negative p16 immunostaining or negative HPV PCR were recorded as negative. Records without any information about HPV testing where considered as unknown. With regard to smoking tobacco, it was defined in terms of cigarettes and cigars, and was reported as smoking status (current/past smoker, and never). Quantification of tobacco smoking was calculated in pack-year and 20 pack-year was chosen as a cut-off point for grouping the patients. Similarly, data on alcohol consumption were obtained and the patients were defined as follows: “current drinker/past drinker” and “never”. Regarding alcohol amount, 20 beverages per week was used as cut-off point to dichotomize the patients into two groups. This information was extracted from the patient electronic files. To facilitate understanding in depth characteristics and risk factors for this disease, we additionally analyzed differences between younger and older patients with regard to gender, sites and subsites, clinical stage, smoking, drinking, and HPV status.

STATISTICAL ANALYSIS

Trends in the incidence rates for the five age-groups were assessed by the annual percent change (APC), average annual percent change (AAPC) and the corresponding 95% CIs, with the Join point Regression Analysis program (version 4.6.0.0), obtained from the National Cancer Institute (http://surveillance.cancer.gov/joinpoint) (18, 19). This analysis program selected the best-fitting log-linear regression model to identify calendar years (i.e. the joinpoints) when APC changed significantly, allowing for the minimum number of joinpoints necessary to fit the data (18). Since

these tumors are very rare in the youngest population aged 20-34 years, splitting up according to gender and clinical stages lead to ESR-values of 0; therefore the year of diagnosis in this group was clustered in four equally spaced calendar (1989-1995, 1996-2002, 2003-2009, 2010-2016).
To investigate differences in patient and tumor characteristics by age category for data of the years 2015 and 2016, Kruskal-Wallis for continuous variables and Pearson Chi-square or Fisher's exact tests with the Monte Carlo simulation for categorical variables were used. For risk factors with significant results ($p<0.05$), adjusted standardized residuals (roughly comparable to a z-score) were converted to chi-square values and the corresponding p-value was calculated and compared to the Bonferroni-adjusted p-value to assess which observation(s) contributed to this finding. Measured data were presented as a median and interquartile range (p25, p75), and count data as N (%). All statistical analysis was performed using SPSS version 22 (IBM Corp. New York, USA, 2012).

RESULTS

A total of 11,739 OPSCC cases were registered in the Netherlands during the period 1989-2016: 7,945 males (68%) and 3,794 females (32%) with a male-to-female ratio of 2.1:1. The young adult groups aged 20-45 years accounted for 486 cases (4%). More than three-fourths of the patients had advanced disease at the time of presentation (76%). Further details on age by gender, and clinical stage are presented in Table 1.

During the study period, the ESR increased from 1.8/100.000 persons-year in 1989-1995 to 2.8/100.000 persons-year in 2010-2016 (Table 2). Table (2) also summarizes the join point analysis on the trend of OPSCC among the Dutch population between 1989-2016. The analysis revealed a clear upward trend in the overall incidence with an average annual percent change (AAPC) of 2.4% [CI 1.8-3.1]. During the same period, the corresponding AAPC was 2.1% per year in males overall and 2.8% per year in females overall. A significant cut-off point was noted in 1997; before which a steep APC increase of 5.3 % was observed. The incidence rate increase was less strong after 1997.

When age-specific trends were analyzed, the incidence rate of OPSCC was stable in those 20-34 years of age, while a significant decline in group aged 35-44 years for both males and females with APC -3.5 and -5.2 respectively, was observed. For the cohort 45-59 years, the incidence rate increased significantly from 1989 to 2000, but showed a decline thereafter, specifically among females (Table 2; see Figure 1 for graphic representation). In the older age groups, the AAPC increased significantly in both genders, though the AAPC were consistently higher in females than

in males. The largest positive AAPC was observed in females aged 75 years and older, followed by females aged 60-74.

When stage-specific trends were analyzed, the overall rates were similarly increased in local and advanced diseases. Specifically, the rate for the local diseased increased by 7.0% per year from 1989 to 1997 and then exhibited stabilization, whereas advanced diseases showed an increase throughout the entire period (Table 2 and Supplementary figure S1).

Table 3 presents data of the last two years of the study period (2015, 2016) and shows the associations between tumor characteristics, patients characteristics and classical risk factors in relation to age groups. The most common site for OPSCC in all age groups was the tonsil (36%), followed by the base of the tongue (30%). The table shows that 79% of the patients were current or former smokers, and 76% were alcohol consumer. It is also apparent from this table that the 60-74 year old patients had the highest level of alcohol consumption; 41% drank > 20 beverages per week. With respect to the prevalence of HPV infection among patients with oropharyngeal cancer, the overall proportion of OPSCC that tested positive for HPV was 31%, 34% was negative and 35% was unknown. Considering variation among different age groups, noticeably, the young adult aged 35-44 years had more than 3 times the rate of being positive than being negative (65% vs 20%). Likewise, in adults aged 45-59 years old, we found 40% of the patients were positive versus 32% who were negative. For the older groups, although a high percentage of the data was missing, percentage of the patients with HPV negative tumors was slightly higher than that who had HPV positive (Table 3). The data also revealed that the proportion and absolute number of the HPV-infected tumors increased from 2015 to 2016 in all age groups except for those 75 years or older (data not shown).

The proportional distribution of smoking ($\chi2 = 38.1$, $P < 0.001$), alcohol drinking ($\chi2 = 29.9$, $P < 0.001$), and HPV-status ($\chi2 = 47.1$, $P < 0.001$) differed statistically significantly by age category. Examination of standardized residuals for tobacco use indicated that the effect was driven entirely by the youngest (20-34 years), the group which was more likely to be a never smoker (Z-residual = 4.2 $\chi2= 17$ P =0.0003) and the late elderly age group (+75 years), for which the opposite was true (Z-residual = 3.2 $\chi2= 10.2$ P = 0.0013). Regarding alcohol consumption, the analysis revealed that significantly lower rates of current or former drinker were reported by patients aged 75 or older (Z-residual = - 4.5, $\chi2 = 20.2$ P =0.00001). Of note, the rate of HPV-positive OPSCC was significantly

higher in patients aged 35-44 years (Z-residual = 3.3, $\chi 2$ = 10.8 P =0.0009) and those aged 45-59 years old (Z-residual = 4.9, $\chi 2$ = 24 P <0.00001) (Table 3).

The interaction between these three common risk factors among all age subgroups was evaluated by post-hoc chi-square tests and revealed different significant associations. Regarding association of smoking with alcohol consumption, the current or former smokers were more likely to be alcohol consumers in the middle and elderly age categories. Besides, the proportion of smokers and alcohol drinkers in the patients aged 45-59 years and those aged 60-74 years old was higher than expected (Supplementary Figure S2a). When we analyzed interaction of HPV status with tobacco smoking or alcohol consumption in different age groups, we found negative associations that were significant as well in the adults and elderly populations. Notably, the smokers/ drinkers patients were more likely to be HPV-negative, and the patients who were never smokers or never alcohol drinker had HPV-positive tumors (Supplementary Figures S2b & c).

Discussion

The overall incidence rate of OPSCC in the Dutch population increased significantly during the past 28 years (AAPC = 2.4%), most notably among females (AAPC= 2.8%). Two previous reports using NCR data are consistent with our findings (6, 20). The current study, however, is the first to investigate and report on trend breaks. A noteworthy finding was that from 1997 the AAPC still showed an increase, though at a much smaller rate than before. Although we do not have a clear explanation why the incidence rates decreased considerably in the late 1990s, we think that this might be a reflection of the decline in the number of people that smoked in the Netherlands which started in the 1960s. Since the impact of tobacco use on cancer incidence becomes manifest only after a latency period of approximately 25-30 years (21), the effect of smoking cessation would therefore become visible in the late 90's indeed. Interestingly, the difference in annual incidence rates between males and females disappeared after 1997 as well. This new gender-specific trend pattern is inconsistent with certain epidemiological studies which have documented considerable increases only in males (22-25), however, it is in accordance with recent studies from Hong Kong and Denmark that have shown very comparable APCs for both males and females (26, 27).

Interestingly, several reports from the United States have shown a rise in the proportion of patients younger than 45 years with OPSCC, specifically among white individual males (28-30). The increasing incidence for this group of patients has been explained by HPV viral infection with more virulent strains and a decreased latency period (28). Practicing oral sex with > 5 partners and French

kissing has also been suggested to play a significant role in oral HPV transmission among those patients (31). Besides, it was observed that most of the young American patients with HPV-related OPSCC were non-smokers/ non-drinkers, but have more marijuana exposure (32). Our data, however, shows contradictory findings with a significant decrease in the annual incidence rate, in particular within the age group 35-44 years (APC of -3.7%), which was quite similar for males (-3.5%). Additionally, in our risk factor analysis, HPV-positive young adults patients were frequent smokers and drinkers. In such a case, it might be difficult to determine the specific or relative contribution of each of these risk factors in this group of patients, if any. This is also in line with what was previously reported by Monsjou et al. in a sample of 54 Dutch patients younger than 45 years, where the authors concluded that HPV association was not exclusively detected in nonsmoking, nondrinking young patients (33). With the reduced incidence rate observed in our study, it is tempting to speculate that the role of HPV viral infection in young Dutch patients may be less prominent. However, whether the interaction of HPV virus with tobacco and alcohol may lead to a biological modification and consequently reduction of its pathogenicity is currently unknown. More research, therefore, is needed to clarify the genetic features of HPV strains in interaction with tobacco and alcohol, for instance, and whether there is any effect on viral load or activity.

The typical profile of HPV-positive OPSCC patients has been established based on a landmark study by Chaturvedi and co-authors. The study showed that the highest number of the patients with this infected tumor were males in their fifties (adult population) (8). Another study compared the incidence of head and neck cancer, in particular for HPV-related OPSCC, among Canadian patients in different age groups for the recent timeframe (1992-2009) (34). The study found a significant increase in HPV-associated OPC, specifically, in patients aged 50-59 years old (APC = 5.4, $p<0.001$). Researchers from England and Australia also showed a younger age at diagnosis of males with HPV-positive OPSCC (35, 36). For those aged 45-59 years, our findings closely mirrored those studies for the period until the late 1990s only. However, in contrast to the findings described above, from 2000 on we found a significant decline in the trends for this subpopulation of Dutch patients which was most prominent in women. It remains speculative what could be the reasons behind this phenomenon, and we do not have an explanation other than the relation to cessation of smoking as argued above.

In the findings reported in the current paper, OPSCC incidence rates were the highest in those patients that were 60-74 years of age at diagnosis. This is consistent with what has been reported

previously in the United States by Zumsteg et al. (22). The study found a significant increase in the age-adjusted incidence of OPSCC in the patients aged 65 years and older with an APC of 2.92 % (95%CI, 2.32-3.51; p< .001). Equally important, a shifted paradigm of the typical HPV-positive OPSCC patients, and increasing prevalence of HPV infection among patients aged 70 years and older with oropharyngeal carcinoma has been reported in the last 10 years in the US (12). This evolving picture is unclear for the Dutch population, in which our data revealed that the lowest proportion of HPV-related OPSCC was found among patients aged 60 years and older. It is important to note, however, that these older patients were also less often tested for HPV, as implied by the larger proportion of unknown HPV-status in this age group. On the other hand, this patient group consists of patients of which the vast majority were heavy smokers and heavy drinkers. Our findings, thus, suggest that tobacco smoking and alcohol drinking are still important factors for OPSCC. Thus, there is still to gain with further national efforts to increase population awareness about these preventable risk factors.

An important aspect which distinguishes the results in the Dutch population from other studies is that the most significant increase in the APC of this tumor was observed in females 60 years or older. Of interest, a very recent study from Germany has also reported that a rising incidence of OPSCC was predominantly observed in female patients, confirming that such a finding is a genuine phenomenon (37). One possible explanation for our observation could be the heavy drinking habit of the Dutch females (22%) when compared to men (14%), especially those above 55 years, as reported in a recent study (38). In support of this finding, the evidence for the carcinogenic effect of heavy drinking on oropharyngeal mucosa has been considered sufficient by the International Agency of Research on Cancer (IARC) Monograph 96, regardless of the smoking status (39). Additionally, WHO reported that Dutch females smoke almost as much as Dutch males, while the association between female ever-smokers and the risk of head and neck cancer was found to be stronger with an odds ratio (OR = 2.33, 95% CI = 1.56 to 3.49) higher than for males (OR =1.65, 95% CI = 1.14 to 2.39)(40, 41). Likewise, analysis for *Cancer Incidence in Five Continents* database has shown a predominant increase in oropharyngeal carcinoma in women in countries where oral and lung squamous cell carcinoma were also increasing, linking the causative role for smoking rather than HPV infection (7). With this background, it seems clear that tobacco and alcohol usage remain important risk factors for OPSCC , besides HPV infection.

To our knowledge, we report the first population-based data on HPV-status among Dutch OPSCC patients. Despite the fact that the data for the risk factors HPV status, smoking, and drinking was

only available for the last two years, it provides essential and critical information. The data reveals that at least 31% of OPSCC in the Netherlands are HPV-positive (2015-2016). Even though some may criticize our result because of the relatively high proportion of unknown cases, one has to keep in mind that the majority of those unknown cases were elderly patients (75%), and it is firmly established that HPV positive OPSCC is a distinct entity which in particular affects the young and middle aged populations (7). Therefore, we think our result may still be fairly accurate and may more or less reflect the reality. This figure also differs from the most recent study that demonstrated HPV prevalence of 48% in Dutch OPSCC patients from a single institution(14). This variation indicates that overestimation of HPV incidence in monocentric studies is a significant limitation and population based studies should remain the standard approach to measure disease distribution. Of note, the estimated percentage in this study differs from the results reported in other countries such as North America (70%), Spain (6.1%), France (46.5%), and United Kingdom (55%), but is comparable to what is reported in Japan (29%) (7, 42-45). This disparity in the viral prevalence between different populations might be a reflection to various methods of viral detection in the older studies, but geographical differences in cultural practices and sexual behavior seem the key factors.

The 5-year survival rate for OPSCC has been reported to improve dramatically with early detection, reaching 75 % when the lesions were small and localized. However, it remains as low as 25 % in metastatic disease (46). Unfortunately, in our data the percentage of the patients exhibiting local diseases (stage I and II) was relatively low, accounting for only 21% of all patients, while 76% exhibited advanced diseases at the time of initial diagnosis. This finding is consistent with the existing literature recounting that OPSCC is often diagnosed at advanced stages(8, 47). In the Wesley et al. study, for example, only 14% of the patients were diagnosed at an early stage (stages I-II), whereas 86% were diagnosed at advanced stages (stages III-IV) (48). Unawareness of the patients due to inaccessibility of the OPSCC lesions and its signs (such as involvement of neck lymph nodes) and symptoms (like sore throat) that are less frequently observed in contrast with other diseases could be one of the reasons for the delayed diagnosis. In addition, the low alertness level among general practitioners (GPs) and the dentists due to its relative rarity may also delay secondary care referral. Hence, considering public education about the warning signs such as painful swallowing or odynophagia (a good initiative is for example the make sense campaign; https://makesensecampaign.eu/en/cancer-information/head-neck-cancer) and advising the GPs and the dentists themselves to engage in further education and training courses may play a key role in the earlier detection of OPSCC and consequently, improving survival rates in OPSCC patients.

The main strength of our study is that it is population-based (covering virtually all cancer cases in The Netherlands) and thus avoids any selection bias of clinical series. At the same time, we have investigated the relevance of the well-known risk factors for OPSCC, in particular HPV infection, which allowed us to explore additional differences between various age groups. Nevertheless, our findings should be understood in the context of some limitations. Firstly, our analysis for the risk factors and evaluation prevalence of HPV viral infection were based on the available data for only two years. Therefore, we cannot relate these findings to changes in incidence rates over time. Secondly, though we could illustrate the differences by age group, we cannot make a definitive statement about cause-effect links. This is because patient and tumor characteristics, including lifestyle habits, were only available at the time of diagnosis of the tumor. Additionally, even though the study has provided a good picture about HPV distribution, no data was available about the survival rate to explore whether or not HPV was an independent prognostic factor. Investigation this association between tumor HPV status and survival will be interesting, especially because the majority of HPV-positive Dutch patients were also smokers and drinkers.

In conclusion, we observed that incidence of OPSCC increased at slower rate in the recent years, except for 35-44 year-olds and those aged 45-59 year-olds which showed a significant decline. The prevalence of smoking and drinking alcohol was quite high in all age groups, while the proportion of HPV-positivity was relatively low, showing that tobacco and alcohol use remain relevant factors in OPSCC. Further studies are needed to elucidate the role of HPV infection in OPSCC and should focus on viral variants and latency period in smokers and alcohol drinkers. Moreover, examining effect of HPV status on OPSCC survival rate at population-based study certainly warrants further efforts .

Supplementary material:

Figure S1 represents graphical presentations of the joinpoint analysis of the clinical stages for all age groups

Figure S2 represent the risk factors interaction among the patients with OPSCC

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Table (1): General characteristics of 11,739 patients with oropharyngeal SCC diagnosed in 1989-2016 by age groups

Variables		All age	Age groups 20-34 y	35-44 y	45-59 y	60-74 y	75+ y
Total	N (row %)	11,739 (100)	31 (0.3)	455 (4)	4,892 (42)	5,108 (43)	1,253 (10.6)
Gender	N (column %)						
	Male	7,945 (68)	23 (74)	329 (72)	3,297 (67)	3,498 (68)	798 (64)
	Female	3,794 (32)	8 (26)	126 (28)	1,595 (32)	1,610 (32)	455 (36)
Clinical stage	N (column %)						
local disease (stage I & II)		2,515 (21)	8 (26)	88 (19)	1,002 (21)	1,142 (22)	275 (22)
advanced disease (stage III & IV)		8,966 (76)	21 (68)	355 (78)	3,788 (77)	3,864 (76)	938 (75)
Unknown		258 (2)	2 (6)	12 (3)	102 (2)	102 (2)	40 (3)

Table 2: Trend in incidence for oropharyngeal SCC in the Netherlands, 1989-2016

Variables	Overall trend (1989-2016)	Join point analysis				ESR/ (100,000)**			
		Trend I		Trend II					
	AAPC (95 CI%) (%)	Year	APC 95CI% (%)	Year	APC 95CI% (%)	1989-1995	1996-2002	2003-2009	2010-2016
Overall	2.4* (1.8, 3.1)	1989-1997	5.3* (3.4, 7.3)	1997-2016	1.2 * (0.7, 1.7)	1.75	2.33	2.49	2.77
Gender									
Males	2.1* (1.3,3.0)	1989-1997	4.5 * (2.0,7.0)	1997-2016	1.2 * (0.5,1.8)	2.54	3.24	3.49	3.82
Females	2.8 * (1.9,3.6)	1989-1996	7.2 * (4.1,10.5)	1996-2016	1.2 * (0.6,1.9)	1.06	1.49	1.56	1.76
Age groups									
20-34 years**	2.2 (-4.8, 9.6)					0.02	0.03	0.04	0.03
M**	1.5 (-3.1, 6.4)					0.04	0.04	0.06	0.05
F**	-0.6 (-17.1, 19.3)					0.01	0.03	0.02	0.01
35-44 years	-3.7* (-5.0, -2.3)					0.90	0.79	0.53	0.44
M	-3.5* (-5.4,-1.5)					1.29	1.10	0.74	0.68
F	-5.2* (-7.6, -2.9)					0.50	0.48	0.32	0.20
45-59 years	1.6* (0.7, 2.6)	1989-2000	5.7* (3.8, 7.6)	2000-2016	-1.1* (-2.1-0.0)	4.16	5.91	5.96	5.57
M	1.9* (0.6, 3.2)	1989-2000	5.9* (3.8, 8.5)	2000-2016	-0.8 (-2.2, 0.6)	5.41	7.77	8.05	7.58
F	1.1* (0.1, 2.2)	1989-2001	4.7* (2.8, 6.7)	2001-2016	-1.7* (-3.0, -0.3)	2.88	4.00	3.83	3.55
60-74 years	3.4* (3.0, 3.8)					5.49	7.14	8.70	11.32
M	2.8* (2.4, 3.2)					8.53	10.35	12.2	15.46
F	4.3* (3.6, 5.1)					2.95	4.28	5.36	7.28
75+ years	2.8* (2.0, 3.6)					3.09	4.21	4.38	5.64
M	1.6 * (0.7, 2.5)					6.14	7.16	7.62	8.63
F	4.4 * (2.8, 6.0)					1.40	2.61	2.50	3.58
Clinical stage									
local disease (stage I& II)	2.1* (1.1, 3.1)	1989-1997	7.2* (4.0, 10.4)	1997-2016	0.0 (-0.8, 0.8)	0.38	0.55	0.52	0.55
advanced disease (stage III & IV)	2.8* (2.1, 3.6)	1989-1996	6.0 * (3.4, 8.7)	1996-2016	1.7* (1.2, 2.3)	1.29	1.72	1.92	2.18
Unknown	-3.6 * (-5.0, -2.2)					0.08	0.05	0.05	0.03

* The APC or AAPC is significantly different from zero (p<0.05), AAPC, average annual percentage change; APC, annual percentage change, M: males, F: females, ** calculated by clustered perio

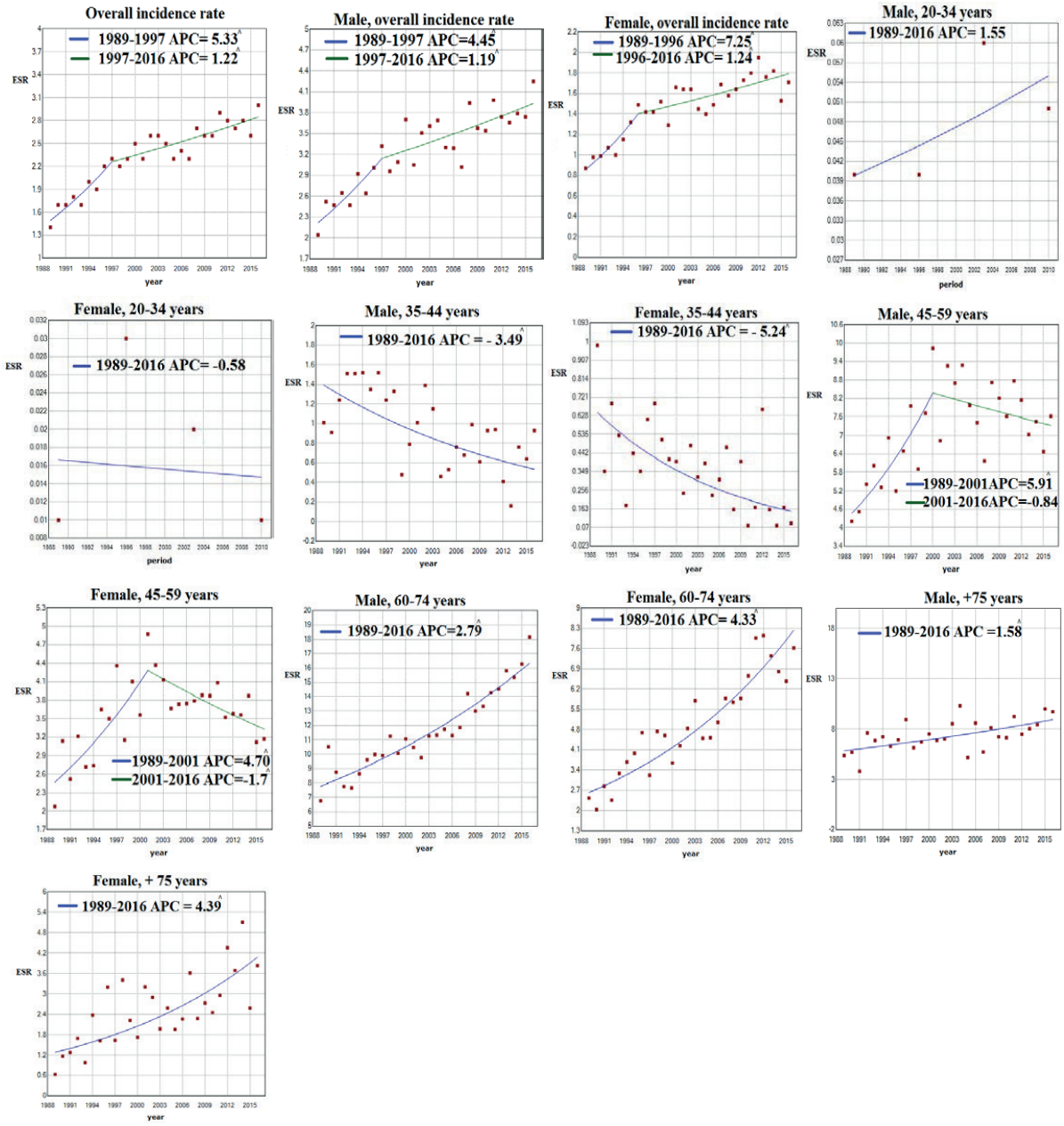


Figure 1: Join point regression analysis shows trend of incidence of oropharyngeal squamous cell carcinoma`(1989-2016), ESR : European age-standardized rate per 100,000 person-years. ^ indicates that the Annual Percent Change (APC) is significantly different from zero at the alpha = 0.05 level. The APC of males and females aged 20-34 years was calculated in period of four equally spaced calendar (1989-1995, 1996-2002, 2003-2009, 2010-2016).

Table (3): Characteristic of oropharyngeal SCC patients and differences of individual risk factors for different age groups for years 2015-2016

		Age groups					
Variables	**All age**	**20-34y**	**35-44y**	**45-59y**	**60-74y**	**75+y**	**P-value**
Total N (raw %)	1243 (100)	2 (0.2)	20 (2)	368 (30)	684 (55)	151 (12)	
Gender N (column%)							
Males	873 (70)	2 (100)	17 (85)	267 (69)	482 (71)	105 (69)	
Females	370 (30)	0 (0.0)	3 (15)	119 (31)	202 (29)	46 (31	0.61**
Anatomical sites N (column%)							
Tonsils	449 (36)	1 (50)	8 (40)	157 (41)	222 (32)	61 (40)	
Base of the tongue	375 (30)	0 (0.0)	6 (30)	135 (35)	197 (29)	37 (24)	
Soft palate &uvula	136 (11)	0 (0.0)	0 (0.0)	38 (10)	82 (12)	16 (11)	**<.001****
Other oropharynx	283 (23)	1 (50)	6 (30)	56 (14.5)	183 (27)	37 (24)	
Smoking status N (column%)							
Current or past	981 (79)	0 (0.0)	13 (65)	311(81)	548 (80)	109 (72) **b**	**<.001****
Never	115 (9)	2 (100) **a**	5 (25)	39 (10)	50 (7)	19 (13)	
Unknown	147(12)	0 (0.0)	2 (10)	36 (9)	86 (13)	23 (15)	
Pack-years N (column%)							
1-20 pack-year	132 (13)	---	9 (69)	53(17)	57 (10)	13 (12)	**<.001****
≥ 21 pack-year	495(50)	---	2 (15)	152 (49)	293 (53)	48 (44)	
Unknown	354 (36)	---	2 (15)	106 (34)	198 (36)	48 (44)	
Median (P25, P75) #		5.5 (1.75, 13,75)	20 (15, 25)	32 (20,40)	40 (25,50)	35 (23,50)	**<.001*****
Alcohol status N (column%)							
Current or past	941(76)	1 (50)	14 (70)	306 (79)	525 (77)	95 (63) **b**	**<.001****
Never	43 (3)	1 (50) **a**	2 (10)	13 (3)	19 (3)	8 (5)	
Unknown	162 (13)	0 (0.0)	4 (20)	65 (18)	140 (20)	48 (32)	
Number of alcoholic beverages per week N (column%)							
1-20	363 (39)	1 (100)	7 (50)	122 (40)	193 (37)	40 (42)	**<.001****
≥ 21	360(38)	0 (0. 0)	3 (21)	112 (37)	216 (41)	29 (31)	
Unknown	218 (23)	---	4 (29)	72 (23)	116 (22)	26 (27)	
Median (P25, P75) #		5 (2,15)	10 (5.5, 24.5)	21 (8, 42)	21 (7,30)	14 (5.75,21)	**<.001*****
HPV status N (column%)							
Positive	383 (31)	1 (50)	13 (65) **a**	156 (40) **a**	177 (26)**b**	36 (23.8)	
Negative	420 (34)	1 (50)	4 (20)	122 (32)	251 (37)	42 (27.8)	**<.001*****
Unknown	440 (35)	0 (0.0)	3 (15)	108 (28)	256 (37)	73 (48.3)	

*chi-square, ** Fisher exact, *** Kurskal wallis test, # Median calculated by interquartile range = Percentile 75th- Percentile 25th , a:The observed count of this cell was significantly higher than expected per null hypothesis, b: The observed value of this cell was significantly lower than expected per null hypothesis. Statistically significant p-values are shown in Bold.

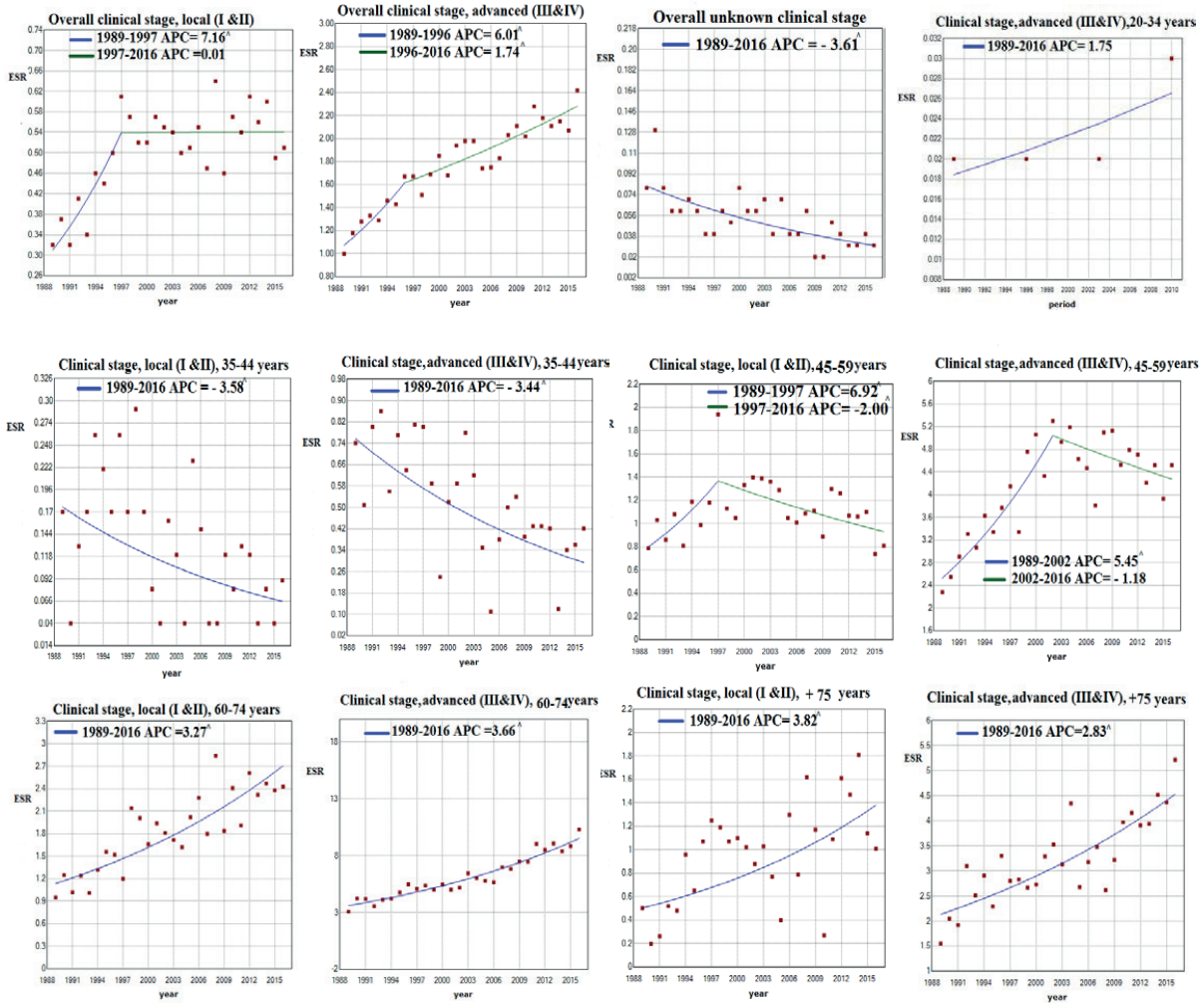
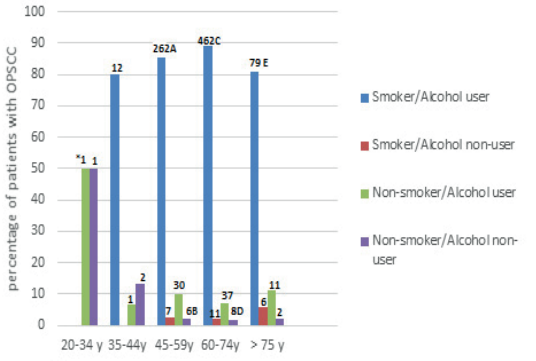


Figure 2: Join point regression analysis shows trend of **clinical stages (Local and advanced)** of oropharyngeal squamous cell carcinoma`(1989-2016), ESR : European age-standardized rate per 100,000 person-years. ^ indicates that the Annual Percent Change (APC) is significantly different from zero at the alpha = 0.05 level. Clinical stage of patients aged 20-34 years was calculated in period of four equally spaced calendar (1989-1995, 1996-2002, 2003-2009, 2010-2016).

Note1 : During the study period of time, several editions of the International Union against Cancer (UICC) TNM classification were used to record tumor stages : 4th edition (1989–1998), 5th edition (1999-2002), 6th edition (2003-2009), and 7th edition (2010-2016)
Note2: clinical stages, local (I & II) could not be calculated for age group 20-34 years



FigS2a: percentage of patients with OPSCC within each of the four conditions of smoking and drinking interactions. A (Z-residual = 5.0 $\chi 2$= 25 P <0.0001), B (Z-residual = 4.20 $\chi 2$= 17.6 P =0.00003), C (Z-residual = 7.5 $\chi 2$= 56.2 P <0.00001), D (Z-residual = 5.6 $\chi 2$= 31.3 P <0.00001) and E (Z-residual = 4.3 $\chi 2$= 23.0 P <0.00001). * patients number. Missing patients were excluded.

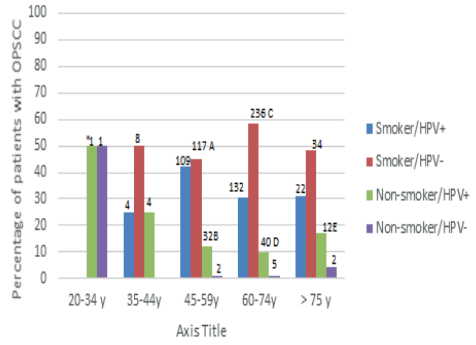


Fig S2b: percentage of patients with OPSCC within each of the four conditions of HPV status and smoking interactions. A (Z-residual= 5.1 $\chi 2$= 26.0 P <0.0001), B (Z-residual= 5.2 $\chi 2$= 27 P = 0.0001), C (Z-residual= 6.0 $\chi 2$= 36 P <0.0001), , D (Z-residual= 8.0 $\chi 2$= 72 P < 0.0001), E (Z-residual= 4.0 $\chi 2$=16 P = 0.0006). * patients number. Missing patients were excluded

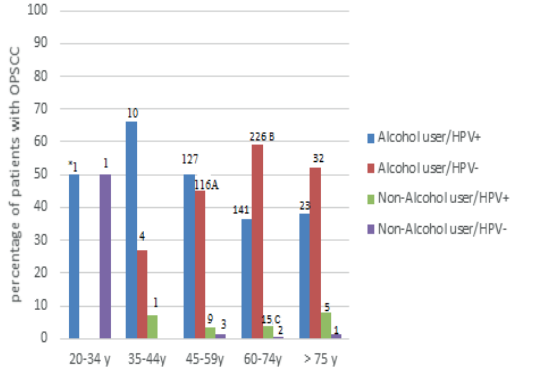


Fig S2c: percentage of patients with OPSCC within each of the four conditions of HPV and drinking interactions. A (z-residual = 3.8 $\chi 2$= 14 P= 0.00014), B (z-residual = 3.4 $\chi 2$= 11.5 P= 0.0006), C (z-residual = 5.0 $\chi 2$= 25.0 P = 0.00001) , * patients number, Missing patients were excluded

Supplementary figures S2 a,b and c present percentage of the patients within each group of the classical risk factors (smoking, drinking and HPV) interactions, including results of post-hoc chi-square analysis

CHAPTER 5

A REVIEW OF THE MOST PROMISING BIOMARKERS FOR EARLY DIAGNOSIS AND PROGNOSIS PREDICTION OF TONGUE SQUAMOUS CELL CARCINOMA

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Abstract:

Background: There is a great interest in developing biomarkers to enhance early detection and clinical management of tongue squamous cell carcinoma (TSCC). However, the developmental path towards a clinically valid biomarker remains extremely challenging. Ideally, the initial key step in moving a newly discovered biomarker towards clinical implementation is independent replication. Therefore, the focus of this review is on biomarkers that consistently showed clinical relevance in two or more publications.

Methods: We searched PubMed database for relevant papers across different TSCC sample sources, i.e. body-fluids (saliva, serum/plasma) and tissues. No restriction regarding the date of publication was applied except for immunohistochemistry (IHC); only studies published between 2010 and June 2017 were included.

Results: The search strategy identified 1,429 abstracts, of which 96 papers, examining 150 biomarkers, were eventually included. Of these papers, 66% were exploratory studies evaluating single or a panel of biomarkers in one publication. Ultimately, based on studies that had undergone validation for their clinical relevance in at least two independent studies, we identified 10 promising candidates, consisting of different types of molecules (IL-6, IL-8 and Prolactin in liquid samples; HIF-1α, SOX2, E-cadherin, vimentin, MALAT1, TP53 and NOTCH1 in tissue biopsies)

Conclusions: Although more exploratory research is needed with newer methods to identify biomarkers for TSCC, rigorous validation of biomarkers that have already shown unbiased assessment in at least two publications should be considered a high priority. Further research on these promising biomarkers or their combination in multi-institutional studies, could provide new possibilities to develop a specific panel for early diagnosis, prognosis, and individualized treatments.

Key words:

Tongue squamous cell carcinoma, liquid-based biomarker, tissue-based biomarker, prognostic biomarker, promising biomarker

Abbreviations:

IL-6: Interleukin-6;

IL-8: Interleukin-8;

HIF-1 α: Hypoxia inducible factor 1 alpha subunit;

SOX2:Sex-determining region y-box protein 2;

RT-PCR: Real-time polymerase chain reaction;

MALAT1: Metastasis associated lung adenocarcinoma transcript 1;

TP53: Tumor protein p53;

NOTCH1: Notch homolog 1, translocation-associated (Drosophila);

REMARK: REporting recommendations for tumour MARKer prognostic studies

Background

Tongue squamous cell carcinoma TSCC is one of the most lethal head and neck cancers worldwide [1]. It is comparatively silent and progresses from a premalignant state into invasive carcinoma without any specific alarming symptoms [2]. This causes delay in diagnosis, eventually leading to poor prognosis. The incidence of this disease is rising in the population, particularly in Western communities among young individuals [3,4,5]. Unfortunately, even with combined treatment involving surgery, radiation and chemotherapy, the 5-year survival rate is still unsatisfactory [6, 7]. One reason could be the marked biological propensity for local invasion and the high incidence of cervical lymph node metastasis at initial diagnosis (40%) [8]. Another is a uniform treatment for all patients with the same clinical and histological features that disregards individual differences in genetic and biological behavior.

Currently, understanding of cancer development and progression is rapidly increasing. Knowledge about specific regulatory pathways and signaling interactions that lead to neoplastic transformation and invasion has been gained. Delineation of these pathways has revealed a multitude of biomolecular changes that could be exploited as biomarkers. A biomarker by definition is an objective measure such as, a gene, a protein, enzyme or hormone that can reflect the entire spectrum of the disease, from the earliest features to the end stages. It can also provide information on how the body responds to any therapeutic interventions; this may help in making treatment decisions [9, 10].

Cancerous cells, or other body cells in response to tumor development secrete or release a subset of biomarkers into tissues and different biological body fluids. The body fluid biomarkers can be detected and evaluated in succession with non-invasive or slightly invasive means, whereas tissues-derived ones need invasive procedures like biopsies. For TSCC, finding a novel, and specific biomarker in body fluids can offer complementary information beyond what is provided by current clinical practice, especially in the field of early detection and diagnosis. Additionally, biomarkers that mirror genetic alterations and proteins expressions on histological slides may play a key role in predicting tongue cancer behavior and determining the treatment plans.

There is a three-level evidence hierarchy for biomarker validation, ranging from exploratory to validated to clinically useful, and to qualify as a useful biomarker it is essential to successfully pass them all. The exploratory biomarker is defined as any biomolecule identified in one discovery publication with targeted or untargeted approaches. This classification results in a large list of

discovery biomarkers that, however, require rigorous validation. Validation is a second and pivotal step to move any biomarker towards clinical implementation, and is based primarily on confirming a discovery biomarker's finding in at least two independent studies [11,12]. To date, despite the proposition of a large number of potential biomarkers of TSCC, none are currently used in clinical practice, and only very few have actually proceeded towards the path of validation.

To our knowledge, this review is the first to list the published literature on both liquid and tissue-based biomarkers in TSCC. Since squamous cell carcinoma of different subsites of the oral cavity is quite heterogeneous, we only considered studies which specifically addressed the tongue locus and in particular the mobile part of the tongue. Our focus was particularly on biomarkers whose clinical significance was described in at least two independent studies. As these might represent promising biomarker candidates, we evaluated the studies with regard to the potential of these biomarkers for early diagnosis and prognosis prediction of TSCC, in which the markers demonstrated a consistent association between their expression and specific clinical outcomes. Moreover, we evaluated them using Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) [13] guidelines for prognostic studies and STARD[14] (Standards for Reporting of Diagnostic Accuracy) criteria for the diagnostic ones. In this way, we aim to help both researchers and clinicians in identifying and pursuing the most promising tongue cancer biomarkers for further evaluation and validation studies.

Materials and Methods:

Search strategy

Potentially eligible studies were identified in a search of US National Library of Medicine electronic database (PubMed), using combination of the following terms: "tongue carcinoma", "tongue SCC", "biomarker", " biological marker", "tissue", "body fluid " "saliva" , "serum/plasma", " immunohistochemistry", "long non-coding (lnc) RNA", and " genetic mutation". No restriction regarding date of publication was applied except for immunohistochemistry (IHC); only studies published between 2010 and June 2017 were included to ensure that all new published evidence on potential markers since last IHC review[15] were encompassed. In addition, PubMed Advanced Search Builder (http://www.ncbi.nlm.nih.gov/pubmed/advanced) was utilized to identify some publications. Results were supplemented with manual searching for relevant citations. The initial search was performed in January 2017 and updated in June 2017.

One author (AAH) examined all titles and abstracts to exclude studies that were beyond doubt irrelevant. Then, AAH and MNH assessed full-text manuscripts of all remaining studies against prespecified eligibility criteria.

Selection of studies

Inclusion criteria

- Studies investigating association(s) between TSCC and biomarkers
- Studies reporting clinical significance(s) for biomarker expression
- Studies investigating biomarker expressions in oral cavity when all samples were taken from the tongue
- IHC studies encompassing multivariate analysis in statistical assessment
- English full-text version available

Exclusion criteria

- Studies investigating biomarkers in different anatomical subsites of oral cavity, and head and neck cancer
- Studies unclear about clinical implications
- Studies exclusively addressing the base of the tongue
- Studies investigating biomarkers only in animals
- Studies investigating micro-RNAs as biomarkers; these were already reviewed[16] recently for their clinical implications in TSCC
- Case reports, letters to the Editor, and systematic reviews

Definition of the level of evidence and promising biomarkers

Biomarkers are usually classified based on the development pipeline, subdivided into 4 phases: exploratory, assay development and validation phase, retrospective validation studies, and prospective validation studies[11, 17,18]. However, since most of the TSCC biomarker studies are still in the exploratory phase with rather small sample sizes, we had to employ an alternative approach, based on the study of Teunissen and coworkers[12], which we slightly adapted (downscaled).

Ranking level of evidence (LoE)

- Negative (-): Study reported no significant association between biomarker expression and clinical values

- Weak (+): One study reported an association between biomarker expression and clinical values
- Intermediate (++): 2 independent studies reported consistent evidence of an association between biomarker expression and clinical values
- Strong (+++): ≥3 independent studies reported consistent evidence of an association between biomarker expression and clinical values

Only biomarkers with an intermediate or strong LoE, i.e. demonstrating a consistent association between their expression and specific clinical outcomes in at least two reports, were considered as promising biomarkers, even in the case that also neutral or opposite predicted outcomes were available for the same biomarker.

Data extraction

Included studies were classified into liquid and tissue-based biomarkers. These were further categorized according to the aforementioned LoE ranking into two groups:

- Group A: studies with negative and weak LoE
- Group B: studies with intermediate and strong LoE

Group B comprised all promising biomarkers, the master variable of interest of the current review. The studies of both groups were arranged according to year of publication, earliest to latest.

Since tissue biopsies were evaluated using various techniques, the tissue-based biomarkers were subdivided as follows:

- Protein biomarkers
- lnc RNA biomarkers
- DNA biomarkers

Information about the biomarker studied, including its usefulness, sample type and size, the method of detection, expression level, type of mutation, and validity indices were listed in table format.

Quality assessment

For the purpose of this review, we first defined prognostic biomarker as a marker has an association with the typical outcomes such as survival rate or recurrence or has an association with the predictor of outcomes like metastasis or tumor grade/size and differentiation. We then started screening the data and found that the vast majority of these studies were prognostic in nature, while a few were diagnostic. Consequently, the quality of the selected biomarkers studies was independently assessed by two authors (A.A.H and M.N.H) on the basis of the criteria as formulated in the Reporting

Recommendations for Tumor Marker Prognostic Studies (REMARK)[13] guidelines for prognostic studies and STARD[14] (Standards for Reporting of Diagnostic Accuracy) criteria for the diagnostic ones. The former comprises of 20 items, and the latter consists of 30 items, in which each item can encompass several aspects in both guidelines. When all aspects of an item were clearly stated in the study, it was given 1 point, 0.5 point was attributed if some but not all aspects were mentioned, and 0 point were given when the item was not reported. Based on the total scores, the studies were subdivided into three groups: studies with a REMARK score of 15-20 or STARD score of 20-30 were assigned as high reporting quality, studies had a REMARK score of 5 -14.5 or STARD score of 10-19.5 were considered to have an average reporting quality, and low reporting quality when the score ≤ 5 for REMARK and ≤ 10 for STARD. Disagreements were resolved by discussion.

RESULTS

A diagram of studies selected for this review after exclusion of irrelevant studies is presented in Figure 1. Seventy-two studies classified biomarkers belonging to group A, while only 24 studies satisfied the criteria for group B. In total, the included studies examined 150 biomarkers: 23 markers in body-fluids, and 127 in tissue. The sample size used in these studies varied between 4 and 202 in group A, and between 17 and 248 in group B. Additionally, quality estimation according to REMARK and STARD (supplementary tables 1 and 2) showed that the overall quality of the included studies was consistent with an average rating.

In thirteen studies, the potential of salivary and blood biomarkers in tongue cancer was evaluated (Table 1). Five of these papers assessed the performance of 14 different markers for early diagnosis[19,20,21,22,23], seven assessed performance for prognosis[24,25,26,27,28,29,30], while the final study, dealing on pro-inflammatory cytokines, assessed both diagnostic and prognostic performance [31]. Within the included studies, the most promising biomarkers were IL-6 and IL-8 that showed consistent evidence for clinical usefulness in detection and diagnosis, and prolactin in prognosis. Test accuracy indices were reported in six studies, wherein sensitivity and specificity for these studies ranged from 65%–100% and 45%–100%, respectively. In two papers [22,20] evidence was provided that measuring a single biomarker is less effective than assessing a specific set of biomarkers, the latter showing enhanced sensitivity and specificity.

A total of 83 studies investigated different tissue-biomarkers, using various techniques (Tables 2-4). Forty-nine papers used IHC to assess expression of 82 proteins and their potential usefulness to predict prognosis (Table 2). Fifty-two proteins showed a significant association, and 13 of them

were confirmed by mRNA expression. Most IHC studies belonged to group A (39, 80%). As can be deduced, five markers were independent indicators for good prognosis, while the majority (28) were adverse prognostic indicators. Group B comprised ten studies, identifying four promising IHC biomarkers: HIF-1α, SOX2, E-cadherin, and vimentin.

Using quantitative RT-PCR, eleven studies evaluated lncRNA expression levels in tongue cancerous tissue (Table 3). Whereas 16 lncRNAs belonged to group A, only MALAT1 belonged to group B and thus represented the solely promising lncRNA biomarker. Studies assessing DNA mutations in TSCC evaluated 22 mutations in either a single gene or both alleles , while one evaluated promotor methylation of specific genes. Eighteen of these studies satisfied group A, and five satisfied group B, identifying TP53 and NOTCH1 as promising mutation markers.
In summary, only 22 biomarkers were evaluated in two or more independent studies, of which only 10 demonstrated a consistent association between their presence and specific clinical outcomes. Of the latter, three were biomarkers for liquid biopsies and seven were tissue-based biomarkers. Collectively, these ten biomarkers qualified as the most promising candidates for tongue cancer diagnosis and prognosis (Fig. 2).

DISCUSSION

Since pathology and radiology, the current keys to TSCC diagnosis and treatment decisions, are essentially visual subjective measures that are labor-intensive with limitations in diagnostic accuracy, there has been an intensified interest in biomarkers as an objective alternative and more accurate tool for early diagnosis, prognosis or personalized treatment. A plethora of TSCC biomarker studies have been published, however, virtually all biomarkers are still in early stages of development, and far from potential application in a clinical setting. This review aimed to drive the acceleration of TSCC biomarker validation by providing an inventory of currently evaluated TSCC biomarkers across different sample sources, including saliva, serum/plasma, and tissues, and by highlighting promising biomarkers that consistently showed clinical relevance in two or more publications.

Overall, we noticed an abundance of studies that described single or multiple biomarkers only in one publication (66%), whereas there has been no corresponding increase in the validated ones. This may be due to the current pressure from journals to only publish innovative research, which prohibits researchers to perform sound repeat studies providing independent confirmation of the initial

identification of a potentially promising biomarker. Since it remains in this exploratory phase pivotal to determine which biomarker is potentially promising and should be prioritized for further steps of confirmation, high-quality studies should be performed. In this regard, although we have noticed that the majority of the studied biomarkers in these discovery studies showed significant results, we observed several shortcomings affecting the reliability of their value. For example, in some publications only the data of a small number of patients are presented, while in others study designs are not the optimal or statistical design was unpowered. Two strategies should be implemented to improve this situation: One should emphasize on validation and confirmation of biomarkers that have already shown unbiased assessment in at least one publication, and the other is to conduct future research based on sound scientific and well-planned study designs so that reporting can be done according to guidelines such as REMARK for prognostic biomarkers[13].

Last year, two other oral cancer biomarker reviews were published (Rivera et al [32]; Almangush et al. [33]). Rivera and co-workers analyzed immunohistochemically identified potential biomarkers for oral SCC at various subsites, thereby however, disregarding the heterogeneity and well-documented variation in genomic and proteomic properties of this malignancy between different regions of the oral cavity [34-36], and consequently risking divergence of biomarker specificity and discriminative ability. Also, since their aim was to identify potential biomarkers per se, many biomarkers were evaluated based on one publication. Last but not least: although a scientifically sound method of biomarker evaluation was followed with a quality assessment (QA) according to REMARK guidelines, this QA only indicates the reporting quality of the study, but not necessarily the potential of the biomarker(s) at hand. Almangush et al., on the other hand, evaluated immunohistochemical biomarker studies in TSCC of three decades, and subsequently performed a meta-analysis of the five most frequently studied prognostic biomarkers. Only cyclin D1 and VEGF-A were identified as potential prognostic factors. However, they assessed the overall survival as the clinical end point based on unadjusted or 'univariate' analysis which ignored other known prognostic variables, such as tumor stage, tumor size, etc.

How does our current review relate to the two reviews described above? First of all, in contrast to both other reviews, we evaluated TSCC biomarkers across different sample sources, including saliva, serum/plasma, and tissues. Using this approach, our study identified 10 promising biomarkers, consisting of a different type of molecules: seven proteins, one lnc-RNA, and two genes (Fig.2). Three of these markers: IL-6, IL-8, and Prolactin were detected in liquid samples, while

HIF-1α, SOX2, E-cadherin, vimentin, MALAT1, TP53, and NOTCH1 were identified in tissue biopsies. Secondly, as is also the case for the Almangush review but in contrast to the Rivera report, our focus on a specific subsite within oral cancer, i.e. TSCC, is a clear advanced approach and thus our results may strongly point to unique molecular alterations. These different approaches could also explain why the Rivera paper mentioned 41 potential biomarkers, in which we merely identified ten. Thirdly, Almangush et al. did a comprehensive investigation for published prognostic biomarkers of the last 30 years, while our IHC studies were limited to the published articles in the last 7 years. Due to the technological breakthroughs in the last decade that have enabled scientists to identify new key genes and proteins in tongue carcinogenesis, we deliberately aimed to draw more attention to the latest pursued proteins such as SOX2. Last but not least, we think that a biomarker review should base its evaluation on reports employing multivariate analysis only.

Notably, these 10 promising biomarkers have demonstrated different clinical values. For example, increased expression of serum IL-6 has been found to effectively discriminate patients with TSCC from controls with an excellent sensitivity[23]. Likewise, in another study, elevated salivary levels of IL-6 and IL-8 were reported to reliably and accurately identify the progression of TSCC from high-risk to neoplasm[31]. This implies increased usefulness of combining these two markers in early detection of new or recurrent cases of TSCC. Nevertheless, one should be aware that increased levels of expression may be caused by sources of inflammation elsewhere, and a vigorous effort thus should be made to determine appropriate cutoff values for each marker to differentiate tongue cancer at different stages from healthy subjects. Furthermore, all biomarkers of this list showed a significant correlation with poor prognosis. In clinical practice, applicability of these biomarkers may range from recommending wider surgical resection margins to adjusting management strategy, e.g the addition of adjuvant chemo-radiation therapy. Another key element to achieve optimal outcome may be through using them as therapeutic targets.

There is no dispute that there is an urgent and yet unmet need for novel diagnostic and prognostic biomarkers to improve TSCC treatment. Therefore, we are convinced that it is timely and highly necessary to integrate all available information about TSCC biomarkers not only from IHC samples but also from other sources. In other words: it could be important to rely on a group of molecules rather than on a single marker, because molecular evidence on multiple levels such genes, proteins, and RNAs may work in concert to prevent or promote the development of the hallmarks of cancer. Only in this sense, it will be possible to form a relatively correct picture about the molecular pathogenesis of this aggressive malignancy and identify which molecules may play a key role and

accordingly, may serve as accurate biomarkers. Just as important, limiting the focus to protein expression in IHC studies only could be insufficient and misleading in the biomarker discovery phase, particularly due to the potential ongoing modifications of proteins by a plethora of post-translation changes. One such example is P53, the most frequent IHC studied protein, which has been reported to have an insignificant value in TSCC prognosis[33],whereas we found its gene to be a strong promising indicator. Furthermore, it should be noted that as yet there is no single method suitable for reflecting the complete complexity of TSCC. Hence, our journey through different samples and various molecules assessed by different assays was in our view an essential step to find molecules with distinct biological pathways such as MALAT1 that merit further thorough investigation and validation.

Validation is a critical step for introduction of any newly discovered biomarker into the clinical practice. However, it is important to realize that there are two aspects of validation: clinical and technical. Clinical validation depends on many parameters, one of which is consistency across studies between specific clinical outcomes and the biomarker evaluated, a policy we adopted in our current study. Of equal importance are other clinical parameters which may influence the strength of a biomarker validation. These include the number of cohorts of a study, whether they are of sufficient size or not, existence of a control group, and what their characteristics are. In parallel, technical validation by using independent methods of biomarker evaluation is another parameter that should be strived for.

One major and underappreciated problem with TSCC biomarker studies which we have found is that several studies used very small samples (few with exceptions). Unfortunately, in current practice it is widely accepted that for validation studies the research must meet rigorous criteria in all aspects, particularly in sample size calculation; however, in discovery studies, such criteria are not mandatory. Indeed, neglecting this epidemiological issue in the discovery studies may have contributed to many false findings. And since the discovery studies form an essential element for the selection of biomarkers to be validated, this may partly explain why not one single biomarker has yet reached the oral oncology clinic. Admittedly, including studies with small subjects in this review may potentially bias the conclusions drawn, because the real performance of these biomarkers may remain unclear. However, we consider our validation approach for the promising biomarkers in which two or more cohorts were included as a useful strategy to minimize this bias.

One might argue that our validation approach to focus on the positive consistent studies and ignore the negative ones is considered as flawed and tenuous, particularly if these negative studies may have a higher quality. Therefore, the quality of the included studies was assessed using REMARK and STARD which are well-established scoring systems to evaluate the quality of prognostic and diagnostic studies, respectively. Nonetheless, it should be mentioned that these two guidelines were primarily developed to assess the quality of reporting rather than to rate the research methodology. According to the evaluation in here, our results showed an average reporting quality for the included studies, which implies that these could be considered trustworthy. As such, we are confident to suggest that our list of promising biomarkers have demonstrated robustness to warrant further validation studies. Notwithstanding, we cannot speculate about the potential for clinical adoption of any of these markers. Further, we noticed that the highest scores were within lncRNA studies. Since all these studies have been published in the recent few years, this might reflect the rise in awareness among researchers about the importance of reporting and transparency in research.

The anterior two-thirds of the tongue (mobile tongue) and the posterior one-third (base of tongue) are commonly considered as two distinct clinical entities, particularly after the recognition of human papillomavirus (HPV) as a risk factor for base of the tongue in 2007[37]. Indeed, for mobile tongue, no such link with any viruses is found in literature. To date, although each subsite of the tongue is unique with different etiological factors, pathogenesis and prognosis, unfortunately, many authors still combine the samples of both loci or report their studies without a clear-cut specification. The scarcity of studies prohibited us to strongly apply this distinction, but we would nevertheless strongly recommend specifically addressing the tongue subsites separately.

Intriguingly, tissue-biomarkers could be investigated for its validity for detection of, and screening for TSCC in body fluids. Identification of specific biomolecules in body-fluids, with a preference for saliva samples, to obtain on-the-spot potent diagnostic and prognostic information with minimal or non- invasive procedures is still a distant dream. Why this propensity for saliva? Firstly, since saliva is in direct contact with tongue cancer, accumulation of released biomarkers is likely to occur. Secondly, saliva is an ultra-filtrate of plasma, which means that blood-circulating biomolecules may be detected in saliva as well. Moreover, saliva may be preferred over serum or plasma since the latter may contain biomarker compounds derived from different sources than the actual TSCC. To evaluate the aspects listed above, biomarker levels should preferentially be simultaneously quantified in both saliva and serum/plasma samples. Finally, since biomarkers in body fluids may reflect the entire heterogeneity of cancerous tissue, a biomarker panel instead of a single biomarker

may increase sensitivity and specificity [20]. For example, a single biomarker like pro-inflammatory cytokine IL-6 or IL-8 that holds great promise is often not unique to TSCC, and no reference level of expression has been reached yet in cancer, so combining these markers, together or with other biomarkers, would likely provide a more robust clarification of true detection or prognosis.

Tissue samples are evaluated with various analytical methods, ranging from simple (such as IHC) to high technology (such as genomics) platforms. IHC is a relatively simple and affordable technique and consequently, the literature is dominated by this assay type. However, IHC suffers from considerable lack of standardization and mostly only qualitative presentation of data, making technical validation extremely difficult. Nonetheless, developments in digital pathology will improve IHC-based analyses. To solidify our results and compensate for some of these limitations, we only evaluated studies that performed multivariate analysis. Genomic approaches (e.g. microarrays, RT-PCR) are more robust and quantitative methods, with minimum analytical variability and thus facilitating technical validation. Nonetheless, these techniques cannot anticipate levels and actions of the effector molecules (proteins) in directing cancer behavior [38]. Thus, an integral approach studying genetic mutations, RNA expression, and protein concentrations in parallel may be warranted.

Finally, it is worth mentioning that biomarker development process is financially very challenging, and moving from one phase to another becomes even more burdensome. Recently, it has been estimated that biomarker research expenditures in the U.S only in two years were over $ 2.5 billion, with nearly 500,000 publications. In contrast with this significant and massive investment in biomarker research, the number of translatable biomarkers to patients care is so far negligible [39]. Regarding tongue cancer biomarkers, we did not find information about (industrial) financial investment, but the pattern appears similar: an overwhelming number of literature studies of potential TSCC biomarkers with no biomarker translation yet to be expected. In this view, we recommend focusing efforts on a selected set of promising biomolecules already in an early phase in order to move clinical biomarker implementation forward in an economically viable manner.

To the best of our knowledge, this is the first and largest review that evaluated specifically TSCC biomarkers across different sources, including saliva, serum/plasma, and tissues in an integral manner. The included studies used various types of assays for analysis, which allowed us to explore more details about the currently evaluated TSCC biomarkers. In addition, based on a staged approach of a biomarker validation in which one publication does not provide a meaningful role of

the biomarkers as a measure of disease activity, unless more consistent evidence is available supporting its utility, we used the wide and comprehensive set of data identified here provided a shortlist of qualifying promising biomarkers. Nevertheless, our findings should be understood in the context of some limitations, which may have introduced some bias in our assessments. Firstly, we did not consider the number of patients tested in our evidence rating of the promising biomarkers due to the scarcity of the subjects in several studies. Secondly, we have included IHC studies only from 2010 onwards, consequently, it cannot be excluded that some confirmatory studies for some protein biomarkers were missed. Another limitation is that our search strategy is based on the PUBMED search engine only, which may not have revealed all relevant studies. Furthermore, validation of a biomarker such as a prolactin that emerged as one of the promising biomarkers in this review was entirely based on several studies from the same authors and this reduces the robustness of the finding. Even though, the authors followed the rule of thumb by increasing number of the patients in the confirmatory studies, further elucidation in different patient cohorts performed by different research groups to evaluate its value in forecasting prognosis should be conducted.

In conclusion, although biomarkers may play an important role in TSCC detection and management, the developmental path towards a clinically valid biomarker is always long and challenging. This study sheds some very critical light on TSCC biomarkers that demonstrated a consistent association between their expression and specific clinical outcomes at least in two publication, thus qualifying as promising candidates. Furthermore, the findings from this work show how important is the performance of the biomarker during the discovery stage because it will guide the selection of the promising markers for validation. Henceforth, it is critical at this stage to use appropriate sample size and study design. Unfortunately, two-thirds of TSCC biomarker studies have not yet advanced beyond the discovery phase. Despite the fact that more exploratory research is needed to identify specific biomarkers for TSCC, rigorous validation of biomarkers that have already shown unbiased assessment in two publications should be considered a high priority. Further research on these promising biomarkers or their combination in multi-institutional studies, could provide new possibilities to develop a specific panel that may yield better assessment of progression of this malignancy at various stages.

Supplementary material

This word file contains two tables assessing the quality of the included studies based on REMARK and STARD 2015 criteria.

"Supplementary information is available at the British Journal of Cancer's website"

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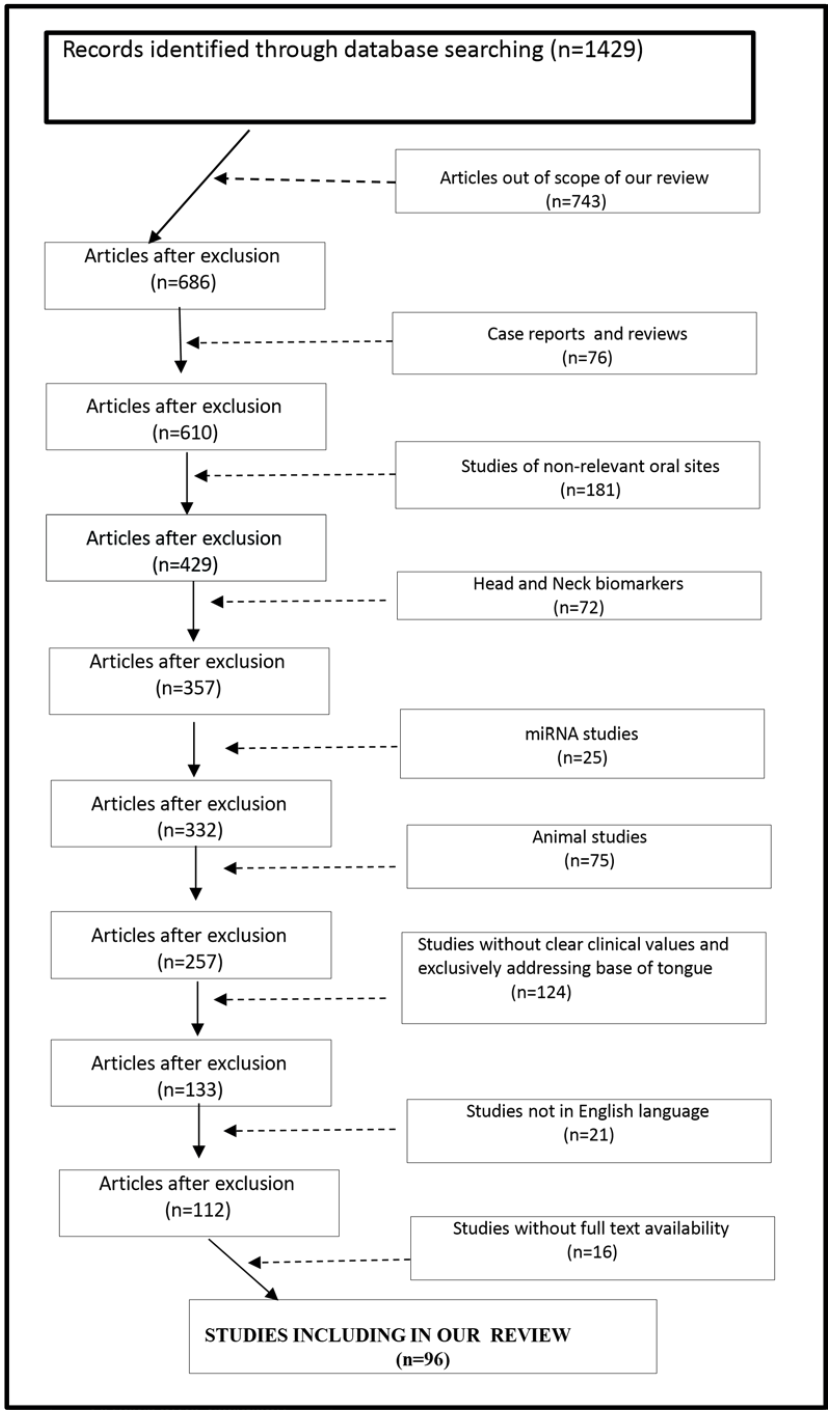

Figure 1: flow chart illustrating studies selected

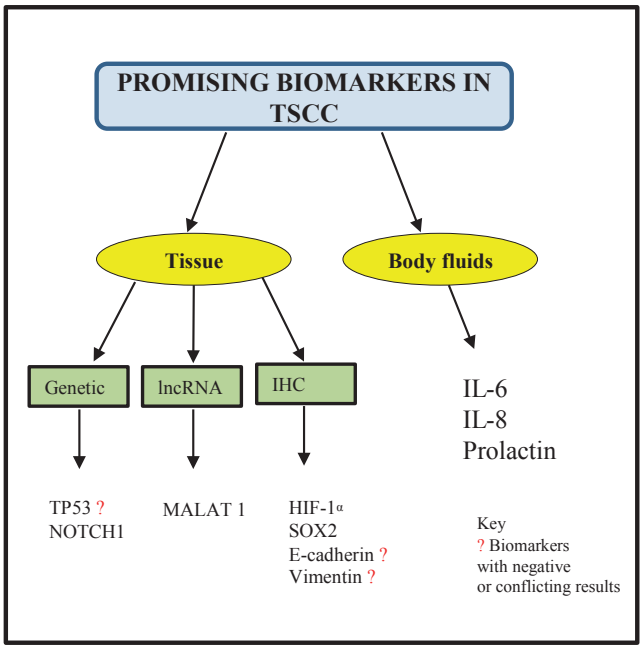

Figure 2: a diagram illustrating the promising biomarkers

Table 1: Summary of body-fluid biomarkers for TSCC

Studied biomarkers	No.Patients	Sample type	Significance of biomarker (s) ‡	Test accuracy indices	Expression	Potential clinical purpose	Level of evidence (LoE) ‡	References
Group A studies								
AMDL DR-70	52	serum	AMDL DR-70	73%/ 93% †	↑	Poor prognosis	+	[24]
SCCA-1	4	serum	SCCA-1	---	↑	Detection	+	[19]
CA125, CA19-9, TPS, CEA, SCC, & Cyfra 21-1	21	Saliva	**Cyfra 21-1, TPS, and CA125**	71%/75% †	↑	Detection &diagnosis	+	[20]
Adenosine deaminase	50	Saliva & serum	**Adenosine deaminase**	---	↑	Detection	+	[21]
Adiponectin	59	serum	Adiponectin	---	↓	Poor prognosis	+	[25]
Syndecan-1	43	Serum	**Syndecan-1**	---	↓	Progression	-	[26]
Group B studies								
Prolactin & TPS	20	Serum	**Prolactin**	100 % ç &75 %	↑	Poor prognosis		[27]
prolactin, TPS, EGF& IGF-1	52	Serum	**Prolactin**	---	↑	Poor prognosis	+++	[28]
Prolactin	37	Serum	**Prolactin**	100%/100% ç	↑	Poor prognosis		[29]
Prolactin	99	Serum	**Prolactin**	---	↑	Poor prognosis		[30]
IL-1a, IL-6, IL-8, VEGF-a and TNF-a	18	Saliva	IL-1a, **IL-6, IL-8,** VEGF-a & TNF-a	---	↑	Detection& poor prognosis		[31]
COL5A1, ABCG1, MMP1, IL-8 and FN1	37	Saliva	COL5A1, ABCG1, MMP1, **IL-8 &** FN1	65%/87% †	↑	Detection	++*	[22]
IL-6	17	Serum	**IL-6**	95%/ 45% †	↑	Detection& diagnosis		[23]

Abbreviation:(-): no significant association between biomarker and clinical value, (+): number of studies with statistical significant outcome = 1; (++): number of studies with consistent outcome = 2 ; (+++):number of studies with consistent outcome ≥3, * the evaluation for biomarkers in bold, ç predictive value,† Sensitivity/specificity, ‡ only significant biomarker(s) used in ranking level of evidence, ↑= increased ↓=decreased

Table 2: summary of proteins biomarkers for TSCC

Tested proteins	Sample size	Sample type	Significant biomarker ‡	Expression	Potential clinical use	Level of evidence (LoE) ‡	References
Group A studies							
Bmi-1, c-myc, and Snail	73	Tissue	**Bmi-1**	↓	Poor prognosis	+	[40]
Foxp3	81	Tissue	**Foxp3 ***	↑	Poor prognosis	+	[41]
RCAS1	49	Tissue	**RCAS1**	↑	Not prognosticator	-	[42]
Metallothionein	49	Tissue	**Metallothionein**	↑	Good prognosis	+	[43]
HDAC-1 and -2	49	Tissue	**HDAC-1**	↑	Not prognosticator	-	[44]
TRB3&p-AKT	128	Tissue	**TRB3& p-AKT ***	↑ ↓	Good prognosis,	+	[45]
MMP-2, MMP-8, MMP-9,& MMP-13	73	Tissue	**MMP-13**	↑	poor prognosis (Invasion depth & tumor size)	+	[46]
GOLPH3	179	Tissue	**GOLPH3***	↑	poor prognosis	+	[47]
FAK and Src	48	Tissue	**FAK and Src**	↑	Not Prognosticator	-	[48]
TLR5	119	Tissue	**TLR5**	↑	Poor prognosis	+	[49]
AEG-1	93	Tissue	**AEG-1***	↑	Poor prognosis	+	[50]
EZH2 &Ki-67	84	Tissue	**EZH2***	↑	poor prognosis	+	[51]
BATF2	202	Tissue	***BATF2**	↓	poor prognosis	+	[52]
FLOT1	181	Tissue	***FLOT1**	↑	Poor prognosis	+	[53]
Eph-A1, -A2, -A4 and -A7	37	Tissue	**Eph –A7**	↑	Good prognosis	+	[54]
LAT1, ASCT2, xCT, 4F2hc &Ki-67	85	Tissue	**LAT1**	↑	Poor prognosis	+	[55]
α –SMA, N-cadherin, vimentin, & LYVE-1	50	Tissue	**α –SMA**	↑	Poor prognosis	+	[56]
p16	167	Tissue	**p16**	↑	Poor prognosis	+	[57]
t-ERK1 and p-ERK1/2	47	Tissue	**p-ERK1/2**	↑	Poor prognosis	+	[58]
PKM2 & LDH5	63	Tissue	**PKM2 & LDH5**	↑	Poor prognosis	+	[59]
LSD1 &Ki-67	63	Tissue	**LSD1***	↑	Poor prognosis	+	[60]
ZEB1 and CA9	84	Tissue	**ZEB1 and CA9***	↑	Poor prognosis	+	[61]
CAFs & Activin A	110	Tissue	**Activin A**	↑	Poor prognosis	+	[62]
MMP2&MMP9	59	Tissue	**MMP9**	↑	Poor prognosis	+	[63]
CAF	178	Tissue	**CAF**	↑	Poor prognosis	+	[64]
Foxc2	61	Tissue	**Foxc2***	↑	Poor prognosis	+	[65]
RKIP	85	Tissue	**PKIP**	↓	poor prognosis	+	[66]
MMP13& TLR9	195	Tissue	**TLR9**	↑	Poor prognosis	+	[67]

VEGF-C& VEGF-A	90	Tissue	**VEGF-C**	↑	Poor prognosis	+	[68]
VEGF-C, VEGFR-3 and podoplanin	65	Tissue	**VEGF-C/VEGFR-3**	↑	Not prognosticator	-	[69]
CB1R and CB2R	28	Tissue	**CB1R**	↑	Good prognosis	+	[70]
VEGF-C, VEGFR-3, CCR7, Nrp1,2 ,MVD,LVD& SEMA3E	80	Tissue	**Nrp1**	↑	Poor prognosis	+	[71]
Securin	93	Tissue	**Securin**	↑	Not prognosticator	_	[72]
HMGA2, Snail, E-cadherin and Vimentin	60	Tissue	***HMGA2**	↑	Poor prognosis	+	[73]
HK2	137	Tissue	**HK2 ***	↑	Poor prognosis	+	[74]
SUZ12	72	Tissue	**SUZ 12**	↑	Poor prognosis	+	[75]
pEGFR	48	Tissue	**pEGFR**	↑	Good prognosis	+	[76]
HA & EGFR†	64	Tissue	**HA**	↑	Poor prognosis	+	[77]
Nrp2, VEGFC, VEGFR3, and Sema3F††	88	Tissue	**Nrp2**	↑	Poor prognosis	+	[78]
Group B studies							
SIP1 & E-cadherin	37	Tissue	SIP1**& E-cadherin**	↑&↓	Poor prognosis (Delayed neck metastasis)	++	[79]
Snai1,Snai2, E-cadherin &vimentin	53+76 (129)	Tissue	**E-cadherin & vimentin**	↓&↑	Poor prognosis		[80]
CXCR4, CXCR12, CA9, E-cadherin & vimentin	47	Tissue	**Vimentin**	↑	poorer prognosis		[81]
Snail, Twist, E-cadherin, and Ncadherin, &vimentin	248	Tissue	**Vimentin**	↑	Poor prognosis	+++	[82]
HIF-1 **α**,HIF-2α TWIST2 and SNIP1	89	Tissue	**HIF-1 α,** TWIST2 & SNIP1	↑	Poor prognosis		[83]
CypA, CD147, HIF-1 α, VEGF-A and VEGF-C	80	Tissue	**HIF-1 α**	↑	Poor prognosis	+++	[84]
HIF-1 **α**, CA-9, GLUT-1, and EPOR	33	Tissue	**HIF-1 α**	↑	Poor prognosis		[85]
HIF-1α & VEGF	49	Tissue	**HIF-1 α ***	↑	poor prognosis		[86]
SOX2	82	Tissue	**SOX2**	↑	Poor Prognosis	++	[87]
ALDH1, CD44, OCT4 & SOX2	66	Tissue	**SOX2**	↑	Poor prognosis		[88]

Abbreviation: (-): no significant association between biomarker and clinical value**,** (+): number of studies with statistical significant outcome = 1; (++): number of studies with consistent outcome = 2; (+++):number of studies with consistent outcome ≥3, ‡ only significant biomarker(s) used in ranking level of evidence, ↑= increased ↓=decreased, * studies confirmed by mRNA, † Electronically published in March, †† Electronically published in June,

Table 3: summary of long non- coding RNAs biomarkers for TSCC

Studied biomarkers	Sample Type	Sample size	Significant biomarkers ‡	Detection method	Expression	Clinical implication	Level of evidence (LoE) ‡	References
Group A studies								
lncRNA UCA1	Tissue	94	**lncRNA UCA1**	qRT–PCR ä	↑	Poor prognosis (Increased risk metastasis)	+	[89]
lnc-AL355149.1-1,lnc-PPP2R4-5, lnc-SPRR2D-1, **lnc-MBL2-4:3**,lnc-MAN1A2-1,lnc-FAM46A-1, lnc-STXBP5-1, lnc-MBL2-4:1	Tissue	32	**lnc-AL355149.1-1** **lnc-MBL2-4:3**	qRT–PCR	↓ ↑	Advanced T stages Poor prognosis (Increased risk metastasis)	+ +	[90]
lncRNA MEG3	Tissue	76	**lncRNA MEG3**	qRT-PCR	↓	Poor Prognosis	+	[91]
lncRNA HOTTIP	Tissue	86	**lncRNA HOTTIP**	qRT-PCR	↑	Poor Prognosis	+	[92]
lncRNA NKILA	Tissue	96	**lncRNA (NKILA)**	qRT-PCR	↓	Poor prognosis (Increased risk metastasis)	+	[93]
lncRNA TUG1	Tissue	27	**lncRNA (TUG1)**	qRT-PCR	↑	Detection	+	[94]
lncRNA TUC338	Tissue	25	**lncRNA TUC338**	qRT-PCR	↑	Enhanced proliferation	+	[95]
lnc RNA 152 (**LINC00152**	Tissue	15 182 } 197	**LINC00152**	qRT-PCR& in situ hybridization	↑	Detection& prognosis	+	[96]
lncRNA 673 (LINC00673)	Tissue	202 15 } 217	**LINC00673**	qRT-PCR	↑	Poor prognosis (Increased risk metastasis)	+	[97]
Group B studies								
MALAT1	Tissue & cell lines (CAL27 and SCC-25)	127	**MALAT1**	qRT-PCR	↑	Poor prognosis (Increased risk metastasis)	++	[98]
MALAT1	tongue cancer cell lines and tissue	30	**MALAT1**	qRT-PCR	↑	Poor prognosis (Increased risk metastasis)		[99]

Abbreviation: (-): no significant association between biomarker and clinical value, (+): number of studies with statistical significant outcome = 1; (++): number of studies with consistent outcome = 2; (+++):number of studies with consistent outcome ≥3, ‡ only significant biomarker(s) used in ranking level of evidence, ↑= increased ↓=decreased, ä qRT–PCR: quantitative real-time polymerase chain reaction

Table 4 : summary of DNA biomarkers for TSCC

Studied biomarkers	Sample size	method	Significant biomarkers‡	Type of mutation	Potential clinical use	Prevalence	Level of evidence (LoE) ‡	References
Group A studies								
TP53	31	FISH	**TP53**	CNV (deletion)	Field cancerization	late-stage tumors 75%	+	[100]
CCND1	23	FISH	**CCND1**	CNV(amplification)	poor prognosis	13 (56.5%)	+	[101]
CCND1	22	FISH	**CCND1**	CNV(Amplification)	Not prognosticator	2 (9.1%)	-	[102]
7q21	16	CGH	**7q21**	copy number gain	metastatic	44 %	+	[103]
MMP-1 -1607 1G/2G and IL-8 -251 A/T	69	FISH	**MMP-1 2G/2G & IL-8 A/A**	SNP in the promoter region	progression & recurrence	38 (%53.6) 8 (%11.6)	+	[104]
Her-2/neu	40	FISH	**Her-2/neu**	CNV	Not prognosticator	1 (2.5%)	-	[105]
EGFR	78	FISH	**EGFR**	CNV	Not prognosticator (no correlation with survival)	35 (54%)	-	[106]
FADD	30	RT-PCR	**FADD**	CNV (Amplification)	Poor differentiation	13 (44.3%)	+	[107]
Telomeres	24	Q-FISH	**Telomeres**	Shortening	field cancerization	----	+	[108]
WIF1 and RUNX3 methylation	76	nested methylation specific PCR method	**RUNX3**	Promotor hypermethylation	Poor prognosis (lymph node involvement)	25%	+	[109]
EGFR	89	FISH	**EGFR**	CNV	Poor prognosis	32 (36%)	+	[38]
FGFR1	123	FISH	**FGFR1**	(amplification) VCN	Not prognosticator	9.3 %	_	[110]
Survivin gene	91	PCR	**Allele C**	Polymorphism at -31 G/C	Advanced stage	23% in T1 and 48% with larger tumor	+	[111]
TP53	115	PCR-RFLP , allele specific PCR & Sanger sequencing	**Pro72 allele**	Pro72Arg polymorphism	high risk of cancer	44 (38.3%)	+	[112]
TP53, STK11, MET, PIK3CA, BRAF and NRF2	66	Sequenom multiplexed LungCarta panel	**MET**	missense	poor loco-regional recurrence	10.6% (11)	+	[113]
CDKN2A	131	Sanger sequencing, FISH & Methylation-sensitive high resolution melting	**CDKN2A**	CNV missense & promotor methylation	Not prognosticator	------	-	[114]
8q11.21, 8q12.2-3, and 8q21.3 , 22q11.23, 16p11.2 & 20q11.2	10	High density SNP array	**20q11.2**	CNV (gain)	metastasis	50%	+	[115]
ACTN4 (protein name: actinin-4)	54	FISH	**ACTN4**	CNV (amplification)	Poor prognosis	6 (12.5%)	+	[116]
Group B studies								
TP53	39	SSCP	**TP53**	Deletion	Poor prognosis (advanced stage &high grade)	21 (54%)		[117]
TP53 & CDKN2A	51	PCR, and direct sequencing on 3730xl DNA Analyzer	**TP53 & CDKN2A**	Point mutation	Poor prognosis	10 (19.6%). 4 (7.8%)	+++	[118]
FHIT, EGFR,LOH, TP53 DNA binding domain	121	Bidirectional sequencing, MSI &LOH analysis	**TP53 DNA binding domain**	Point mutation	Poor prognosis	18%		[119]
TP53 & NOTCH1ẍ	50	exome sequencing, SNP genotyping, CNVs & LOH	**TP53 & NOTCH1**	----	poor prognosis (nodal metastasis) poor prognosis (Poorly differentiated tumor)	38 % 4 %		[120]
NOTCH ẍẍ	60	whole-exome & targeted deep sequencing	**NOTCH1**	Point mutation	poor prognosis (high recurrence)	5 %	++	[121]

Abbreviation:(-): no significant association between biomarker and clinical value, (+): number of studies with statistical significant outcome = 1; (++): number of studies with consistent outcome = 2; (+++):number of studies with consistent outcome ≥3, ‡ only significant biomarker(s) used in ranking level of evidence, ↑= increased ↓=decreased, ẍ: several genes were included in this integrated analysis, ẍ ẍ: mutational landscape analysis, Q-FISH: Quantitative Fluorescent in situ hybridization, RT-PCR: real time polymerase chain reaction, CGH: Comparative genomic hybridization, Sequenom multiplexed LungCarta panel: panel of assays for somatic mutation profiling, SNP: single nucleotide polymorphism, SSCP: single stranded conformation polymorphism, MSI: Microsatellite instability. LOH: loss of heterozygosity, CNV: copy number variation,

Supplementary table 1: Evaluation of the included studies based on REMARK criteria

		REMARK																							
		Introduction	Materials and Methods											Results								Discussion			
			Patiens		Specimen cha	Assay methods					Statistical analysis			Data		Analysis and presentation									
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20				
Biomark	Ref	State the mark	Describe	Describe	Describe type	Specify t	State the	Precisely	List all ca	Giverati	Specify al	Clarify h	Describe	Report di	Show the	Present u	For key	Amongr	If done, r	Interpret	Discuss	Score	Quality		
AML 70	[24]	1	.5	0	1	.5	.5	1	1	0	.5	1	0	.5	.5	1	0	0	1	.5	0	10.5	Average		
Adipo	[25]	1	.5	0	.5	.5	.5	1	1	0	.5	.5	0	.5	1	1	1	.5	0	1	1	12	Average		
Syn	[26]	1	.5	0	1	.5	.5	1	1	0	.5	.5	0	1	.5	.5	0	0	0	1	1	10.5	Average		
prol 20	[27]	1	.5	.5	1	.5	.5	1	1	0	.5	1	0	.5	.5	1	0	0	1	1	1	12.5	Average		
prol52	[28]	1	.5	.5	1	.5	.5	1	1	0	.5	1	0	.5	.5	1	0	0	0	1	1	11.5	Average		
Prol37	[29]	1	.5	.5	1	.5	.5	1	1	0	.5	1	0	.5	.5	1	0	0	0	1	1	11.5	Average		
Prol99	[30]	1	.5	.5	1	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11.5	Average		
Bmi	[40]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	0	0	1	1	12.5	Average		
Fox3	[41]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	0	0	1	1	12.5	Average		
RCAS	[42]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
Metallotl	[43]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
HDAC-1	[44]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
TRB3	[45]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average		
MMP-13	[46]	1	1	.5	.5	.5	.5	1	1	0	.5	1	.5	.5	1	.5	0	0	0	1	1	12	Average		
GOLPH3	[47]	1	1	1	.5	.5	.5	1	1	0	.5	1	.5	.5	1	.5	1	0	0	1	1	13.5	Average		
FAK and	[48]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	0	0	0	1	1	12	Average		
TLR5	[49]	1	.5	.5	.5	.5	1	1	1	0	.5	1	0	1	1	0	1	1	0	1	1	13.5	Average		
AEG-1	[50]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13	Average		
EZH2	[51]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
BATF2	[52]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
FLOT1	[53]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11.5	Average		
Eph-A7	[54]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11.5	Average		
LAT1	[55]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	1	0	1	1	13	Average		
α –SMA	[56]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
p16	[57]	1	1	1	.5	.5	1	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	15	Average		
p-ERK1/	[58]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
PKM2 &	[59]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
LSD1	[60]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	0	0	1	1	12.5	Average		
ZEB1 an	[61]	1	.5	0	1	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average		
Activin A	[62]	1	.5	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
MMP9	[63]	1	1	.5	1	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	1	1	1	13	Average		
CAF	[64]	1	.5	.5	.5	.5	.5	1	1	0	.5	.5	0	.5	1	.5	0	0	0	1	1	10.5	Average		
Foxc2	[65]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
PKIP	[66]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
TLR9	[67]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	0	0	1	1	12	Average		
VEGF-C	[68]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	0	0	1	1	12.5	Average		
VEGF-C	[69]	1	1	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14.5	Average		
CB1R	[70]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
Nrp1	[71]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	0	0	1	1	12	Average		
Securin	[72]	1	1	0	1	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	0	0	1	1	12.5	Average		
*HMGA2	[73]	1	.5	0	1	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
HK2	[74]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	1	0	1	1	13	Average		
SUZ12	[75]	1	1	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
pEGFR	[76]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
HA	[77]	1	1	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	0	0	1	1	13	Average		
Nrp2	[78]	1	1	1	.5	.5	1	1	1	0	.5	1	0	.5	1	1	0	0	1	1	1	14	Average		
E-cadher	[79]	1	1	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	1	0	1	1	13.5	Average		
E-cadher	[80]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
Vimentin	[81]	1	1	.5	.5	.5	1	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	12	Average		
Vimentin	[82]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13	Average		
HIF-1 α	[83]	1	1	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	.5	0	1	1	13	Average		
HIF-1 α	[84]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	.5	0	1	1	12.5	Average		
HIF-1 α	[85]	1	1	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	.5	0	1	1	13.5	Average		
HIF-1 α	[86]	1	.5	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	.5	0	1	1	14.5	Average		
SOX2	[87]	1	.5	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	14	Average		
SOX2	[88]	1	.5	0	1	.5	.5	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	13.5	Average		
UCA1	[89]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	0	0	0	0	1	1	10.5	Average		
lnc-AL35	[90]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	.5	1	0	0	0	0	1	1	10	Average		
MEG3	[91]	1	.5	1	1	.5	1	1	1	0	.5	1	1	.5	1	.5	1	1	0	1	1	15.5	High		
HOTTIP	[92]	1	.5	1	1	.5	1	1	1	0	.5	1	0	.5	1	1	1	1	0	1	1	15	High		
NKILA	[93]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	.5	.5	1	1	1	0	1	1	12.5	Average		

TUC338	[95]	1	.5	.5	1	.5	.5	1	1	0	.5	1	0	.5	0	.5	0	0	0	1	1	10.5	Average
LINC001	[96]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average
LINC006	[97]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average
MALAT	[98]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	0	0	0	0	0	1	1	9.5	Average
MALAT	[99]	1	.5	.5	.5	.5	0	1	0	0	.5	1	0	.5	0	.5	0	0	0	1	1	8.5	Average
CCND1	[101]	1	.5	1	1	.5	.5	1	1	0	.5	1	0	.5	.5	0	0	0	0	1	1	11	Average
CCND1	[102]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average
7q21	[103]	1	1	.5	.5	.5	.5	1	.5	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average
MMP	[104]	1	1	1	.5	.5	.5	1	1	0	.5	1	0	.5	1	1	1	0	0	1	1	13.5	Average
Her-2	[105]	1	.5	.5	.5	.5	1	1	0	0	.5	1	0	.5	0	.5	0	0	0	1	1	9.5	Average
EGFR	[106]	1	.5	1	1	.5	1	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	12.5	Average
FADD	[107]	1	.5	.5	1	.5	1	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	12	Average
RUNX3	[109]	1	.5	.5	.5	1	.5	1	1	0	.5	1	0	.5	1	1	1	0	0	1	1	13	Average
EGFR	[38]	1	.5	.5	.5	.5	.5	1	1	0	.5	1	0	.5	1	.5	0	0	0	1	1	11	Average
FGFR1	[110]	1	.5	0	.5	.5	.5	1	1	0	.5	1	0	1	1	.5	0	0	0	1	1	11	Average
AlleleC	[111]	1	.5	0	.5	.5	.5	.5	0	0	.5	.5	0	.5	1	.5	0	0	0	1	1	8.5	Average
MET	[113]	1	1	.5	1	.5	.5	1	1	0	.5	1	0	1	1	.5	0	0	0	1	1	12.5	Average
CDKN2A	[114]	1	1	1	.5	.5	1	1	1	0	.5	1	0	1	1	.5	0	0	0	1	1	13	Average
20q11.2	[115]	1	.5	.5	.5	.5	.5	1	0	0	.5	0	0	.5	1	.5	0	0	0	1	1	9	Average
ACTN4	[116]	1	1	1	.5	.5	1	1	1	0	.5	.5	0	.5	1	1	1	1	0	1	1	14.5	Average
TP53	[117]	1	1	1	.5	.5	.5	1	1	0	0	.5	0	.5	1	.5	0	0	0	1	1	11	Average
TP53 & C	[118]	1	1	1	.5	.5	1	1	1	0	.5	.5	0	.5	1	1	1	1	0	1	1	14.5	Average
TP53	[119]	1	.5	0	1	.5	1	1	1	0	.5	.5	0	.5	1	.5	0	0	0	1	1	11	Average
TP53ä	[120]																						
NOTCH	[121]	1	1	1	1	.5	.5	1	1	0	.5	1	0	.5	1	.5	1	0	0	1	1	13.5	Average

ä: was not evaluated because we could not get the additional files that contain the clinical information

Supplementary table 2: Evaluation of the included studies based on STARD criteria

		STARD 2015																																				
		TITLE/ABSTRACT/ KEYWORDS		Introduction		Methods																	Results											Discussion		Other info				
						Study design	Participants				Test Methods							Analysis																						
		1	2	3	4	5	6	7	8	9	10a	10b	11	12a	12b	13a	13b	14	15	16	17	18	19	20	21a	21b	22	23	24	25	26	27	28	29	30		
Biomarker	**Ref**																																			**Score**	**Quality**
SCCA-1	[19]	0	.5	1	1	.5	0	1	0	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	6.5	Low
CA125, CA19-9, TPS, CEA, SCC, & Cyfra 21-1	[20]	1	1	1	1	.5	0	1	.5	0	1	0	0	0	0	0	0	1	.5	0	1	0	0	1	1	0	0	0	.5	0	0	1	0	0	0	13	Average
Adenosine deaminase	[21]	0	.5	1	1	.5	1	1	.5	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	10	Average
IL-1a, IL-6, IL-8	[31]	0	.5	1	1	.5	1	1	.5	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	10	Average
COL5A1, ABCG1	[22]	1	.5	1	1	.5	0	1	0	0	1	0	0	0	0	0	0	1	.5	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	1	12.5	average
IL-6	[23]	1	1	1	1	.5	1	1	.5	0	1	0	0	0	0	0	0	1	.5	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	13.5	Average
lncRNA TUG1	[94]	0	1	1	1	.5	0	1	1	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	9	low
TP53	[100]	0	1	1	1	.5	0	1	0	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	9	low
Telomer	[108]	0	.5	1	1	.5	0	1	0	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	7.5	low
Pro72	[112]	0	1	1	1	.5	0	1	1	0	1	0	0	0	0	0	0	0	.5	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	11	average

Item:	
1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)
2	Structured summary of study design, methods, results, and conclusions
3	Scientific and clinical background, including the intended use and clinical role of the index test
4	Study objectives and hypotheses
5	Whether data collection was planned before the index test and reference standard
	were performed (prospective study) or after (retrospective study)
6	Eligibility criteria
7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)
8	Where and when potentially eligible participants were identified (setting, location and dates)
9	Whether participants formed a consecutive, random or convenience series
10a	Index test, in sufficient detail to allow replication
	Reference standard, in sufficient detail to allow replication
11	Rationale for choosing the reference standard (if alternatives exist)
12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory
12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory
13a	Whether clinical information and reference standard results were available to the performers/readers of the index test
13b	Whether clinical information and index test results were available to the assessors of the reference standard
14	Methods for estimating or comparing measures of diagnostic accuracy
15	How indeterminate index test or reference standard results were handled
16	How missing data on the index test and reference standard were handled
17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory
18	Intended sample size and how it was determined
19	Flow of participants, using a diagram
20	Baseline demographic and clinical characteristics of participants
21a	Distribution of severity of disease in those with the target condition
21b	Distribution of alternative diagnoses in those without the target condition
22	Time interval and any clinical interventions between index test and reference standard
23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard
24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)
25	Any adverse events from performing the index test or the reference standard
26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability
27	Implications for practice, including the intended use and clinical role of the index test
28	Registration number and name of registry
29	Where the full study protocol can be accessed
30	Sources of funding and other support; role of funders

CHAPTER 6

PROFILE OF NATIVE AND RADIATION-INDUCED C-MET EXPRESSION IN TONGUE SQUAMOUS CELL CARCINOMA: IS C-MET A POTENTIAL CANDIDATE FOR TARGETED THERAPY APPROACHES?

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Abstract

Background: Nanoparticle-mediated drug delivery via tumor cell-selective surface receptors is a novel strategy that may significantly improve safety and efficacy of cancer therapeutic molecules, particularly in combination treatment modalities. c-Met has been investigated in several tumors and a five-fold increase in its expression after irradiation was observed. We assessed whether c-Met is likewise abundantly expressed in tongue squamous cell carcinoma (TSCC), with special emphasis on surface expression to allow nanoparticle targeting.

Methods: Six TSCC cell lines were exposed to therapeutic dosages of gamma radiation. C-Met protein levels were assessed over time by western blot and flow cytometry. Radio sensitivity of the cells was determined by viability assay. Gene expression, cell cycle, and wound healing assays were also used to better understand c-Met functions.

Results: Abundant c-Met total protein was present in all cell lines, however, on average only 8.3% of the cells displayed c-MET expression on their surface. Abrupt downregulation of c-Met surface expression occurred one hour after radiation in all but one cell line (4.5% including, 1.8% without this cell line), however, surface expression returned to similar levels (10.5%) 48h post-radiation in all cell lines. Intracellularly, the highest level of expression was found at day 5 after radiation exposure. C-Met mRNA expression increased 24h post-radiation. No c-Met ligand production by the cell lines and consequently no phosphorylation of c-Met was observed. Expression levels of c-Met protein appeared to correlate with enhancement of proliferation and invasion ability , but no correlation was found between radiation resistance and c-MET expression levels.

Conclusion: These results provide novel insights into the dynamic changes in the intracellular and extracellular c-Met profiles in native and radiation-exposed TSCC cells. Unfortunately, the relatively low surface expression percentages disfavor the use of c-Met for nanoparticle targeting, and shows the importance of surface expression analysis of cancer targeting candidates prior to developing targeted therapies based on total protein analysis.

Key words: Tongue squamous cell carcinoma (TSCC) , c-Met expression level, radiation effect, targeted drug delivery.

Abbreviation: Tongue squamous cell carcinoma (TSCC), mesenchymal-epithelial transition factor (c-Met), epidermal growth factor receptor (EGFR), phosphorylation c-Met (p-Met)

Introduction

Mobile tongue is the most commonly involved site for carcinoma in the oral cavity (1). Lately, most cancer registries in the western world have reported a marked increase in the incidence rate of this type of carcinoma, especially among young individuals. Worryingly, mobile tongue cancer characterizes by an aggressive clinical behavior, in which 40% of all patients already have cervical lymph node metastasis at initial diagnosis (2). This, indeed, is one of the reasons for treatment failure and unsatisfactory survival so far.

Radiotherapy is an important modality used for patients with tongue squamous cell carcinoma (TSCC) as a part of their primary treatment and has shown a success rate similar to surgery when the disease in stage I and II, though no clinical trial has made a direct comparison between them yet (3). Additionally, for patients with locally advanced lesions in stages III and IV, radiation along with chemotherapy are major components of the treatment modality to control the disease progress (4). Thus far, these conventional treatments are not efficient enough and often fail to eradicate the cancerous cells. One reason is that although higher dosages of radiation and/or medications would be necessary to kill the neoplastic cells, this is in practice not possible because increasing doses will ultimately cause irreversible damages to the normal tissues and deteriorate the patients quality of life (5). Hence, targeted drug delivery is a potential solution to improve the efficacy and safety of cancer therapeutic molecules. One of the potential cellular surface receptors that could be suitable for use in targeting drug delivery in TSCC is mesenchymal-epithelial transition factor (c-Met). Strikingly, in head and neck cancer, increased c-Met expression has been reported in 52%-68% of cases (6). Furthermore, the overexpression of this receptor specifically in TSCC has been shown to correlate with enhancement of *in vivo* and *in vitro* metastasis (7).

An important dimension of c-Met biological feature was a five-fold increase in its expression after exposure to ionized radiation in a set of cell lines from several solid tumors, including breast, lung, colon, and prostate carcinoma (8). As far as we know, this has not yet been determined for TSCC. Our central aim in this study was to enrich the knowledge about this transmembrane receptor by identifying the radiation response of c-Met, its subsequent dynamic changes in the sub compartmentalization (intracellular or within the cell membrane) of the receptor, its phosphorylation and cellular ligand (hepatocyte growth factor, HGF) production, and the relation between c-Met expression and proliferation and invasive behavior. Together, this will shed light on

whether c-Met may be used for a promising targeted drug delivery for TSCC and if so, what would be the most optimal time frame to apply c-Met targeted therapy as an adjuvant therapy to radiation treatment.

Materials and methods

Cell lines, culture conditions and irradiation

Six human tongue carcinoma cell lines (Cal-27, SCC-25, SCC-15, VU-SCC-120, VU-SCC-040, UM-SCC-47) were used in this study. The first three cell lines were purchased from ATCC, while the others [VU-SCC-120, VU-SCC-040 and UM-SCC-47 (HPV-positive)] were kindly provided by the Prof. Brakenhoff lab (Cancer Center Amsterdam, The Netherlands). SCC-25 and SCC-15 were routinely grown in Dulbecco Modified Eagle Medium (DMEM) and Ham's F-12, supplemented with 10% fetal bovine serum, 400-ng/mL hydrocortisone, penicillin (100 IU/mL), and streptomycin (100 µg/mL). The other four tumor cells (Cal-27, VU-SCC-120, UM-SCC-040, and UM-SCC-47) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin. Cells were incubated at 37˚C in a humidified incubator containing 5% CO2 and passaged at exponential growth prior to confluence.

Cells were irradiated at room temperature in a Gammacell® 220 Research Irradiator (MDS Nordion, Ontario, Canada) at doses varying from 2 to 6 gray (Gy).

Western blot analysis

Cells were plated in 6-well plates and irradiated at a dose of 4 Gy, then protein was extracted from the cells using RIPA bufferlysis before radiation, 1-hour, 24-hours, 48-hours, and 5 days post-radiation. Protein concentrations were measured with BCA protein Assay Kit (Pierce Chemical Co., USA), and 20µg from each sample was separated on a SDS-PAGE gel and transferred to a PVDF membrane by electroblotting. After blocking the membrane with 5% nonfat dry milk in TBS with Tween, it was incubated with the primary antibodies: Rabbit- anti MET (1:1000 ; Cell Signaling, #8198), mouse-anti EGFR (1:1000; Santa Cruz, sc-373746), rabbit- anti phospho MET (Tyr1234/1235) (1:1000; Cell Signaling Technology, #3077), and mouse and rabbit anti- β-actin (1:1000 Abcam, ab8277 and ab6709). Subsequently, the membrane incubated with secondary goat-anti-mouse and goat-anti-rabbit immunoglobulins (IRDye 680RD and 800 CW ; Li-Cor Biosciences). Detection bound antibodies were analyzed with an Odyssey infra-red imaging system (Li-Cor Biosciences).

To evaluate the potential of c-Met to be phosphorylated in response to hepatocyte growth factor (HGF), the cell line VU-SCC-120 was randomly selected, seeded and treated as previously described. Cells were stimulated with 50 ng/ml HGF (Thermo Fisher Scientific, Ghent, Belgium) during 10 min immediately before lysis. Subsequently, 20 µg of protein was size-separated on a SDS-PAGE gel and transferred to a PVDF membrane by electroblotting. After blocking the membrane in 5% nonfat dry milk in TBS with Tween, it was incubated with the primary antibodies: rabbit- anti phospho MET (Tyr1234/1235) (1:1000; Cell Signaling Technology, #3077). Detection was analyzed as previously described.

Flow cytometry

Intracellular c-Met detection

Intracellular c-MET expression was determined by first plating cells in 6-wells plates ($1x10^6$ cells/well). Twenty-four hours later the cells were irradiated with a single dose of 4-Gy. The cells were washed with PBS and harvested at five time intervals (pre-, 1-hour, 24-hours, 48-hours, and 5 days post-radiation) using cell dissociation buffer and collected into tubes containing complete media on ice. Cells were washed 2 times with Cell Staining Buffer, fixed in cold 2% paraformaldehyde for 15 minutes at room temperature, and permeabilized in cell staining buffer containing 0.25% saponin (Sigma-Aldrich) for 30 min at room temperature. After 2 washes with Cell Staining Buffer, cells were incubated with Alexa Fluor 488 conjugated rabbit-anti Met (1:100; Cell Signaling, #8494) for 30 min at 4 °C in the dark. Flow cytometry data were acquired using the BD FACSCelesta and analyzed with FlowJo™ Software (Tree star, Ashland, OR, USA)

Extracellular c-Met detection

For detection of c-Met expression on the cell surface, $1x10^6$ cells were seeded per well of a 6-well tissue culture plate. Twenty-four hours later the cells were irradiated with a single dose of 4-Gy, harvested at five time points (pre-, 1-hour, 24-hours, 48-hours, and 5 days post-radiation) by cell dissociation buffer and collected into tubes containing complete media on ice. After 2 washes with Cell Staining Buffer (PBS with 1% BSA), cells were incubated with Alexa Fluor 488 conjugated rabbit-anti Met (1:100; Cell Signaling, #8494) for 30 min at 4 °C in the dark. Control cells were incubated with secondary antibodies only. The sample data were acquired using (BD FACSCelesta™ flow cytometer (BD Bioscience, USA) and analyzed with FlowJo™ Software (Tree star, Ashland, OR, USA).

RNA isolation and real Time PCR

Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific) and 750ng of total RNA was used for First Strand cDNA using Revert Aid First Strand cDNA Synthesis Kit k1612 (Thermo Fisher Scientific), both according to manufacture instructions.

Real time PCR on 5x diluted cDNA was performed with a Roche LightCycler 480 II device using Cybergreen I Mastermix (Roche). The primers used for analysis are listed in Table 1. Standard dilution method was used for quantification of expression of each gene. Relative gene expression of c-Met and HGF were normalized to normalization factor (NF) of YWHAZ and B2M housekeeping genes according to following equation: NF =√(concentration YWHAZ * concentration B2M).

Table 1. Primer sequences used for PCR

Target gene		Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)
B2M	Forward	5' TCTGGCCTGGAGGCTATCCAG 3'	56	202
	Reverse	5' AGAAAGACCAGTCCTTGCTGAA 3'		
YWHAZ	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	56	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'		
c-Met	Forward	5' GTCCTGCAGTCAATGCCTCTC 3'	56	291
	Reverse	5' GTATTCATCGTGCTCTCACTT 3'		
HGF	Forward	5' TCAGCAAAGACTACCCTAA 3'	56	190
	Reverse	5' CTCCACTTGACATGCTATT 3'		

Table 1. Primers used for the gene expression analyses showing the oligonucleotide sequences, annealing temperature and product size. B2M: Beta-2 microglobulin, YWHAZ: 14-3-3 protein zeta/delta, Met: tyrosine-protein kinase Met, HGF: hepatocyte growth factor.

Viability assay

To determine cell viability, cells were seeded at the optimal density on 96-wells plates, grown for 24 hours, and then the plates were irrated at doses of 2, 4, and 6 Gy. Cell were incubated for 72 hours and then cell viability was assessed using alamar blue (Invitrogen; Thermofischer) according to manufacturer 's instruction. Fluorescence was measured at 540 nm using a Bio Tek Synergy ^{TM} microplate reader (Bio Tek Instruments,lnc., Winooski, VT), and the results were analysed using Graphpad Prism version 8.2.1.

In vitro wound healing assay

Cell migration was investigated using a scratch assay. Duplicate 6-well plates were prepared with each of the six cell lines seeded in one well at a density of 1×10^5/well and grown to confluence in a complete medium. A sterile 200µl-pipette tip was used to make a wound across each cell monolayer. Culture medium was discarded, and the cells were washed three times with PBS to remove the cell debris. Fresh medium was added to the cells, then one plate was exposed to 4 Gy radiation while the other one was used as a control. Multiple photographs were taken at 0 hr and 24 hrs post-radiation under phase contrast microscopy with Zeen software. The efficiency of the wound healing process was determined by calculating the area of the cell gap at the indicated times (0 hr and 24hrs), using ImageJ software. Two images were used for each wound at each experimental point and the experiment was always carried out in duplicate. The results are expressed as percentage of healing at 24 hrs with respect to zero time.

Cell cycle assay

Two cell lines (SCC-15 and VU-SCC-120) were randomly chosen to investigate the impact of radiation on their cell cycle. Cells were seeded and treated with 4 Gy for 24 hours. The next day, cells were washed twice with ice-cold PBS and fixed with 70% ethanol at 4˚C overnight. Then, the cells were incubated in 0.5 ml PBS containing 50 µg/ml RNase A for 30 min at room temperature. After that, PI was added to achieve a final concentration of 200 µg/ml for 30 min on ice in the dark. The resultant suspension was then subjected to flow cytometry analysis using the BD FACSCelesta and analyzed with FCS express V6 (De Novo Software, Ontario, Canada). The percentage of cells in the G0/G1, S and G2/M phases was calculated.

Statistical analyses

Statistical differences were determined by ANOVA and Student's t test and analyzed by GraphPad Prism V8.2.1. Differences were considered statistically significant if P-values were 0.05 or less.

Results

C-Met is upregulated in TSCC

To investigate whether c-Met could be a suitable candidate for targeted nano-particle delivery in TSCC, while sparing normal tissues, we first explored its mRNA expression level from publically available data on a genomic visualization platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) The analysis was performed on 26 mobile TSCC samples and 12 normal tongue cell samples. A significant difference by one-way analysis of variance (ANOVA) was observed in favor of TSCC (Figure 1 A). The details information about microarray analysis and samples can be found at GEO Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9844) (9).

Radiation induced changes in c-Met protein expression

To obtain a comprehensive picture of the c-Met expression level in response to irradiation in the TSCC, we investigated the expression of this protein at three different levels and at different time intervals: (i) the total amount of c-Met protein expressed, (ii) the intracellular c-Met expression, and (iii) the level of cell surface c-Met protein.

Total amount of c-Met protein expressed

Western blot was performed to determine the overall c-Met synthesis after treatment with a single fraction of 4 Gy radiation at 4 time points, along with control (prior to radiation, 1-hour, 24-hours, 48-hours, and 5 days post-radiation). All six TSCC cell lines expressed c-Met prior and after radiation. Figure (1B) shows that after exposure to the radiation and normalization using β-actin levels, four of these cells exhibited strong expression of c-Met protein (Cal-27, SCC-15, VU-SCC-120, and VU-scc-040), while the cell line of SCC-25 exhibited weak expression. It was found that VU-SCC-040 peaked to approximately 1.9 times the level of pre-radiated control cells at 1-hour after radiation, while SCC-15 peaked to 2.1 times of untreated cells at around 24 hours. With regard to the HPV + cell line (UM-SCC-47), the c-Met level remained unchanged up to 48 hours, when it almost doubled, suggesting that radiation influences the expression level of the c-Met.

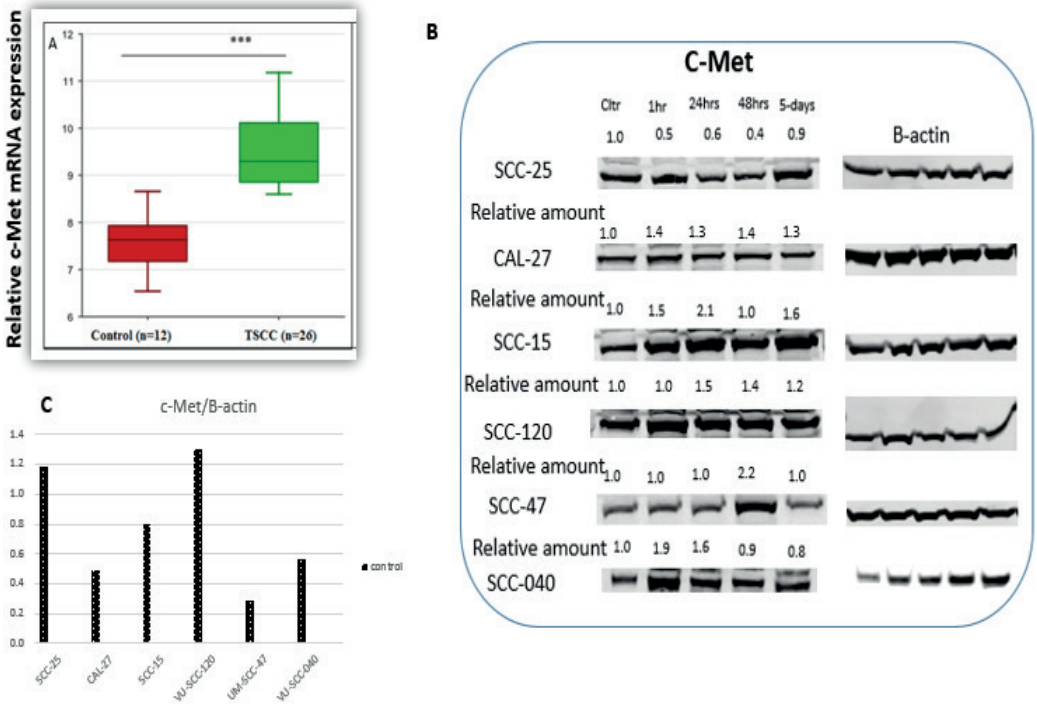

Figure 1: c-Met expression in TSCC prior and after radiation. In (A), we analyzed a public data and found the expression level of c-Met is significantly higher in the tumor than the normal tissue (*** $p < 0.000001$). (B)Time-course changes in c-Met expression in a panel of 6 TSCC cell lines. C-Met is expressed in all cells prior to irradiation. After 4 Gy, relative intensity increases clearly in SCC-15 and VU-SCC-040. (C) Bar graph represents quantification of c-Met protein normalized by β-actin only before irradiation

Intracellular c-MET expression

Flow cytometry analysis of intracellular c-Met expression showed only one population and consequently, median fluorescence intensity (MFI) was used as the qualitative measure for c-Met profiling changes. We observed that the intracellular profile of c-Met in the six cell lines showed a peak induction at around 5 days after exposure to irradiation. Meanwhile, we noticed that the 48-hour time point was the common time between these cell lines where they showed downregulation of c-Met protein, with the exception of Cal-27 cells (Figure 2).

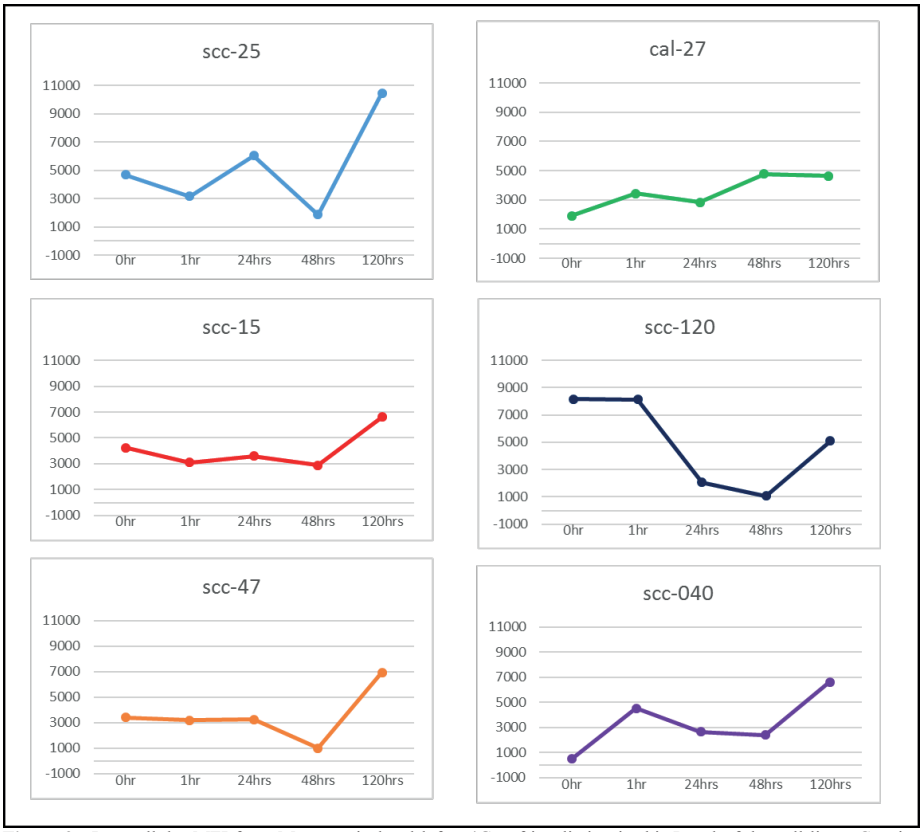

Figure 2: Intracellular MFI for c-Met protein level lafter 4Gy of irradiation in this Panel of the cell lines. Consistant increase is obvious on day 5 time points.

Cell surface detection of c-MET

The panel of cell lines invariably showed two populations (positive and negative c-Met surface expression) on flow cytometry analysis. To gain insight into the dynamic changes of the surface expression levels, we assessed the percentage of cells positive for c-Met on their surface, as well as their mean fluorescence intensity (MFI). In figure 3 we observed that exposure to radiation induced a striking reduction in the percentage of the positive cells at around the 1-hour time point in five of the cell lines, save VU-SCC-120. In fact, in the VU-SCC-120 cell line, 1-hour after irradiation was the time point when we noted the highest percentage of the positive cells and correspondingly the highest MFI value. Interestingly, the percentage of the positive cells for c-Met expressions were more gradually increased in other five cell lines (VU-SCC-040, UM-SCC-47, SCC-25, SCC-15 and Cal-27), and reached a peak at 48-hours time point after radiation exposure. Analogous changes

were detected in the MFI values only in three of the cell lines (Cal-27, VU-SCC-040, and SCC-25). Importantly, we observed for HPV + cell lines (UM-SCC-47) and SCC-15 that the intensity of c-Met protein signals was the highest prior to radiation exposure. Overall, these results point to heterogeneity of the response between the cells. Gating and other details are provided in supplementary data (Figure S1).

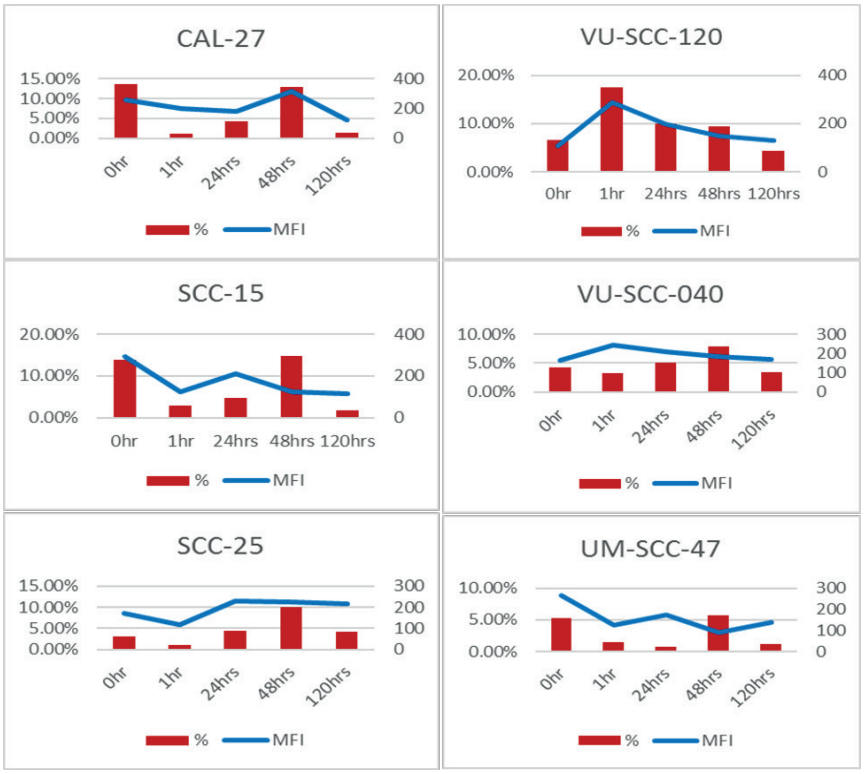

Figure 3: Percentage of the positive cells for c-Met on the cell surface after 4Gy of irradiation in this Panel of the cell lines. Consistant increase in the percentage of the positive cells is obvious at 48-hours time points. One exception is cell line (VU-SCC-120). Corrosponding increase in MFI with higher percenatge of the positive cells at 48-hours is noted for three cell lines (VU-SCC-040, Cal-27, and SCC-25). Regarding VU-SCC-120, 1 hour time point is the only time ,in which upregulation of c-Met expression is observed clearly.

Gradual increase in c-Met mRNA expression level

The noticeable quantitative alterations in the intracellular and extracellular protein expression led us to wonder whether those changes were reflections to modulated localization of this receptor or as a result of mRNA synthesis. Hence, to verify the observed difference in the protein level at different time intervals, we next evaluated the gene expression of c-Met, and its ligand (HGF). Three time intervals in accordance with the time chosen for protein analysis were selected in order to investigate the correlation of gene and protein expression. The results revealed that gene expression was not in accordance with the protein levels at the indicated time points. This may point to non-transcriptional mechanisms underlying protein up/down regulation. Nonetheless, the highest gene expression for the c-Met that was noticed at 24-hours may indicate gradual upregulation of the gene with time after irradiation. However, the opposite results was noticed for the SCC-25, in which the level of the mRNA downregulated significantly from untreated to 1-hour and markedly diminished at 24-hours. The results shown in Figure 4 are representative of at least 3 independent experiments. Together, this might reinforce the idea of internalization of this receptor within the first hours of exposure to radiation, while the late overexpression is more likely to be transcriptionally dependent.

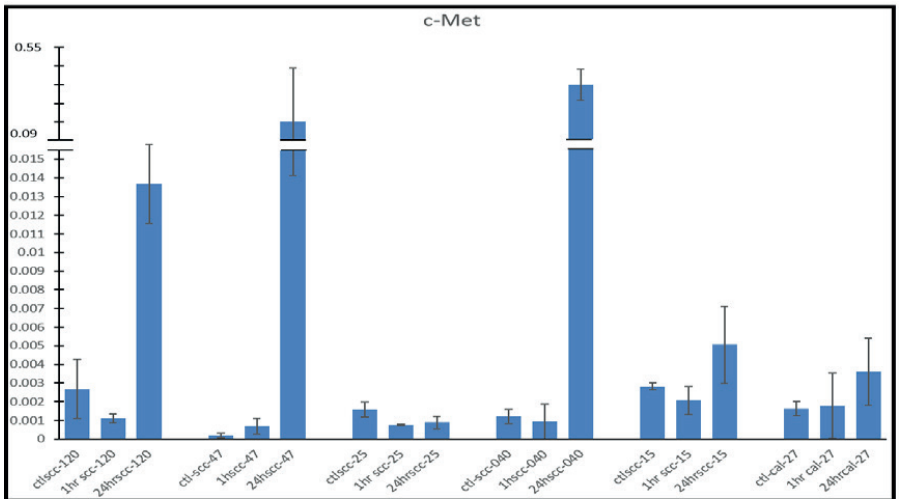

Figure 4: qRT-PCR result for c-Met RNA show increased expression in most cell lines at 24hrs after radiation exposure.

C-Met phosphorylation is functional, but only occurs upon HGF stimulation

c-Met phosphorylation has been reported to induced upon exposure to irradiation in the absence of HGF. We, therefore performed western blot to detect p-Met (Tyr1234/1235) in the absence and

presence of its selective ligand (HGF). We found that lack of the p-Met expression was a constant finding in all cells all time points in case of absence of the ligand (Data not shown). However, Figure 5 shows that on irradiated VU-SCC-120 cells, HGF stimulated more phosphorylation at 24hrs in comparison to the control not radiated cells. Overall, this suggests that presence of the ligand is necessary for functional c-Met in TSCC.

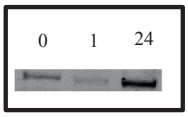

Figure 5: HGF-triggered c-Met tyrosine phosphorylation at three time points for only VU-SCC-120 cell lines. The p-Met overexpression is strongly shown 24-hours after radiation.

HGF is not secreted by TSCC cells

Regarding HGF, there is considerable debate whether the cancer cells secrete this growth factor or it is the function of stromal cells such as fibroblasts. In our analysis, HGF was not expressed by any of the TSCC cell lines, providing further evidence that this growth factors is likely to be secreted by cancer- associated fibroblasts (CAF). Nonetheless, it is also possible that the HGF amount is very small to be detectable by our technique (Data not shown).

Various c-Met expression levels cannot explain innate sensitivity of the cells to the radiation

It is currently accepted that c-Met contributes to acquisition of resistance to radiotherapy in some tumors. To link assessment of the protein in the previous section to the character of the cells either being radio sensitive or resistant, we did viability assays on all cell lines of our panel. In fact, we did not find such a direct link. For instance, in the radioresistant cell lines such as SCC-15 and SCC-25, and relative sensitive VU-SCC-040 and UM-SCC-47, we noticed various patterns of c-Met expression after radiation, indicating and supporting individuality of the cancer cells (Figure 6).

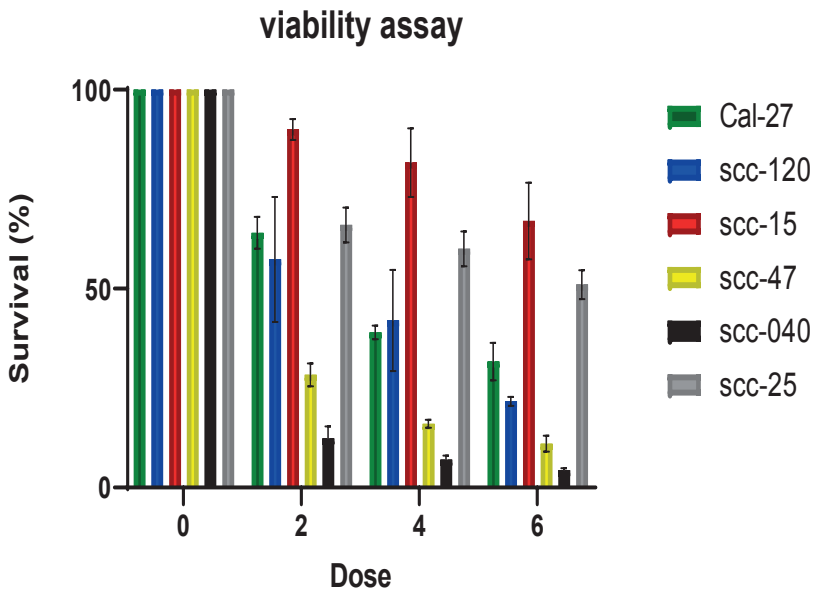

Figure 6: Determination cell viability of panel of TSCC cell lines in response to irradiation by Alamar blue. SCC-15 is the most resistant cell lines and VU-SCC-040 is the most radiosensitive cell line.

Correlation of c-Met expression with its functions

To assess functionality of c-Met, we performed an *in vitro* wound healing assay in four cell lines. In accordance with protein expression, the cell lines with strong overexpression (VU-SCC-120 and VU-SCC-040) covered the wounded area more readily than the cell lines with weak or stable expression of c-Met. This in an indicator how c-Met is a key player in invasion of the TSCC (Figure 7a).

Cell cycle profiling was performed in two cell lines (SCC-15 and VU-SCC-120) to determine whether c-Met expression reprograms the cell cycle. Of note, both of the cell lines were fixed after 1hr of exposure to radiation because these cell lines showed strong expression at that time point as stated above. The data showed an increase in the proportion of the cells at G2/M phase in the radiated cells compared to the control from 19 % to 31% in SCC-15 and from 18 % to 25 % in VU-SCC-120, indicating an enhancing role of this protein for the proliferation (Figure 7b).

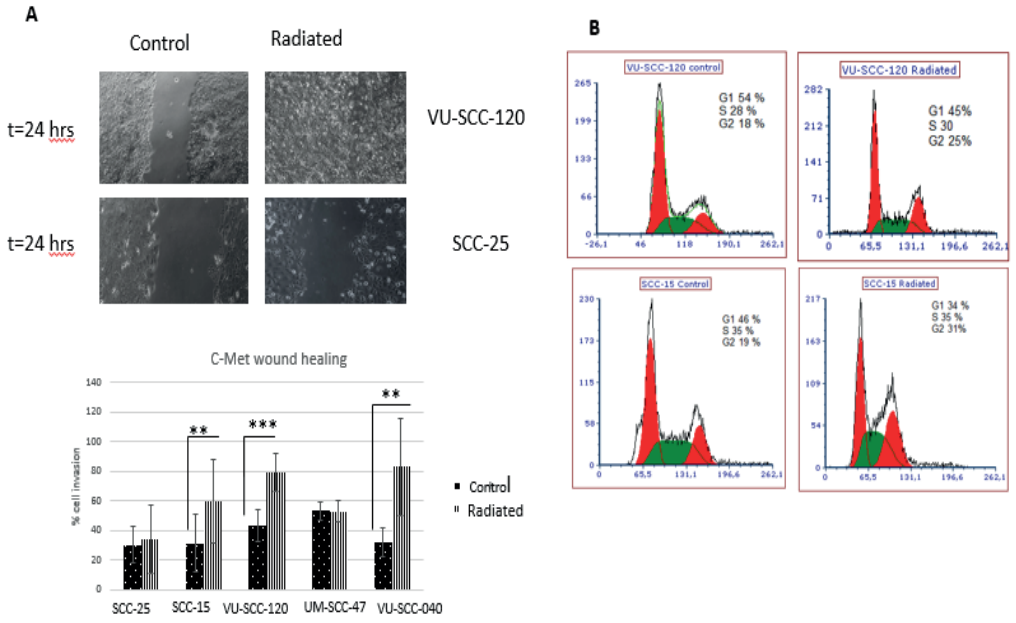

Figure 7: A correlation between expression level of c-Met proteins and its enhancement for invasion (A) and proliferation ability (B). Wound healing assay reveals that the cells show high overall expression are the cells show also higher percentage of invasion ability (VU-SCC-120, VU-SCC-040 AND SCC-15). Representative pictures for the cell line with high invasive potential (VU-SCC-120) and low invasive potential (SCC-25). Bar graphs represent the mean ± S.D. Investigation of the proliferation ability after irradiation through proliferation assay by flow cytometry indicate to the increasing in the proliferation rate upon radiation exposure.

Altered expression level of c-Met and EGFR expression

Importantly, the cross talk between c-Met and EGFR upon exposure to radiation and sometime their co-expression have been suggested by several studies (10-12). In our panel of cell lines, we found in the majority of the cell lines that the highest expression of EGFR was noticed 1hr after radiation, but then decreased to the lowest level at 24hrs. However, it is worth noting that we found a weak expression of EGFR in the most radio sensitive (VU-SCC-040) cell line and strong and sustainable level of its expression in the radio resistant ones (SCC-15, SCC-25 and Cal-27) (Figure 8a).

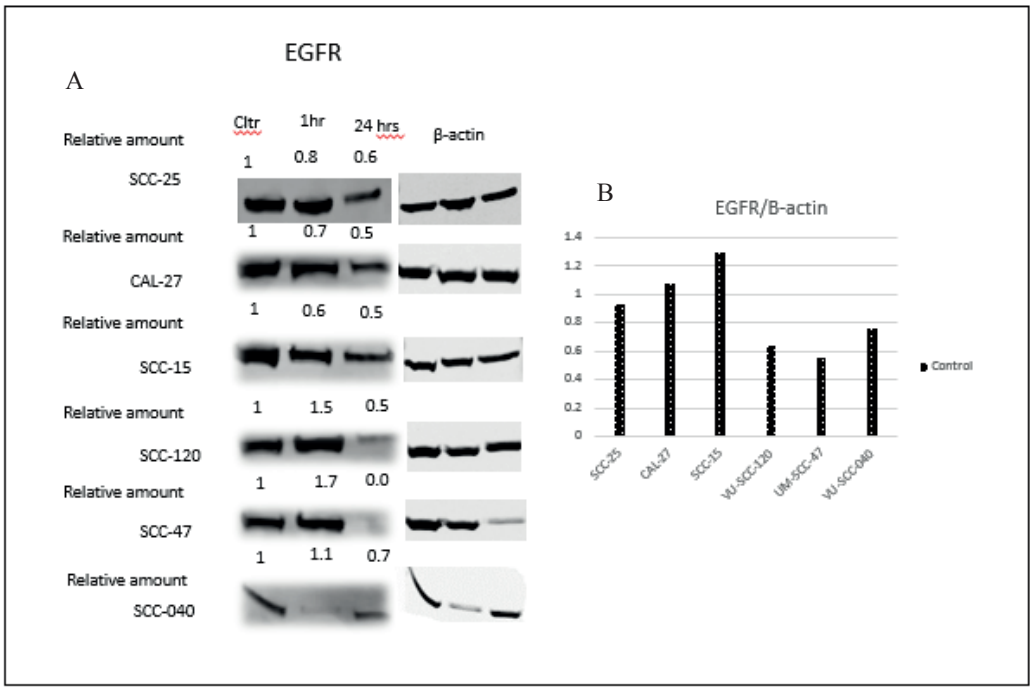

Figure 8: Changes in EGFR expression in the panel of the cell lines before and after 4Gy irradiation. (A) EGFR increases by 1hr and decreases to the lowest level at 24 hrs after irradiation. (B) Bar graph represents quantification of EGFR protein normalized by β-actin only before irradiation

Discussion

Targeted drug delivery using nanotechnology has been designed to allow accumulation of high dosages of medications at tumor sites for superior effect, while sparing healthy tissues for fewer toxicity. Basically, one of the important steps to achieve this is a thorough investigation of targeting surface candidates that are highly expressed on cancer cells when compared to normal cells. Based on published literature data on total protein analysis, c-Met appeared a good candidate for our quest to identify suitable surface markers (13, 14). However, the present study shows that although c-Met is well abundant at the total protein level, the fraction located on the cellular surface is rather low, not inducible and actually strongly declining shortly after radiation treatment, and again at 5 days post-radiation. Moreover, expression levels of c-Met protein appeared to correlate with enhancement of proliferation and invasion ability, but no correlation was found between radiation resistance and c-Met expression levels.

Total c-Met expression, response to radiation

On the basis of our results and measuring the total c-Met production, all tested TSCC cell lines expressed relatively high amount of c-Met protein prior to exposure to the radiation. C-Met is an important tyrosine kinase receptor that plays a significant role in proliferation, migration as well as invasion of tumor cells (15, 16).

Surprisingly, upon exposure to radiation, there was a marked variation in the overall c-Met expression between the selected cell lines. Although results from De Bacco et al. (8) support an evident role of c-Met overexpression in sustaining radioresistance in breast carcinoma and glioma, we could not demonstrate this in our panel of TSCC cell lines. We found that the most radioresistant cell line, SCC-15, and the most radiosensitive cell line, VU-SCC-040 as determined by the viability assays, showed comparable strong c-Met protein upregulation after irradiation. Also the low levels of total c-Met protein in other cell lines such as relatively radioresistant SCC-25 might indicate that c-Met expression is, at least in tongue squamous cell carcinoma, not clearly related with radio resistance.

We determined that synthesis of c-Met mRNA occurred significantly 24-hours after exposure to the radiation. It is interesting to note that a recent study of Jahn and co-authors found in an in vivo model a significant correlation between upregulation of c-Met m RNA and acquisition of epithelial-mesenchymal transition (EMT) phenotypes. Our results confirm this association, in which the highest level of mRNA was observed in the cell lines VU-SCC-040 and VU-SCC-120, for which we found the highest potential of invasion. This is also in accordance with observations by Lim et

al., showing that overexpression of c-Met protein acted directly through activation of matrix metalloproteinase (MMP) 1, 2, and 9 in enhancement of the *in vivo* and *in vitro* TSCC metastasis (7).

Considering the behavior of HPV+ cell line (UM-SCC-47), these cells showed the lowest expression level of c-Met upon exposure to radiation. In fact, these findings do not support an earlier study which found HPV E6 to significantly induce c-Met overexpression through downregulation of wild-type P53 in head and neck cancer (17). However, this is comparable to a study by M.J.Kwon et al. that reported a significant negative association between P16 positivity, which is an indicator of HPV-related head and neck cancer and c-Met overexpression (18). Admittedly, our observation is based on one cell line which is not enough to confirm the result. Notwithstanding, this conflict in results could be partially because of ionized radiation activates or suppresses different biological regulators, including c-Met. Further studies with more HPV+ cell lines are needed.

Intra- and extracellular expression of c-Met, response to radiation

Successful development of a targeted drug delivery requires extensive research particularly on expression levels of the targeted candidates on the cell surface, as well as its expression dynamics on the subcellular level. Therefore, the present study for the first time not only measured overall c-MET production, but also its expression at both intracellular and cell surface locations.

Surprisingly, despite the high overall c-Met expression, we determined that under control circumstances, in all cell lines the percentage of the cells expressing c-Met on their surface did not exceed 20%. Moreover, we observed that (1) a strong reduction in this percentage became evident 1-hour after radiation and again at the 120h time point; and (2) a more or less opposite c-Met expression level was found in the intracellular compartment, with in particular a consistent intracellular accumulation at the late (between 48 and 120 hours) post-radiation period.

The acute phenomenon of downregulation of c-Met surface expression leads us to hypothesize that this might be a result of a progressive internalization of the c-Met receptor into the intracellular compartment. Abrupt removal of the receptor from the cell surface (internalization) is an essential mechanism used by the cells to prevent sustained stimulation. Internalization of receptor tyrosine kinase such as c-Met may be accelerated by ligand binding on the cell surface (19). For this purpose, we first verified that c-Met was functional and could be activated by its ligand, Hepatocyte Growth

Factor (HGF) by studying c-Met phosphorylation upon HGF exposure. Secondly, since so far only fibroblast derived cells were proven to secrete HGF and activate c-Met in a paracrine mechanism for head and neck cancer (20), we assessed whether TSCC cells may themselves produce HGF by analyzing its mRNA expression. We concluded that HGF is neither before nor after exposure to the radiation expressed by TSCC cells lines. Putting it all together, a possible explanation for c-Met immediate internalization after irradiation might be the direct effect of the radiation itself as suggested by McRobb et al. These authors have reported induction of CD 166 trans-localization from the intercellular junction into the apical surface by ionized radiation, which could be the case for c-Met as well (21). Independent of the ligand, internalization has also been demonstrated to be mediated by other mechanisms such as acetylation of the receptor which warrants further investigation (19).

Implications of the current findings

The current study has demonstrated clearly that when targeted delivery is aimed for, it is of pivotal importance not to trust solely on total protein data of a candidate surface marker, but to determine in particular its surface expression and its dynamic changes when combined with other therapies such as radiation. This was exemplified by our c-Met analyses, where a high abundance of total c-Met protein, but a low percentage of surface expression and its further reduction upon radiation together suggest that c-Met may be an unfavourable target for targeted delivery of TSCC, and may actually perform even worse in combination with the current standard of treatment, i.e. radiotherapy. Nevertheless, it is important to realize that although c-Met may not be suitable for tumor cell surface targeting, it may still be an appropriate target for TSCC tumor treatment since c-Met inhibition may counteract its intracellular actions and subsequent effects on the tumor phenotype (e.g. increased invasiveness, proliferation, etc.).

Another consideration, and a limitation of the current study, is that all experiments were performed in the absence of HGF. HGF may influence the dynamics and relative distribution of c-Met over the intracellular and extracellular compartments. For example, it has been described that HGF enhanced invasiveness of cells from various carcinoma's, whereas c-Met silencing by siRNA or inhibition of its kinase activity by treatment with PHA665752 or JNJ-38877605 counteracted radiation-induced invasiveness, promoted apoptosis, and prevented cells from resuming proliferation after irradiation in vitro (7). With this in mind, upcoming experiments will be performed with HGF added to the culture media.

An alternative surface marker may be the epidermal growth factor receptor (EGFR). Cross talk between c-Met and EGFR has been suggested in several studies because of their common

downstream signaling pathways. This also has been implicated in acquiring resistance against their inhibitors. Therefore, recent efforts focus on vertical targeting therapy which combines two or more inhibitors for tyrosine kinases, e.g. against EGFR and c-Met (11),(22-24) . Xu et al., for example, have compared applying gefitinib alone versus gefitinib together with crizotinib, and found enhanced effects against cancer in vivo with the dual inhibitors (25). Our preliminary experiments studying EGFR expression showed EGFR accumulation in time in radioresistant cell lines upon radiation exposure, while we could hardly detect EGFR protein around 24-hours in the most radiosensitive cell lines (VU-SCC-040, UM-SCC-47, and VU-SCC-120). This finding broadly supports the work of other studies in this area suggesting the essential role of EGFR in inducing radioresistance (26, 27), and encourages performing further research on EGFR extra- and intracellular expression profiling.

Last but not least: That the most radiosensitive cell line SCC-40 also acquires the highest invasive potential upon radiation is a striking observation that makes one wonder if radiation may in some cases actually have a worsening rather than a curing effect. In that regard, it should be considered to design dual-targeted nanoparticles that deliver intracellularly acting agents able to block invasion-promoting molecules such as matrix metalloproteinases to counteract these adverse effects.

In conclusion, our analysis provides novel insights into the dynamic changes in the intracellular and extracellular c-Met profiles in native and radiation-exposed TSCC cells. Unfortunately, the relatively low surface expression percentages disfavor the use of c-Met for nanoparticle-mediated targeted delivery, and shows the importance of surface expression analysis of cancer targeting candidates prior to developing targeted therapies. Further research is warranted to identify more suitable tumor cell surface markers for nanoparticle surface targeting.

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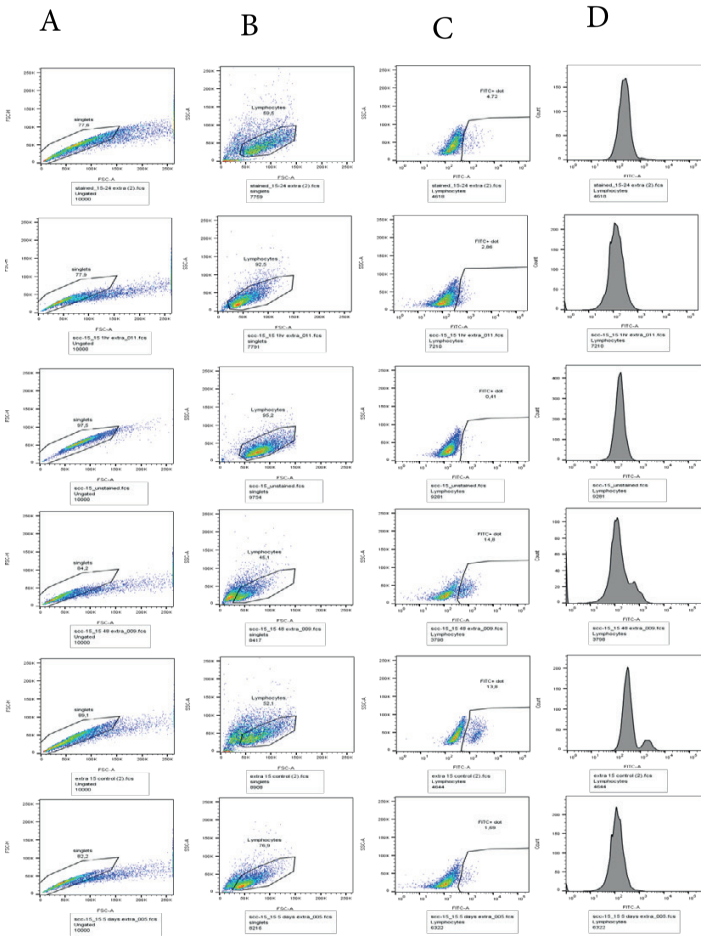
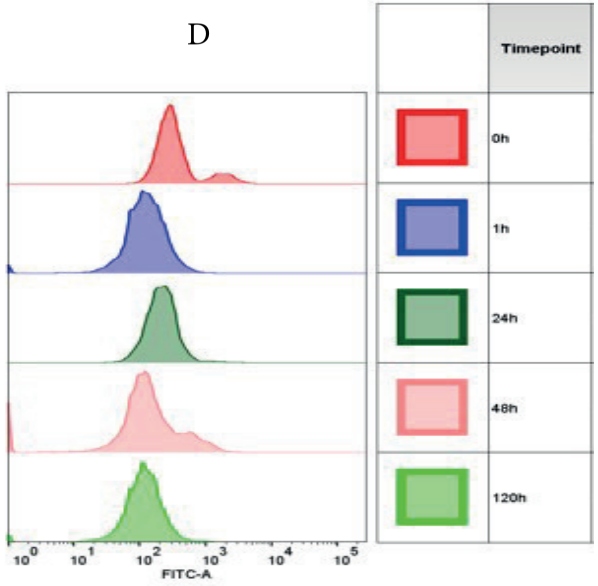
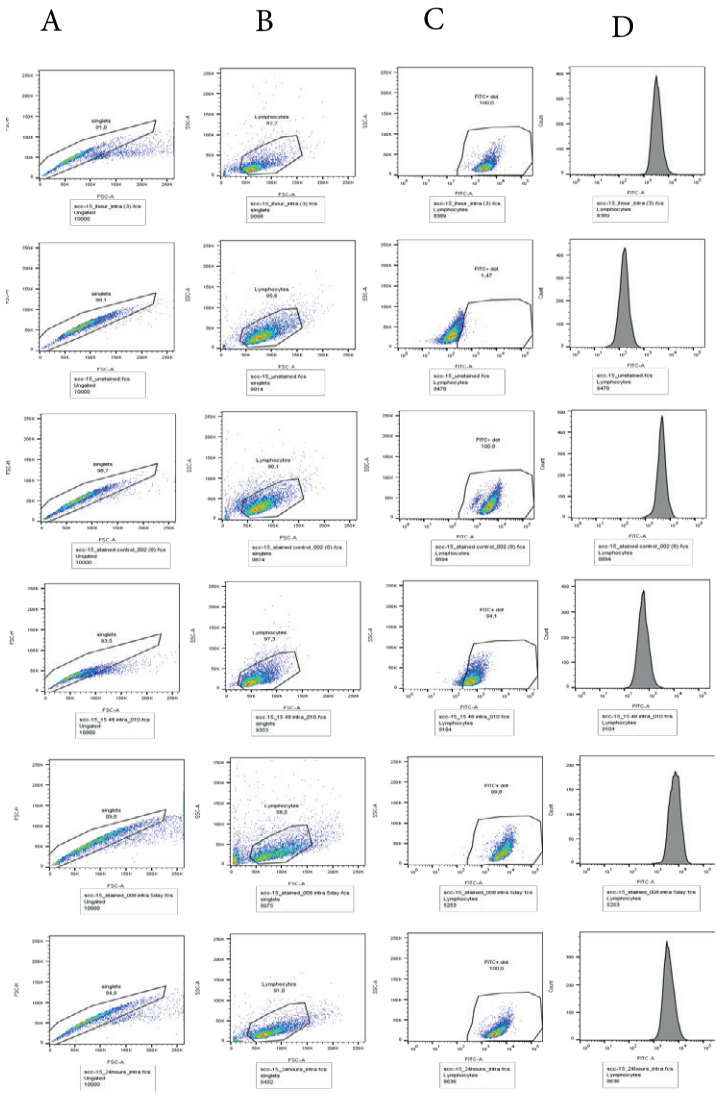
Figur S1: Gating starategy for flow cytometry analysis for the six TSCC cell lines at 5 time points, A-D. A Doublet cells were excluded based on forward scatter hight(FSC.H) vs forward scatter area (FSC.A). B, cells were selected based on (FSC.A) vs side scatter area(SSC.A). C, positive cells were selected based on (SSC.A) vs FITC. D, showed data in gray and color histogram. Percentages and median flourescence intensity (MFI) were measured. This startergy was followed for both extracellular and intracellular analysis. Analysis for all six cell lines are shown.







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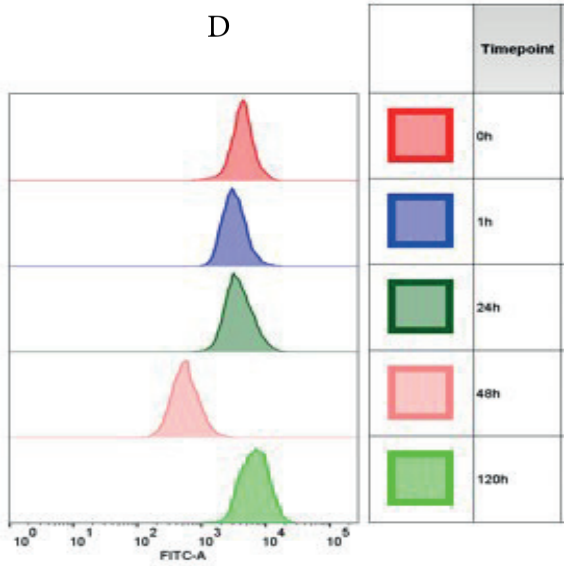

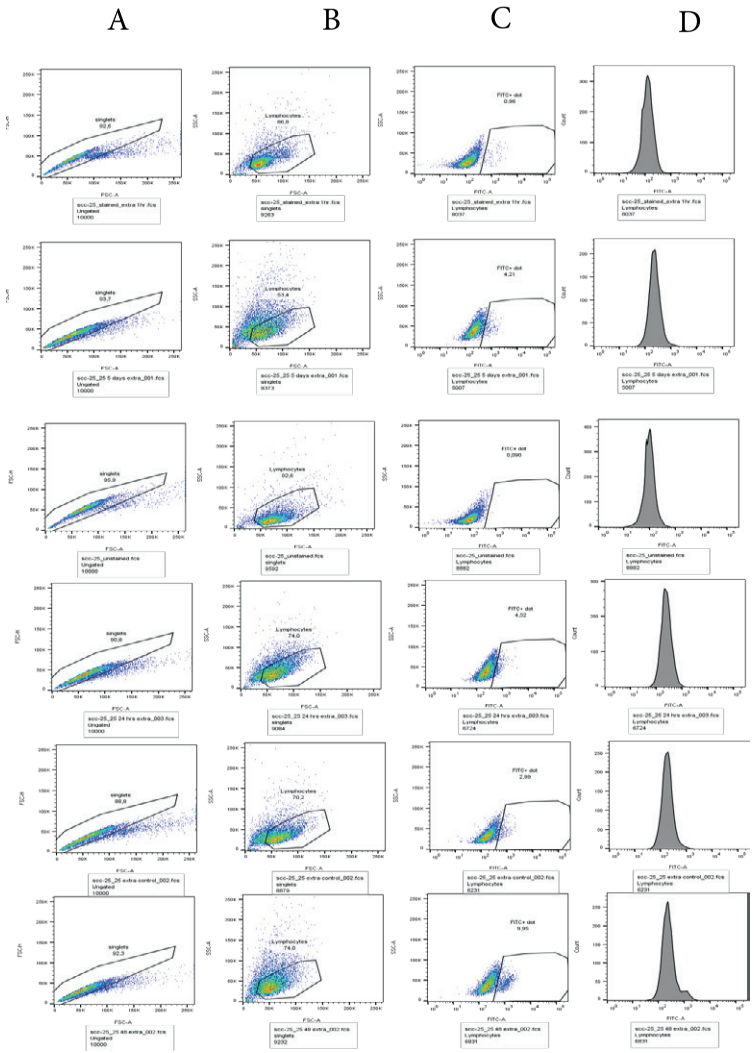

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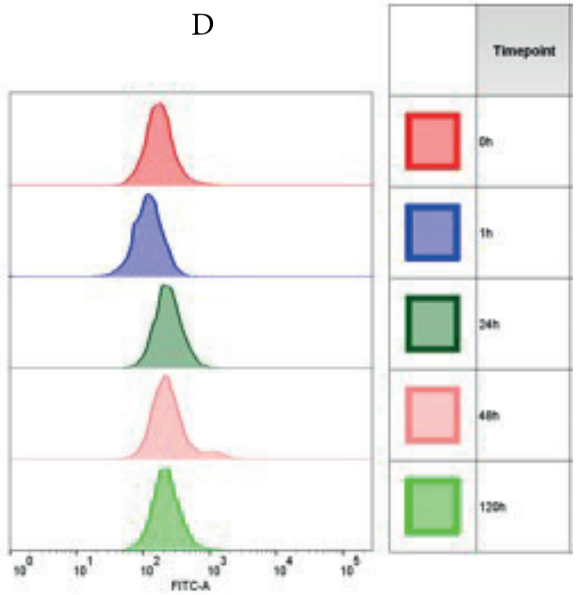

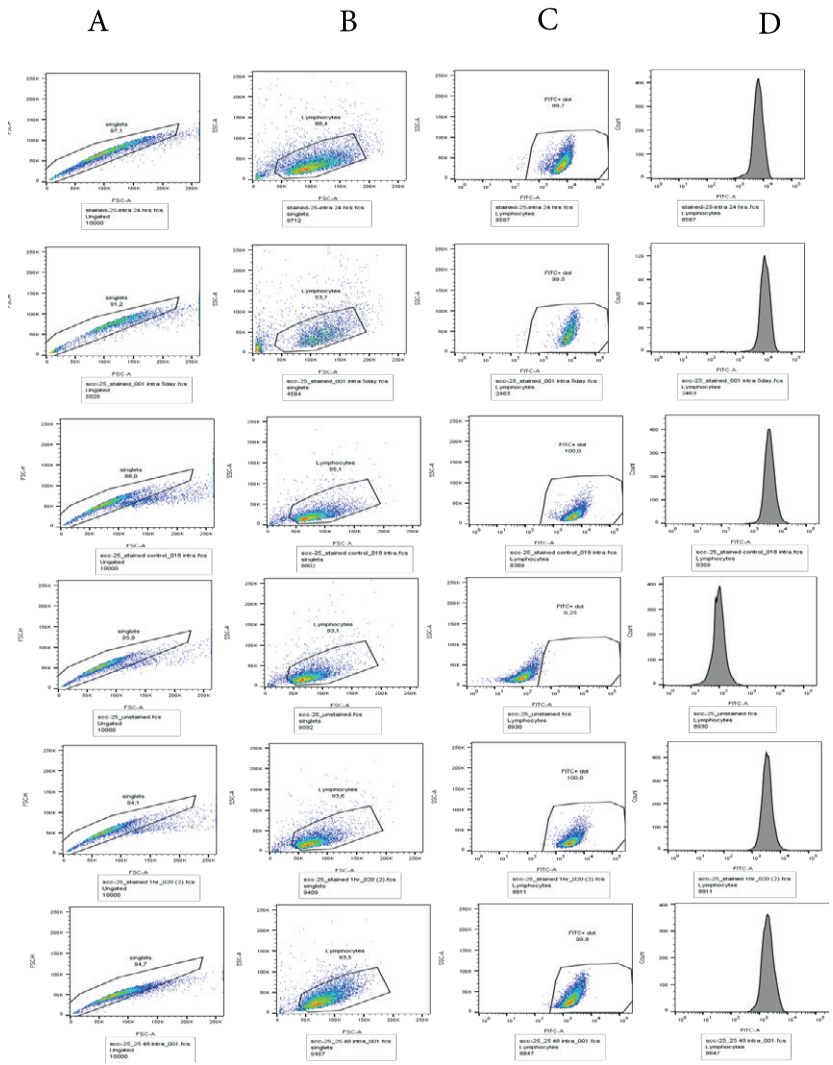

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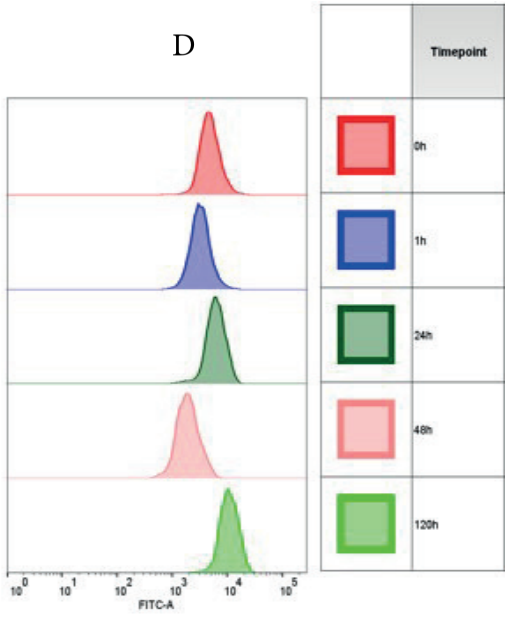

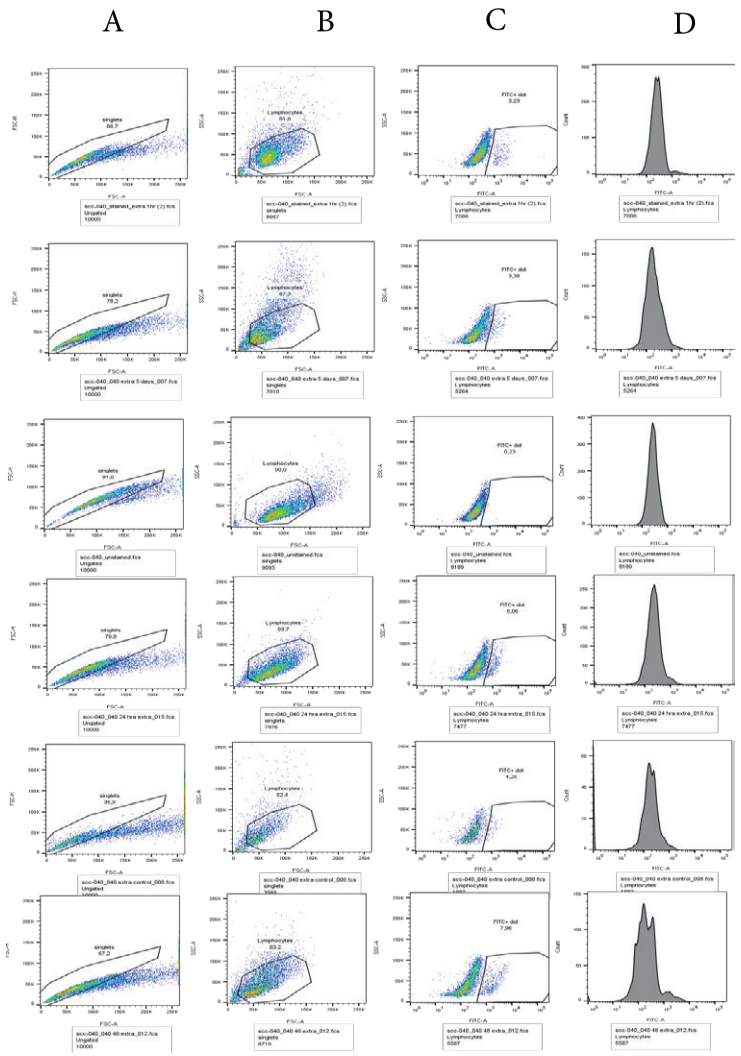

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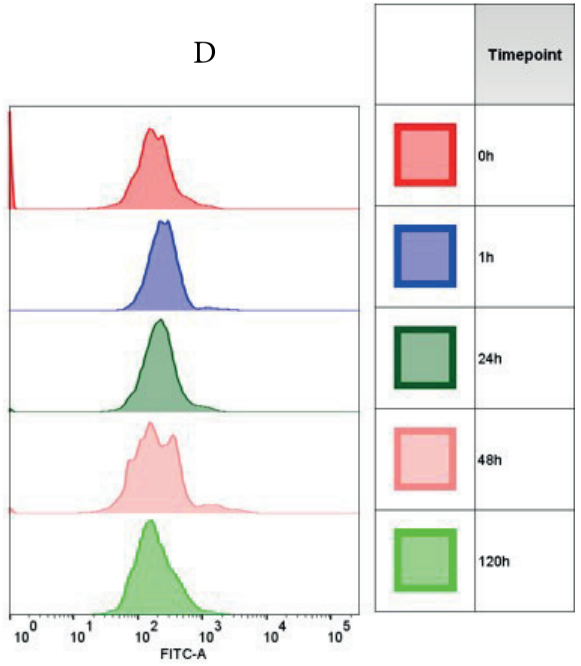

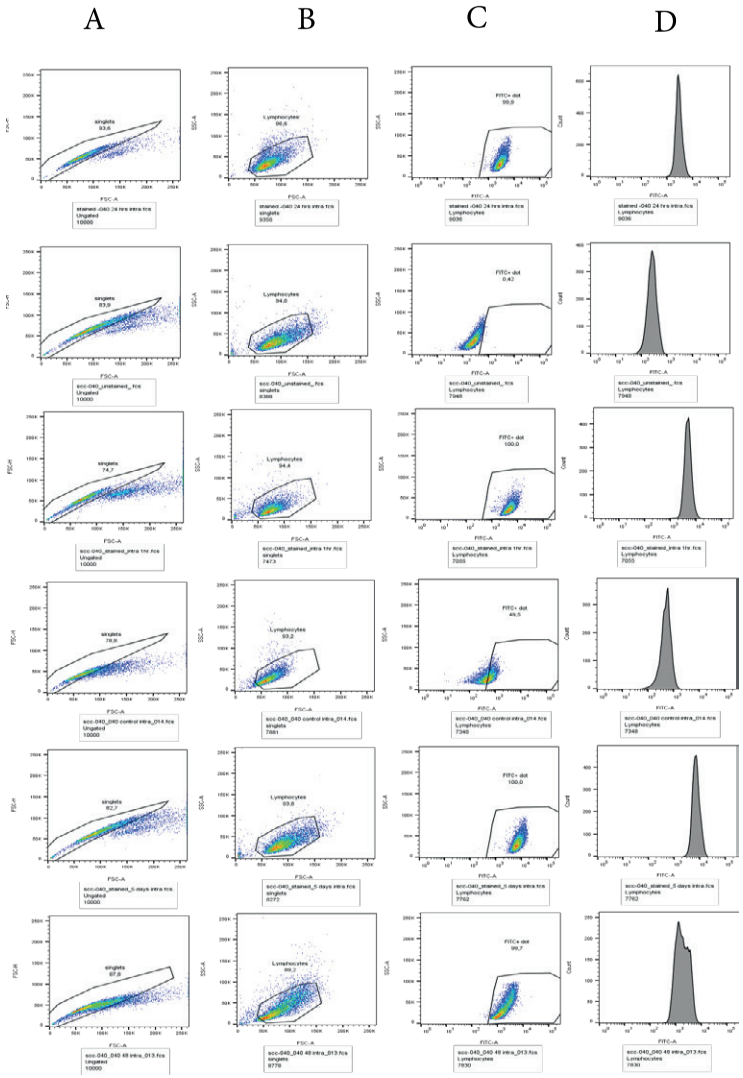

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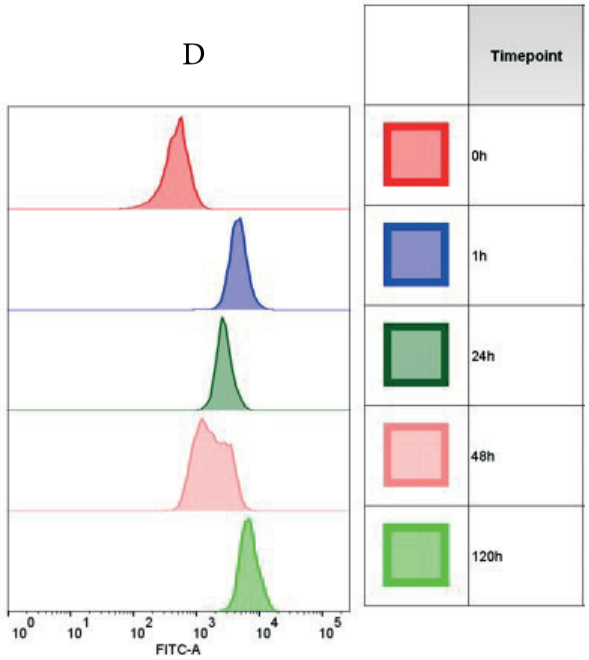

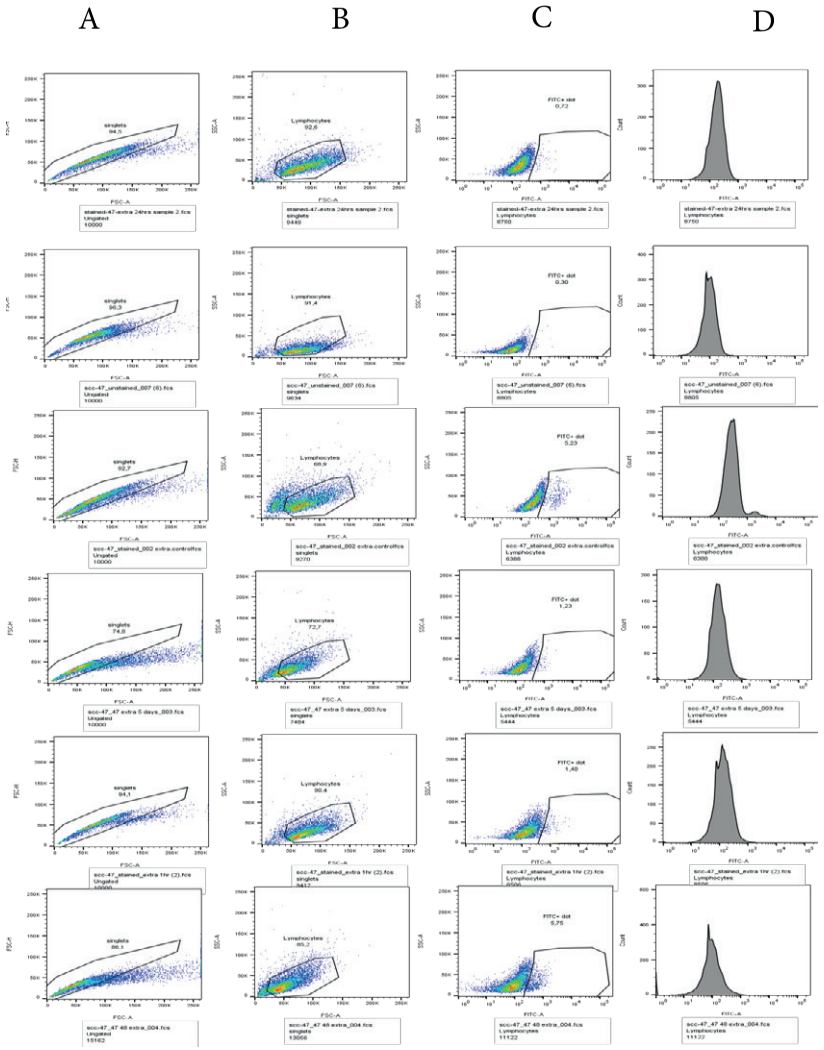

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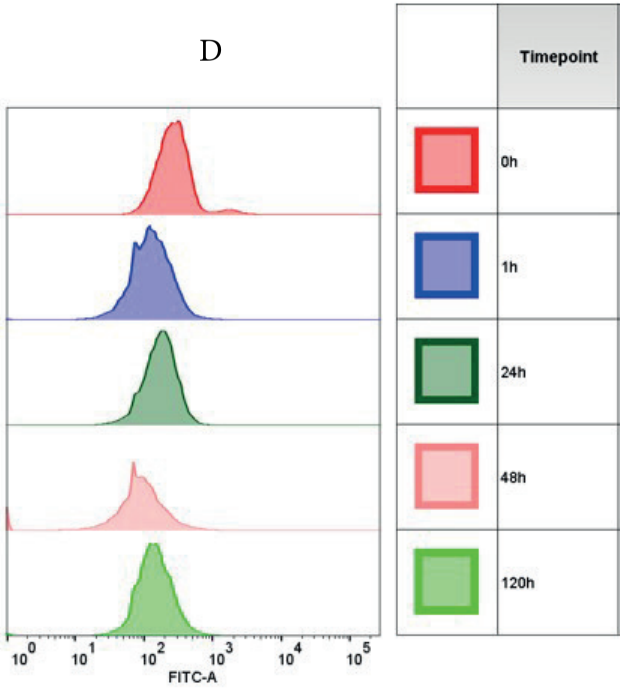

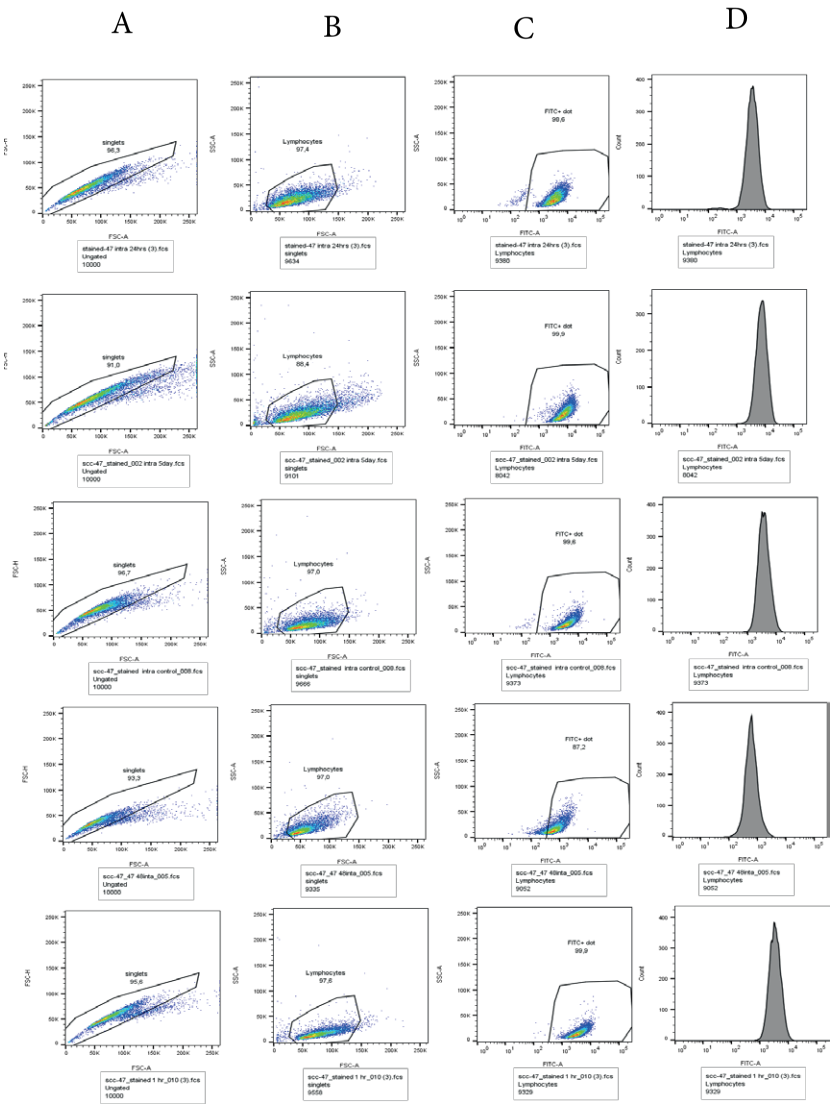

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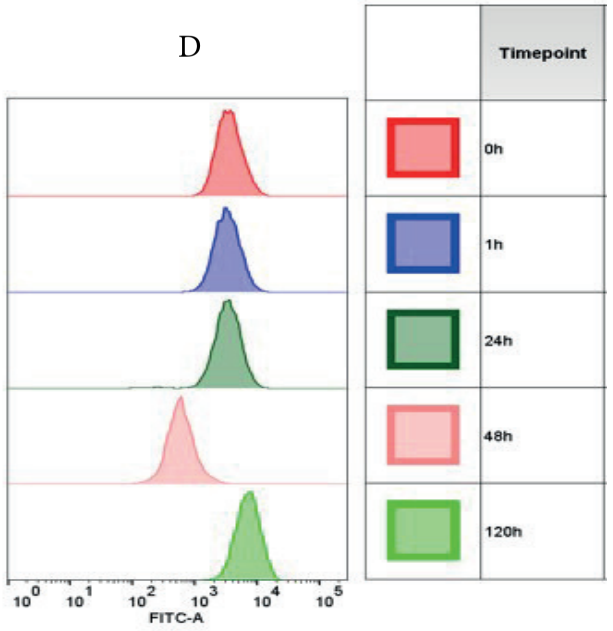

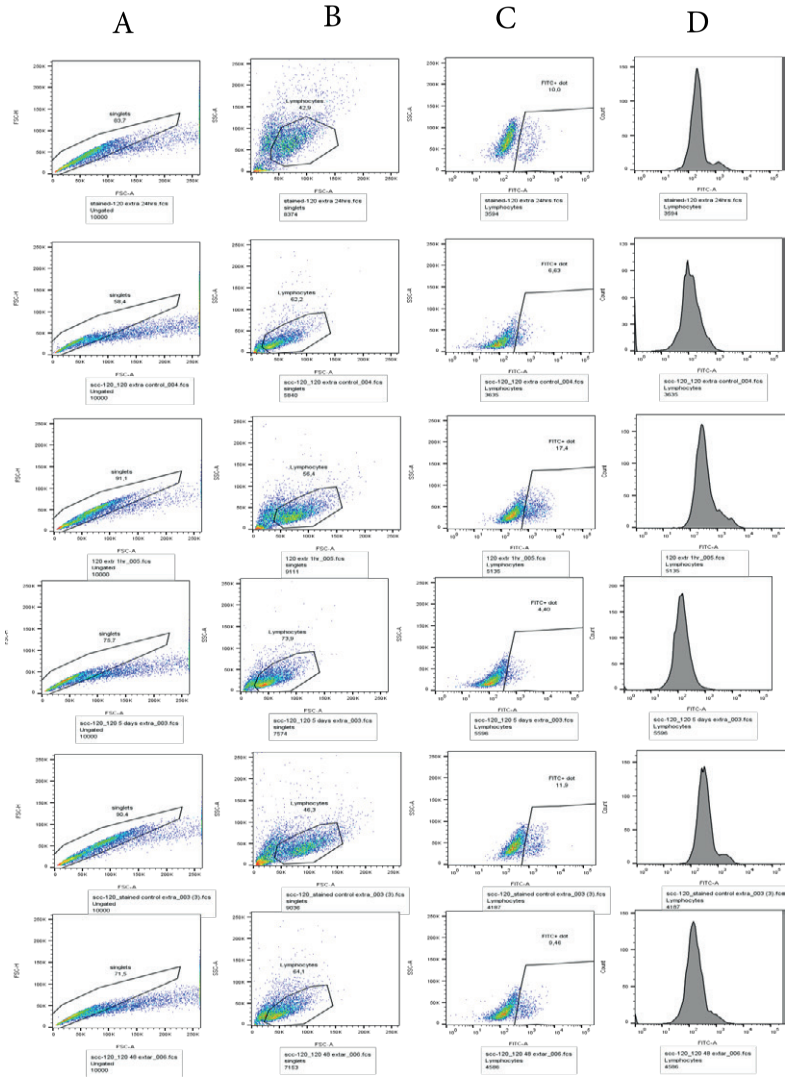

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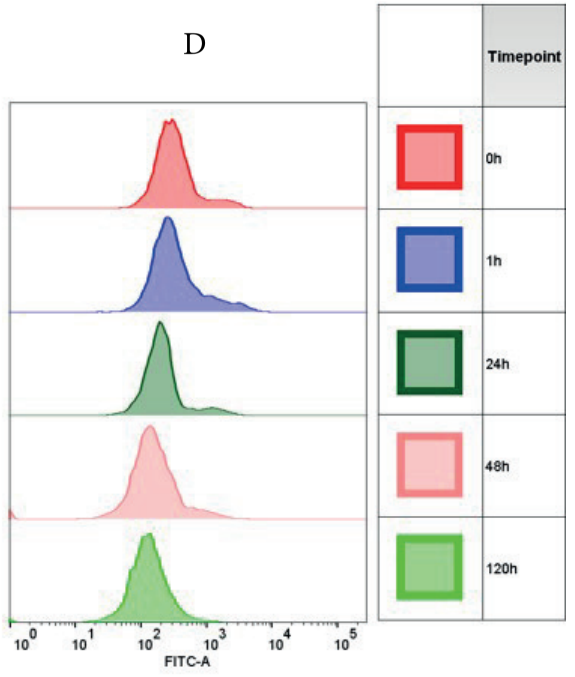

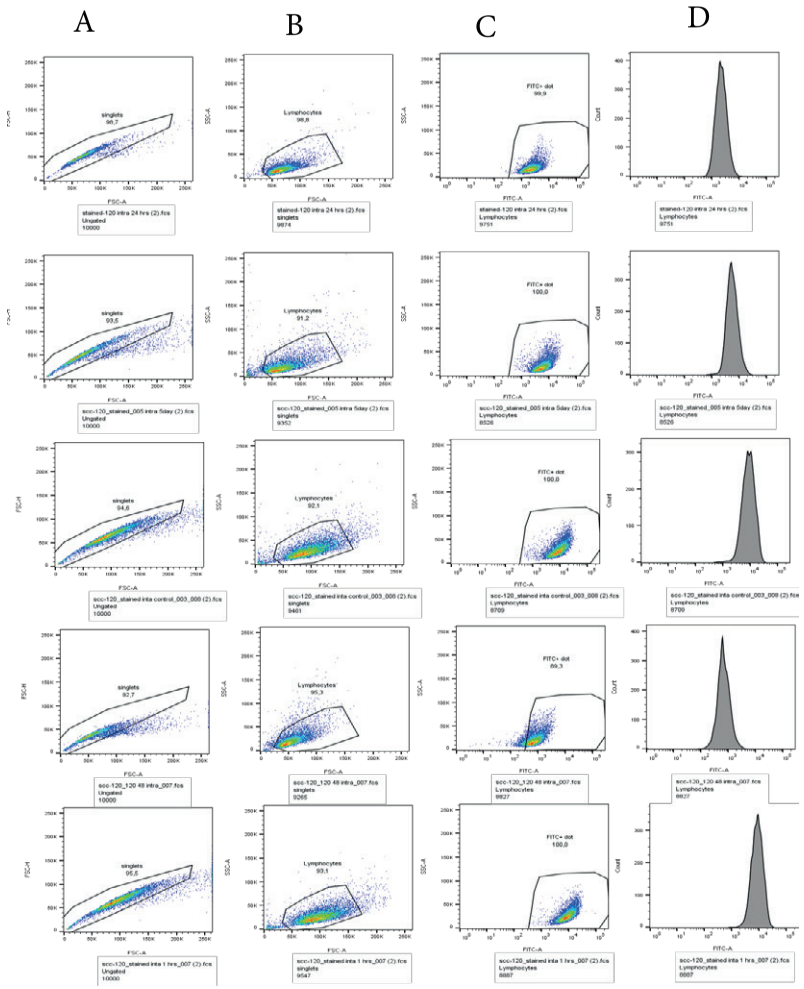

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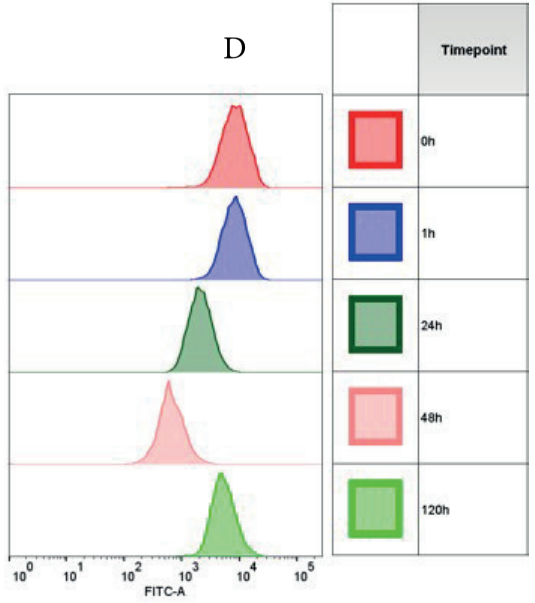

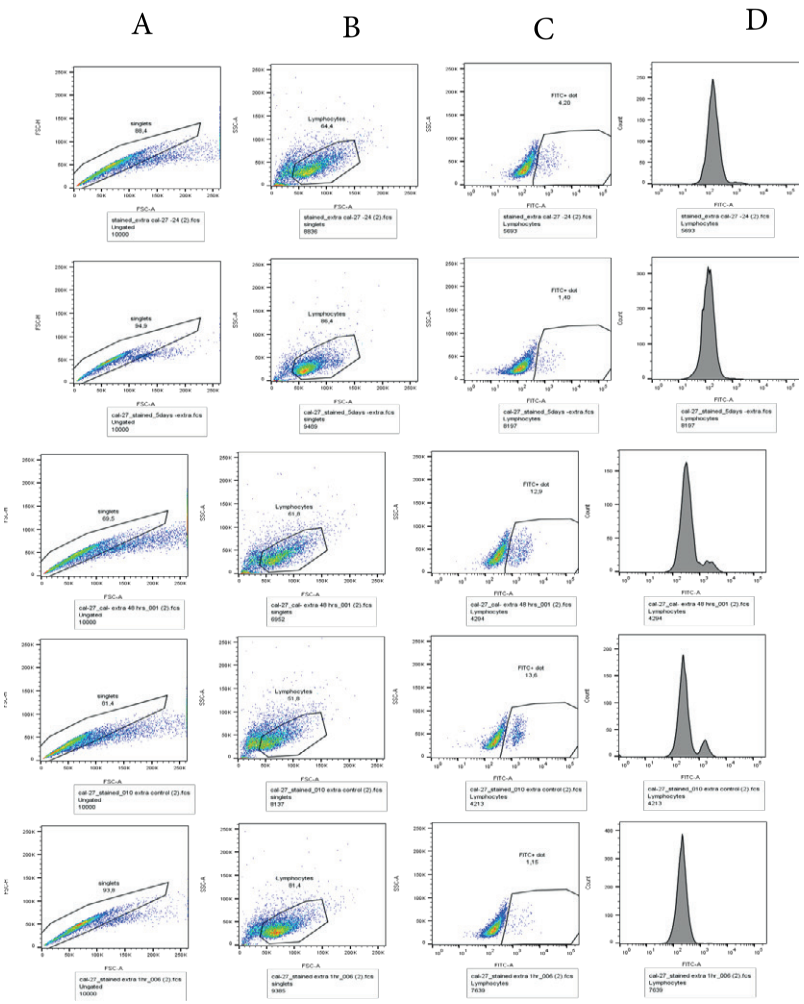

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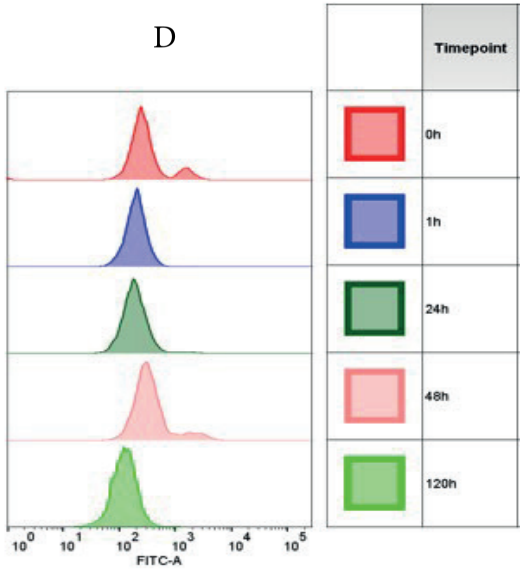

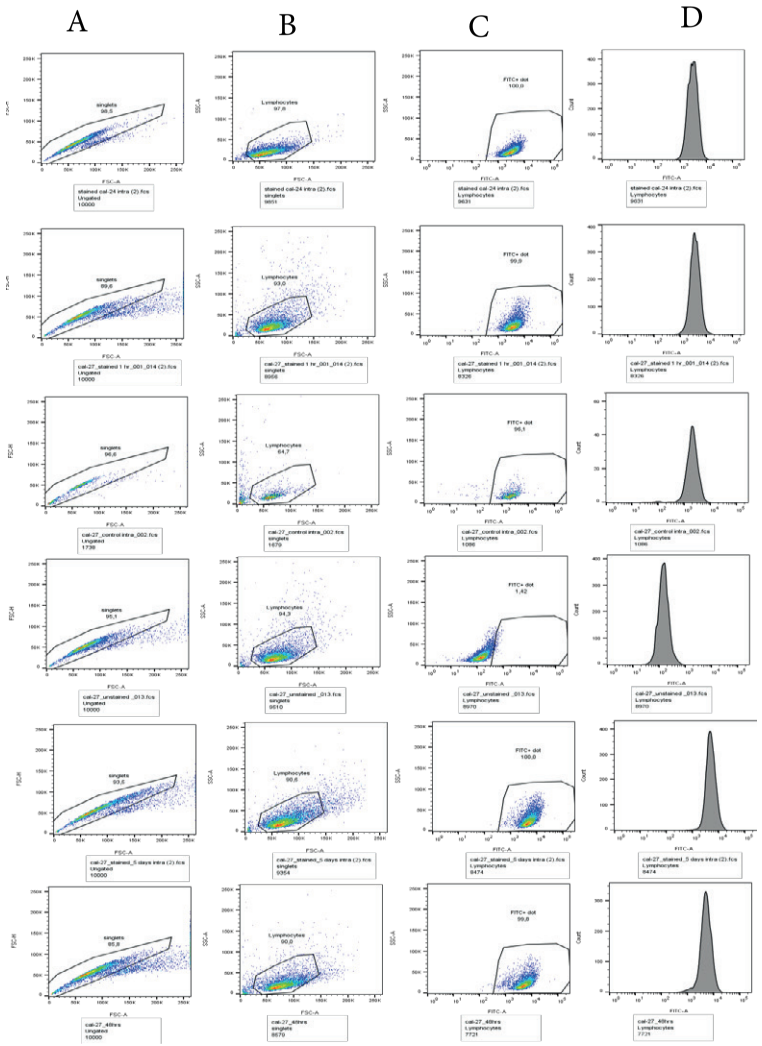

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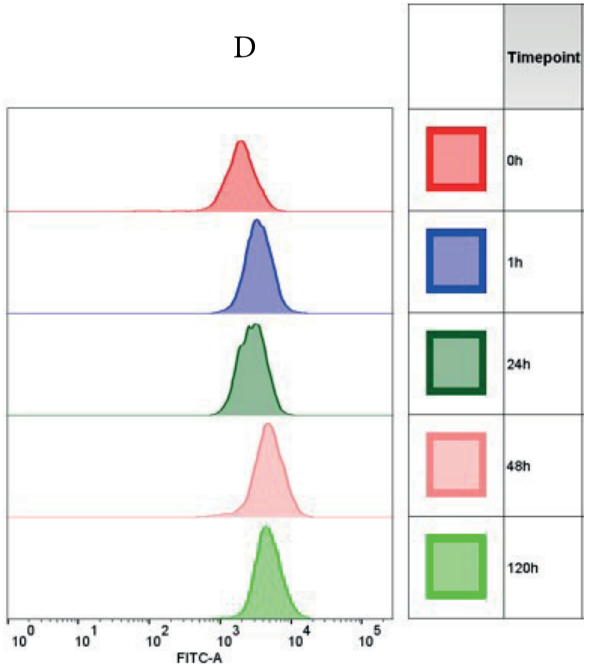

CHAPTER 7

General discussion and Future perspectives

General discussion

This thesis contributes to various aspects of epidemiology of oral and oropharyngeal squamous cell carcinoma (OOSCC) with an emphasis on young individuals in terms of trend of incidence and risk factors. It also provides insight on the importance of tongue carcinoma biomarker validation to accelerate their applicability in clinical practice. Finally, it takes a novel approach to explore the intracellular and cell surface expression of the important oncogene c-Met in a panel of tongue carcinoma cell lines after irradiation. These findings and their significances are presented and discussed in the related manuscripts included as thesis chapters (2-6), while in this section overall conclusions and future perspectives will be discussed.

Young-onset oral and oropharyngeal carcinoma

Within the last three decades, despite declining classical head and neck squamous cell carcinoma incidence rates in many industrialized countries in parallel with successful anti-tobacco campaigns, the population younger than 45 years start to experience a higher burden of incidence of this disease, particularly in the oral cavity and oropharynx subsites (1-4) . Epidemiology and the general characteristics of OOSCC in the young patients; however, remain largely unrecognized for several reasons. Firstly, OOSCC is typically characterized as a disease of aging, with longstanding exposure to conventional risk factors, including tobacco, betel nut, and/or alcohol (5, 6). Consequently, a lesser degree of attention has been given to young adults worldwide, particularly in terms of assessing the incidence rate of newly diagnosed cases. Secondly, the significant variability in the "young" definition (7, 8) results in a discrepancy and inability to compare the published literature in order to reach a robust conclusion about the incidence of this malignancy among this distinct group of the patients globally. Finally, the data which is trustable for mining from cancer registries is only available in the developed countries, while OSCC, for instance, is very common in the developing countries (9). As a result, the world literature could not provide an inclusive depiction of the OOSCC incidence rate in this population, thus hampering appropriate guidance of national and international policymakers and public health services. A major contribution made by this thesis has been to provide an in-depth overview of the global and national incidence of the OOSCC in this young patient group, taking partially into consideration the problems addressed above. On a nationwide level, the studies reported in this thesis may serve as the status report that increases awareness of these two malignancies in this young population, and direct the HNC oncology

researchers in the future to adopt the Dutch definition of young adults oncology, which has been determined at ≤ 35 years old. Detailed discussion in regard to these aspects has been provided in the chapters (2-4).

Gender differences in young patients

In cancer research in general, investigation of gender differences could result in relevant and valuable information that may help in identification the etiology of the diseases and understanding the underlying mechanisms. In classical OOSCC, it is well-known that these type of malignancies are most common in men, and closely related to heavy smoking and drinking habits (10, 11) . Surprisingly, a different picture has been observed in the young population with respect to OSCC. In multiple studies, most incidences of OSCC in the young population occur in women with no history of tobacco and/or alcohol abuse. What is more, the disturbing trend toward an increase in the young adults is noticeable only in specific sub-sites such as mobile part of the tongue (12-14). Based on the results of this thesis, the young Dutch patients showed strikingly different findings. The substantial increase in oral tongue carcinoma in the Netherlands was limited to men aged 20-34 years old, and a high proportion of those men were tobacco and alcohol co-users. Another important finding of this thesis is that due to the subgrouping of the young patients in two age cohorts (20-34 and 35-44 years old), it was shown for the first time that tongue carcinoma is predominant in women over men at ages 35-44 years, but not in the younger age group. Direct comparison between our studies and the published reports with the aim to confirm whether there is a specific age and gender-sub site association is difficult given the difference in grouping intervals used in the available literature. On the one hand, our finding of a higher incidence in the men younger than 35 years old may partly be explained by genetic influences on the metabolisms of the tobacco and alcohol to promote carcinogenesis (15), though many believe that the risk of cancer increases substantially with long duration of exposure to these factors. On the other hand, the early life exposure to environmental carcinogenic factors has been documented to increase susceptibility to cancer formation, inflicting high rate of cell proliferation and incompetent DNA repair in the young individual (16-18). The reason behind the high prevalence of tongue carcinoma in young women with ill-defined etiology remains unanswered still. Nonetheless, since OSCC is generally more common in men as compared to women, some authors suggested a protective role of the primary female sex hormone estrogen in malignant transformation, though the supportive data are sparse. An obvious example of this is that estrogen has been documented to cause a reduction in hepatocellular carcinoma incidence for women (19). Yu et al., have identified oophorectomy at an earlier age (≤50 years) as an important risk factor for hepatocellular carcinoma in women, while

post-menopausal hormone therapy use functions as an additive protective factor (20). Similarly, the protective role of estrogen in colorectal cancer has been suggested, though the exact mechanisms are not precisely known (21, 22). Chlebowski et al., have provided evidence that the use of hormone replacement therapy use was associated with a 30% decline in colorectal incidence in post-menopausal women (23). Regarding oral cancer, a study from Hungary provided initial evidence about the significant association between the time of initiation of menopause and the risk of developing this malignancy (24). The earlier the onset of menopause, the higher the risk of oral carcinoma. Such results led us to hypothesize that increasing risk of tongue cancer in women with age range from 35 to 44 years could be due to spontaneous or induced premature menopause. This item should preferentially be recorded in cancer registries as well. The "estrogen" hypothesis certainly warrants further investigation. Also the evaluation of possible interactions between genetic factors and such hormonal changes may add further value.

HPV

To date, more than 220 HPV genotypes have been discovered and generally they are classified into high-risk (HPV-16, HPV-18) and low-risk genotype groups (25, 26). HPV, specifically HPV-16, is now a well-established risk factor in HNC, and in particular for OPSCC (27). HPV has been detected in more than 65% of oropharyngeal carcinoma in US (28). Comparable results have also been reported in other countries such as Sweden (29) and Australia (30). In spite of the fact that HPV is currently recognized as the main reason beyond the drastic increase in OPSCC prevalence over the last decades, it is also an independent prognostic factor for better survival upon treatment (31). Unfortunately, virtually all of the estimations for HPV prevalence among oropharyngeal carcinoma were based on monocentric study designs, which may not reflect the realistic situation across the country. For the first time, this thesis presented population-based data regarding prevalence of HPV status among the Dutch population which could serve as a valuable reference to evaluate any future preventive measures. The data showed that HPV-related oropharyngeal carcinoma is approaching 31%. This figure is considerably lower than the figures reported from the national datasets of the New Zealand (77.9 %), and Denmark (62%) (32, 33). Our finding is also consistent with a recent systematic review indicating that Spain and the Netherlands had lower prevalence of HPV- related oropharyngeal carcinoma than Europe as a whole (34). In agreement with suggestions of Hennman et al., a possible reason for this disparity might be a good sexual education in the Netherlands, though no data are available to confirm this statement (35). Up to now, the way of oropharynx HPV acquisition is still unclear; however, oral sex practice, multiple partners at earlier age and open mouth kissing were found to be involved (36) . So far, the only country in the world that has reported

these practices and their correlations with the risk of developing oropharyngeal carcinoma is the US (37). In fact, even though the study found the lifetime risk of developing OPSCC was low overall, the prevalence of oncogenic oral HPV was highest among men who currently smoked and had ≥5 lifetime oral sexual partners (14.9%, 95% CI = 11.4–19.1). In addition, it is worth noting that our data demonstrated a significant incidence reduction in OPSCC in the Dutch population aged 35-59 year old. As said, this is in contrast to recent epidemiological data collected worldwide indicating that there is a dramatic global rise in oropharyngeal carcinoma in this age category (38). Up to date, we do not have a clear explanation for this discrepancy.

Optimizing tongue carcinoma treatment

The mobile part of the tongue is the most commonly affected cancer sub-site intraorally (39). It is also characterized with an aggressive clinical behaviour and historically has the poorest prognosis (40). Indeed, delayed diagnosis of malignancy in this sublocation may result in devastating complications such as occurrence of metastasis, and the need for multi-treatment modalities which will eventually lead to accumulation of side effects and reduced quality of life. Therefore, one of the potential solutions to overcome this issue is to find reliable and validated biomarkers to aid in early detection, prognosis prediction and disease monitoring. Indeed, finding well-validated biomarkers becomes a necessity with the emergence of advanced high-throughput omics technology that allow detection of mutated genes and protein dysregulations underlying cancer development and progression. For TSCC, despite the fact that multiple biomarkers were investigated and proposed for clinical use, there is no relevant biomarker available in the clinic yet. There are several issues to be addressed here that may explain this failure. One of the major problems we found in this thesis is that a plethora of the studied biomarkers are still in the early discovery stage, while only 10 biomarkers have been validated in one or two reproducible studies (**chapter 5**). Furthermore, ideally during the early stages of biomarker discovery, broader populations should be collected prospectively on the basis of clear inclusion and exclusion criteria in a cohort or a case-control study design. However, the majority of the studies are based on retrospective collection methods and samples of convenience which both have several sources of bias. Of interest, with regard to prognostic biomarker studies in particular, Simon et al., have suggested what they called a retrospective-prospective study design, using archived specimens of completed prospective clinical trials as an alternative solution to improve validation of robustness of the biomarker (41). Thus, following this kind of study design especially in academic hospitals that have large and well-equipped biobanks will contribute significantly in speeding up the clinical implementation of

suitable biomarkers. Last but not least, in context with the heterogeneity of the tongue carcinoma in mind, searching for a combination of biomarkers in different tissues samples rather than a single molecule may be pivotal in deducing tumor characteristics and provision of a higher extent of accuracy.

Recently, the theme of nanotechnology has raised a lot of attention and it is expected to be able to cause a significant shift in cancer treatment. To pave the way for the future and using liposome nano technology in targeted therapy in tongue carcinoma, it is important to search for surface cell receptors that can clearly distinguish between neoplastic and normal cells. It would be even more useful if this targeted therapy can be combined as an adjuvant therapy with the currently used therapeutic options. Radiation therapy is a major component of treatment modalities in tongue carcinoma. However, this type of therapy is associated with a high risk of toxicity and some complications that reduce the patients' quality of life. To optimize the effect of radiation while minimizing its side effects on healthy tissues, there is a need for novel approaches that enhance specific intracellular delivery of the currently used medications such as radiosensitizers to the cancerous cells. Tyrosine kinase receptors are the largest group of growth factors that orchestrate the majority of the biological pathways in cancer cells. Besides, this groups of receptors have an extracellular domain that is suitable for druggable targets (42). An important receptor of this family is c-Met which has been suggested by several studies to be overexpressed upon irradiation (43). Interestingly, in our approach where we did not only look at total protein expression but in particular to the percentages of the receptor present on the cell surface as well as intracellularly, we found that the majority of c-Met was localized intracellularly and that the surface expression showed a dynamic pattern with rapid surface downregulation followed by a largely increased surface expression after 48h after irradiation. Moreover, we determined that EGFR expression showed different patterns compared to c-Met with the exception of the most radioresistant cell lines, in which both receptors were highly expressed. This might indicate that both c-Met and EGFR proteins are responsible for radioresistance, and may mediate this in a redundant manner. Thus, developing targeted inhibitors for both receptors simultaneously would improve the outcome efficiency, especially in radioresistant tumors. Concomitantly, the simultaneous expression of these receptors on cancerous cells would increase the specificity of the targeted drug delivery, whereas normal cells could evade the therapy. Moreover, and in contrast to previous studies, we found the tongue carcinoma cell line panel was unable to generate c-Met ligand (HGF), and consequently, no phosphorylation in this receptor could be detected. However, since HGF may influence the dynamics and relative distribution of c-Met over the intracellular and extracellular compartments, (REF De bacco et al) it

may be that in the *in vivo* tumor environment where presence of HGF may be expected will show different results. Designing *future* experiments should take this possibility into account and all evaluations should therefore be performed both in the absence and presence of HGF.

The extracellular surface receptor should ensure targeting of the TSCC cancer cell specifically, in order to deliver the treatment moiety efficiently. As an intracellular target, one should select molecules or processes that determine the cancer phenotype and/or its resistance to current treatments. In cancerous cells of several solid tumors, including HNC, the G1/S check point is abrogated because of a deficiency in TP53. Hence, these cancerous cells depend totally upon the G2/M check point wherein the WEE1 kinase plays a key role in the DNA damage response (DDR) process. The DDR process is a series of events that collectively inhibit mitotic entry in cells with damaged DNA to allow DNA repair mechanisms to occur prior to re-entry into cell cycling, thus ensuring cell survival (44, 45). Several WEE1 inhibitors (PD0166285, PD0407824, and AZD-1775) have been developed and tested for efficacy (46-50). We are currently exploring the efficacy of WEE1 inhibitor (AZD-1775) as a radiosensitizer in a preclinical study in TSCC cell lines. In case this inhibitor will show promising results, the ultimate step will be to develop dual-targeted liposomes, with a surface targeting moiety protruding from the outer shell, and encapsulated AZD-1775 to selectively target and eradicate TSCC tumor cell with high specificity and reduced toxicity.

Future strategies

It is known that quantitative assessment of disease burden is the main tool to set priorities for public health and policy decision makers. Therefore, the main aim of this dissertation was to evaluate the incidence rate of oral and oropharyngeal carcinoma in all age groups, with particular emphasis on the patients younger than 45 years old. Surprisingly, we found the global incidence of these tumors in this population to be alarming. Likewise, the epidemiological pattern of these diseases showed that the incidence rates are increasing in the Dutch males aged 20-34 years, while it decreased in the 35-44 years age group. An important approach for the future is carefully monitoring the prognosis of this population. Till now, the risks and prognoses for young OOSCC patients remain controversial. Some studies advocated that the young age groups have a more aggressive cancer behavior and subsequently an inferior prognosis, while in other studies the young patients presented with a better prognosis instead. More specific and detailed evaluations may be an important step in the future and may result in a shift of treatment guidelines for young patients.

Identification of avoidable risk factors is a critical issue in planning prevention strategies. In this thesis we only shed some light on the prevalence of three well known risk factors (smoking, drinking alcohol and HPV infection) in all Dutch patients, however, still with special emphasis for the young age groups. Interestingly, these three risk factors were prevalent in all age groups, including 2/3 of the young patients. A good line of future research could be the comparison of these risk factors in the patients younger than 45 years with an age- and sex-matched cohort in a cross-sectional study with a well-prepared survey, covering in details all information regarding starting age of indulgence in these habits and their exact amount. This is paramount to draw sound conclusions about direct or indirect associations of these risk factors with these malignancies. Importantly, the joint exposure to smoking and drinking may also imply concomitant exposure to other risks such as addiction materials, low physical activity, and unhealthy dietary habits, which should also be included in the questionnaire. Regarding HPV infection, oral sex practices and open mouth kissing have been suggested as potential routes of oral and oropharyngeal viral acquisition. In the Netherlands, in fact, no information is yet available evaluating the young Dutch manners in this regard. Elucidation of the frequency and extent of such behaviors will be useful in two directions. This firstly may help in understanding the current low observation of HPV infection prevalence for the Dutch community that distinguishes the Netherlands from other European countries and many countries of other continents. Besides, this could facilitate and highlight correlations between behavioral patterns, risk factors and oral and oropharyngeal carcinoma.

To date, the age group classifications for cancer patients have been based on arbitrarily chosen age ranges, which may be an invalid approach. With the recent advances in second-generation sequencing that allow a comprehensive characterization of whole-genomic alterations, an accurate alternative grouping of populations according to similarities and differences in their genome profile is at reach. This may be particularly useful for categorization of cancer patients younger than 45 years, because cancer is fundamentally an accumulation of genetic mutations, and early identification of genetic alterations may attribute to more accurate prognosis prediction profiles and subsequent treatment strategies for these patients.

The field of biomarker identification is evolving and growing rapidly. In this thesis after assessment of published tongue carcinoma biomarker studies, we found that the majority of the studied biomarkers are still in the discovery phase. Fortunately, there were 10 promising biomarkers that

validated their expression consistently with some clinical relevance. Clearly such results mean there is a need to reassess the strategy followed in this field, otherwise entering biomarkers in oral oncology clinic will not happen in the near future. Simply, this thesis suggests two pathways for future researches. First, there should be more focus on the reproducibility of the already discovered biomarkers, taking into account these designed studies must have larger sample size. Second, because of the heterogeneity and complexity of this type of cancer, searching for combination panels of biomarkers could benefit more accurate identification of the disease state, patient characteristics, and appropriate treatment regimens for the individual patient.

Finally, our choice for c-Met as one of the tyrosine kinase families to be investigated after radiation in tongue cancer was based on previous studies exploring these receptors in many malignancies. Hence, conducting proteomic analysis for cell surface markers after irradiation, might support c-Met as a promising candidate and will likely also lead to identification of other promising candidates, In fact, as stated above, the complexity and heterogeneity of the cancer requires searching for multiple compounds. For that, from our study we suggest further investigation about the interaction between c-Met and EGFR and their expression patterns simultaneously. This will result in more knowledge about their behavior and the probability of using both as targets to enhance the liposome specificity toward the cancer cells, and avoid the normal tissues. As indicated above, an interesting intracellular targeting moiety may be AZD-1775, re-imposing sensitivity for, and thereby efficacy of radiation therapy. Another interesting candidate which deserves attention may be an siRNA or small molecule inhibitor against the Y1003 gene product, which is responsible for internalization of c-Met receptor to ensure longer period of surface expression. These novel targeted treatment moieties incorporated in nanotechnology-based delivery vehicles may spur more effective adjuvant treatment options for improved TSCC survival in TSCC in the future.

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CHAPTER 8

Summary

SUMMARY

Oral and oropharyngeal squamous cell carcinoma (OOSCC), as described in the introduction section, are the most common HN malignancies that usually occur in elderly patients, having significant death rates. Within the last three decades, many studies suggest that OOSCC incidence is increasing in patients younger than 45 years old in several countries. However, the few number of cases in this young group, compared to the elderly patients, results in marginalization for this population in most of the epidemiological studies. Lack of sufficient data could eventually lead to disastrous outcomes particularly for the young cancer patients, with regard to prevention strategies and clinical interventions. Therefore, the best approach to start measuring these disease burdens in this age group specifically was to estimate their global occurrence (or incidence) and characteristics. In **Chapter 2** of this thesis we conducted a systematic review covering four decades, to include as much as possible of the published literature which intentionally or not estimated the incidence of OOSCC in patients younger than 45 years. The study did not only include population based studies, but also the proportion literature, and both estimations ultimately revealed a significant increase in incidence of these two malignancies worldwide. Further, the observations of that study indicated two unique gender-subsite associations. First, a significant increase in the mobile tongue carcinoma was clearly seen in women in their thirties or forties of age which were neither smokers nor drinkers, i.e. not exposed to the classic risk factors. The second association was the remarkable increase in tonsils and base of the tongue cancer in white, high class society men. Surprisingly when analyzing the data for Western countries, we found a significant reduction in incidence of these tumors in the Netherlands only.

The results of chapter 2 motivated us to know more about the incidence of these malignancies in the Dutch population. To create deeper understanding of these diseases, we also investigated the prevalence of the conventional risk factors such as smoking, drinking and HPV infection at the population level, and made gender specific estimates for each age group. **In chapter 3,** we studied for the first time the trend of incidence of oral squamous cell carcinoma by join point analysis regression that provides a complete evaluation for rate changes throughout the years. In that study we also for the first time classified the young Dutch patients into two subgroups (20-34 years and 35-44 years). The results showed a significant increase in annual incidence for patients younger than 35 years, while an opposite observation was found for the other young group (35-44 years). Additionally, a profound and surprising reduction in annual percentage changes for the adults population (45-59 years) since 1997 onwards was noticed . For the elderly patients (60 years and more), the incidence is increasing, with double rates in the women compared to the men. Regarding

the prevalence of smoking and drinking among the patients, the overall percentages were high for all age groups. However, it is important to keep in mind that we evaluated only risk factor data of the last two years of the studied period (2015,2016) because those were the years when the Netherlands Cancer Registry (NCR) launched risk factor data collection. In chapter 4, we focused on the incidence of oropharyngeal SCC and evaluated the related risk factors smoking and drinking, as well as, for the first time, the prevalence of HPV infection, in a population-based study. To study trend changes during the entire studied period of time which ranged from 1989-2016, we used join point regression software. As indicated previously, the NCR began collecting data for the classical risk factors of OOSCC as part of a national initiative toward a comprehensive registration in 2015. Therefore, the risk factor data for OPSCC were only available for the last two years of the studied period (2015,2016). Our results showed a significant decline in the annual percentage changes for the young patients with ages 35-44 years old and for those aged 45-59 years since 2000 onwards. In patients older than 60 years, incidence rates increased overall, with an annual percentage change for women being consistently higher than men. Importantly, we found that the percentage of Dutch patients with HPV-related oropharyngeal carcinoma is approximately 31%. Overall, the study found that the vast majority of the patients were tobacco smoker and alcohol drinker, which makes a pivotal role for HPV infection in the Dutch patients less likely.

Apart of the epidemiology, one aim of this thesis was to contribute in improving the outcome of the patients affected with tongue squamous cell carcinoma (TSCC). This is because TSCC is characterized by an aggressive clinical and biological behavior which is, however, often only diagnosed at a late stage and accordingly has the worst prognosis among all head and neck cancers. Currently, the highly improved understanding of the molecular pathways involved in malignant transformation facilitates the discovery of many valid biomarkers in different cancers. A biomarker by definition is an objective measure such as, a gene, a protein, enzyme, or hormone that can reflect the entire spectrum of the disease, from the earliest features to the end stages. It is important to mention that the journey of any biomarker from the bench to clinic is a very long and challenging one. At the simplest level, effectiveness of the biomarker cannot be measured by only one discovery study, but by the reproducibility of the results in different and independent populations. Hence, the initial key step to bring a newly discovered biomarker towards clinical implementation is independent replication. In **Chapter 5**, we assessed the validity level of the published studies concerning tongue carcinoma biomarkers. We included the relevant papers across different TSCC sample sources, i.e., body fluids (saliva, serum/ plasma) and tissues. Unfortunately, we noticed an

abundance of studies that described single or multiple biomarkers only in one publication (66%). Nonetheless, 10 biological markers demonstrated a consistent association between their presence and specific clinical outcomes. Collectively, these 10 biomarkers qualified as the most promising candidates for TSCC diagnosis and prognosis. Further research exploring the validity of these biomarkers in a prospective manner using single biomarker or a panel of biomarkers is urgently needed.

Although conventional treatment (chemo radiation therapy) is a commonly used modality for treating advanced TSCC, it often fails to eradicate the neoplastic cells. One reason is the need to deliver a higher dose of the radiation or drugs to kill the cancerous cells, but this ultimately will cause an irreversible damage to normal tissue cells as well. Hence, a potential solution is to enhance the selective intracellular delivery of the current medications in a higher dose to the tumor cells together with radiotherapy, thereby keeping the normal cells unaffected. This could be achieved by finding a suitable receptor which is highly expressed on the targeted tumor cells prior to, or upregulated after exposure to radiation, but absent or only present at low levels in normal tissues. In an attempt to identify a candidate receptor, we performed **in chapter 6** an analysis for c-Met expression upon exposure to irradiation. As a matter of fact, this receptor has been investigated in a set of cell lines of several tumors and a five-fold increase in its expression upon radiation exposure was observed, particularly in the cells showing radiation resistance. As a first step, we determined the intrinsic relative radiosensitivity character of the cells, using viability assays, by exposure of a panel of 6 TSCC cell lines to 4Gy of ionized radiation. Next, we investigated the c-Met expression pattern in our panel thoroughly by means of western blot and flow cytometry. In contrast to previous studies, we found variation in the overall expression of the c-Met that was not related to the intrinsic radiosensitive or radioresistant nature of the cells. Regarding the cell surface expression patterns, all but one of the cell lines showed abrupt downregulation in this receptor expression, but then increased with time. The remaining cell line showed an opposite pattern. For the intracellular expression, most of the cell lines showed a gradual increase in c-Met with time, peaking at day 5 after radiation which was obviously connected to mRNA synthesis. Since the cross talk between c-Met and EGFR has been widely demonstrated, we also investigated the expression of EGFR on the same cell lines. Strikingly, only in the radio resistant cell lines we found consistently c-Met and EGFR co-expression. Last but not least, we observed that the most radiosensitive cell line SCC-40 also acquires the highest invasive potential upon radiation. In conclusion, our analysis provides novel insights into the dynamic changes in the intracellular and extracellular c-Met profiles in native and radiation-exposed TSCC cells. Unfortunately, the relatively low surface expression percentages

disfavor the use of c-Met for nanoparticle-mediated targeted delivery, and shows the importance of surface expression analysis of cancer targeting candidates prior to developing targeted therapies. Further research is warranted to identify more suitable tumor cell surface markers for nanoparticle surface targeting.

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"*Gratitude is not only the greatest of virtues, but the parent of all others*."
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"*Better than a thousand days of diligent study is one day with a great teacher*."
Japanese Proverb

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"*A real friend is one who walks in when the rest of the world walks out.*"

Walter Winchell

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"*No matter how far we come, our parents are always in us.*"

Brad Meltzer

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أنتم هديتي بالدنيا

Curriculum Vitae
List of publications

CURRICULUM VITAE

Aisha Al-Jamaei was born in a small village, around 30 km from Sana'a, the capital city of Yemen. After finishing her primary school, Aisha had to stop at that level of education, because the secondary school was for only boys, due to the significant gender disparities in the country, during that period. Aisha was determined that she would not put up with such circumstances and continued chasing her dream of being well-educated. She finished her secondary schooling with very good marks, allowing her to join the Faculty of Dentistry, Sana'a University. In 2006, she got a scholarship from a German organization to study Masters and specialized in the subject of Oral Medicine and Pathology in Jordan. Aisha Al-Jamaei successfully completed her master's degree with excellent grades. Based on these academic credentials, she was appointed as a permanent lecturer at Sana'a University, Yemen. In 2013, in order to improve her clinical skills and knowledge, she spent 3 months of clinical training in Oral Pathology and Medicine in India. In January 2015, she got a Ph.D. scholarship from Islamic Development Bank, but because of the war, her Ph.D. admission was delayed. Aisha Al-Jamaei could start her Ph.D. in April 2016 at VU University Medical Center, in the Department of Oral and Maxillofacial Surgery / Pathology, which culminated in the creation of this thesis. While pursuing her Ph.D., she got a European Diploma certificate in Oral Medicine in September 2018.

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A review of the most promising biomarkers for early diagnosis and prognosis prediction of tongue squamous cell carcinoma.
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"Life without dreams is like a rainbow without colors"

Greyson Chance