Towards Personalized Medicine in Head and Neck Cancer

Insights into Biological Age, Oncogenic Pathways, and the Tumor Microenvironment.

Martine F. van der Kamp

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Colophon

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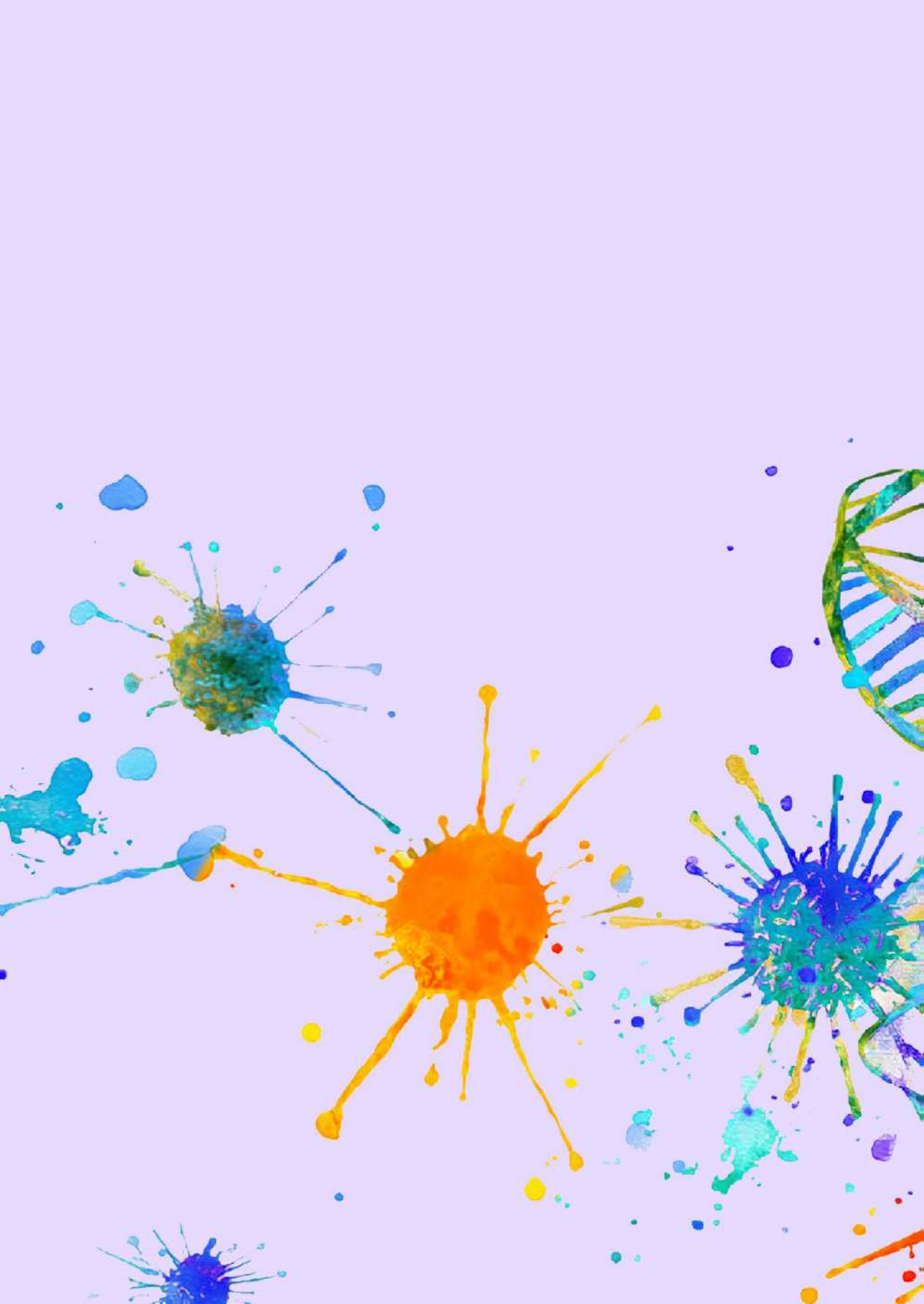

CHAPTER 1

General introduction and outline of this thesis

Martine F. van der Kamp

General introduction

Epidemiology and risk factors

Head and neck cancer is the sixth most common malignancy worldwide, resulting in approximately 890,000 new diagnoses and 450,000 deaths annually.[1] These tumors can arise in various anatomical regions, including the oral cavity, pharynx, larynx, nasal cavity, paranasal sinuses, and salivary glands, and include a variety of histopathological tumor types. Most head and neck malignancies are squamous cell carcinomas (SCC), originating from the mucosal linings of the upper aerodigestive tract. Among these, the oral cavity, pharynx, and larynx are the most affected sites. Therefore, this thesis concentrates on head and neck squamous cell carcinoma (HNSCC) originating from these primary anatomical locations.

Established risk factors for the development of HNSCC include heavy alcohol and tobacco consumption.[2] The synergistic effect of combining these two significantly increases the risk of HNSCC.[2]
Various viral infections are also associated with an elevated risk of HNSCC, including the Epstein-Barr virus (EBV), which gives an increased risk in developing nasopharyngeal SCC.[3] Nasopharyngeal carcinoma is characterized by distinct geographical distribution and is particularly prevalent in east and southeast Asia. The etiology is thought to be multifactorial, due to an interaction of EBV infection, environmental factors (such as the high consumption of preserved foods and smoking), and genetic predisposition.[4]
Infection with high-risk strains of the Human Papillomavirus (HPV), particularly HPV-16 and HPV-18, is widely acknowledged as a significant risk factor for oropharyngeal SCC.[5,6] There has been a notable rise in HPV-related oropharyngeal SCC, especially among males in developed countries and occurring at younger ages.[7] While specific data on this trend in the Netherlands are unavailable, Al-Jamaei et al. report that the prevalence of HPV-positive oropharyngeal SCC is higher among younger patients compared to HPV-negative oropharyngeal SCC.[8] This trend is likely driven by geographic differences in sexual behaviors, such as oral sex and multiple partners, which facilitate HPV transmission. Recently, however, increased incidence of HPV-related oropharyngeal cancer has also been noted in older age groups.[9] This rise is thought to be directly linked to higher rates of oral HPV infections and subsequent HPV-driven cancer in this population.
Other risk factors such as inherited diseases like Fanconi anemia, Dyskeratosis Congenita and Bloom's disease are characterized by specific genetic mutations that predispose individuals to various cancers, including HNSCC.[10,11] Additional contributors to the risk of HNSCC include family history, betel nut chewing, diet, occupational exposure, poor oral hygiene, radiation and immunosuppression.[2,12]

Prognosis

HNSCC represents a complex and challenging group of malignancies with diverse prognostic factors. One pivotal determinant of prognosis is tumor stage, globally assessed through the Tumor, Node, Metastasis (TNM) staging system established by the UICC AJCC.[13] This system describes the anatomical extent of disease per tumor site, incorporating conventional parameters such as tumor size, invasion, regional lymph node involvement, and distant metastasis.

Distant metastasis (DM) is an important prognostic factor in the TNM system, with reported incidences in HNSCC patients ranging from 3% to 52%.[14] The lungs, followed by bone and the liver, are the most frequently involved organs in metastatic disease.[14,15] The development of distant metastasis is frequently associated with an unfavorable prognosis.

HPV-related oropharyngeal SCC exhibits distinct biological behavior and is associated with a markedly improved prognosis compared to HPV-negative counterparts. Recognizing these differences, recent updates to the TNM staging system have incorporated pathological factors such as depth of invasion, bone invasion, and extra-nodal extension due to their significant impact on prognosis. These additions have enhanced the prognostic accuracy of the staging system.[16,17]

However, various histopathological biomarkers have not yet been integrated into the TNM system, as they remain subjects of ongoing investigation and debate. Examples include perineural invasion, tumor hypoxia, and features of the tumor microenvironment (TME). Continued research is necessary to determine their clinical relevance and utility.

Genetic and molecular analyses have advanced efforts to delineate clinical variations in HNSCC. Emerging biomarkers include EGFR, TP53 mutations, Bcl-2, ERCC1, VEGF, cyclin D1 amplification, specific gene signatures, and various components of the TME.[13,18,19] Although these biomarkers demonstrate potential, their application in routine clinical practice remains unestablished.
In addition to tumor-specific characteristics, 'host-related' prognosticators are gaining attention for their influence on outcomes. Factors such as patient age, performance status, and comorbidities are increasingly recognized as significant determinants of prognosis.[20] As research continues to advance, integrating these emerging biomarkers and host-related factors into staging systems may further refine prognostication and treatment strategies for HNSCC.

Despite advancements in treatment modalities, the improvement in survival rates for HNSCC has been modest. Subgroup analyses reveal enhanced survival rates across most age groups and anatomical sites, with the exception of elderly patients (>75 years) and laryngeal tumors.[21] Notably, the increased incidence of HPV-associated HNSCC has contributed to improved survival rates.[22]

Treatment

HNSCC poses a significant clinical challenge, with more than 60% of patients presenting at advanced stages (stage III and IV).[23] Advanced stage disease is generally characterized by local invasion and/or the presence of lymph node or distant metastases, necessitating comprehensive and aggressive multimodality treatment strategies.

Treatment for advanced HNSCC consists of a combination of surgery, radiotherapy, and systemic therapy. In cases with curative intent, the treatment goal is to achieve locoregional tumor control while preserving critical functions such as swallowing, airway protection, and voice quality. Conversely, in the palliative setting, the focus shifts towards symptom management, improving quality of life, and slowing disease progression. The intricacies of HNSCC management demand a multidisciplinary approach, tailored to the anatomical site, tumor stage, and individual patient factors, including performance status and preferences of the patients. In modern times, after considering all these factors, shared decision-making gains more popularity.

In recent decades, treatment decisions have undergone a paradigm shift, placing greater emphasis on the tumor's (sub)site and stage. In oral cavity SCC, for example, surgical resection, often complemented by adjuvant therapy, is the cornerstone of treatment. Early-stage disease is often managed by minimally invasive surgery. Oropharyngeal SCC can be effectively managed through transoral robotic surgery and laryngeal SCC with transoral laser microsurgery. Treatment of advanced stage disease generally involves a combination of radiotherapy and systemic therapy, with surgery reserved for select cases. In laryngeal SCC, treatment decisions depend on the functionality of the larynx. Preserving swallowing and airway function supports the recommendation of (chemo)radiotherapy in advanced-stage disease. However, when laryngeal function is impaired or anticipated to be lost post-treatment, a total laryngectomy becomes the recommended approach.

Adjuvant systemic therapy, including chemotherapy, targeted therapy, and immunotherapy, is administered to patients with a heightened risk of locoregional recurrence, primarily in the context of advanced-stage disease.

Age specific treatment

Notable shifts in HNSCC are occurring, driven by an increasing life expectancy that contributes to a growing population of older individuals diagnosed with cancer. Currently, the majority of HNSCC cases are identified in patients aged 60 and above, indicating a growing trend among older patients.[24] Previous studies have highlighted variations in treatment approaches for younger and older HNSCC patients, with advanced age serving as a predictor for receiving non-conventional treatments.[25,26] As age increases, older HNSCC patients are less likely to undergo surgical interventions, multimodality approaches, and tumor-directed treatments.[27–29] This observed trend contrasts with evidence from various studies demonstrating that older patients can tolerate more aggressive treatments, presenting comparable response rates and survival outcomes.[20] Within this group of older patients, a central challenge emerges — determining the most efficacious treatment strategy with minimal toxicity. Balancing these factors becomes essential to optimize therapeutic outcomes and enhance the quality of life for older patients with HNSCC.

Frailty screening and geriatric assessment

The determination of age poses a challenge, as the term "older" lacks a consistent definition and may appear conventional in estimating an individual's suitability for specific therapy. Chronological age, being a numerical value, fails to provide accurate insights into a patient's physical and mental condition. Recognizing this limitation, the concept of biological age offers a more precise estimation of a patient's overall condition. This measure, often synonymous with 'frailty,' can be identified through the Comprehensive Geriatric Assessment (CGA). The CGA is a multidisciplinary, multidimensional diagnostic and treatment process, that evaluates an individual's functional status, comorbidities, cognition, psychological state, social support, nutritional status, and medication.[30]

Frailty, defined as "*a state of increased vulnerability to poor resolution of homeostasis following a stressor, which increases the risk of adverse outcomes*," serves as a clinical reflection of an individual's biological age.[31] It is established that frail or biologically older patients face an increased risk of adverse treatment outcomes and loss of functioning.[32] Notably, HNSCC patients often exhibit frailty or a biologically older status, partly attributed to their unhealthy lifestyle.[33] In the specific context of HNSCC, frailty has been associated with an increased frequency and severity of postoperative complications, prolonged hospital stays, higher readmission rates, worse overall survival and quality of life.[34–36]

In light of these findings, utilizing a patient's biological age instead of chronological age has demonstrated superior predictive value for treatment tolerance in both oncological surgery and medical oncology.[37,38] This perspective underscores the importance of considering the multifaceted dimensions of aging when determining treatment strategies for HNSCC patients.

Tumor microenvironment

In recent years, there has been a growing focus on the TME due to its potential as a source of prognostic biomarkers and targets for immunotherapy. The composition of the TME varies between tumor types and sites, but generally encompasses innate immune cells, adaptive immune cells, stromal cells, blood vessels, and extracellular matrix components.[39,40] The interaction between the tumor and the TME can lead to either tumor suppression or tumorigenesis, as detailed in Table 1.1 (adapted with permission from Peltanova et al.[40]

Two main components of the TME that are further explored in this thesis are Tumor Infiltrating Lymphocytes (TILs) and Tumor Associated Macrophages (TAMs).

Table 1.1. Systematic overview of cell populations and their functions in the TME of HNSCC.

Cell type	Markers (human)	Increased production	Activity	Function
M1 TAMs	$CD68^+$	IL-12, IL-23, TNF-α, CCL-5, CXCL9, CXCL10, CXCL5	anti-tumor	Contribution to the Th1 response, inhibition of proliferation, cytotoxic activity
M2 TAMs	$CD163^+$	IL-1ra, IL-10, TGF-β, arginase-1	pro-tumor	Promotion of tumor progression, angiogenesis, suppression of T cell antitumor immune response
N1 TANs	$CD11b^+$, $CD14^+$, $CD15^+$, $CD16^+$, $CD62L^+$, $CD66b^+$	ICAM1, TNF-α	anti-tumor	Cytotoxic activity, increased NET formation
N2 TANs	$CD11b^+$, $CD14^+$, $CD15^+$, $CD16^+$, $CD62L^+$, $CD66b^+$	CXCR4, VEGF, MMP-9	pro-tumor	Promotion of angiogenesis, invasion
MCs	$CD117^+$, $CD203c^+$, $FcεRI^+$	Histamine, heparin, chondroitin sulfate E, PGD_2, tryptase, chymase, CPA1, LTC_4, GM-CSF, MMPs, IL-4, TNF-α, cathepsin G	pro-tumor	Promotion of angiogenesis, ECM degradation, stimulation of cancer cell proliferation, recruitment of immune cells

Table 1.1. (continued)

Cell type	Markers (human)	Increased production	Activity	Function
MDSCs	CD11b$^+$, CD33$^+$, CD14$^+$, CD15$^+$, CD16$^+$, HLA-DR$^-$	NO, ROS, iNOS, arginase-1, PD-L1, MMP-9	pro-tumor	Immunosuppression, inhibition of T cell activation and proliferation, promotion of angiogenesis, degradation of ECM
NK cells	CD3$^+$, CD16$^+$, CD56$^+$	IFN-γ, TNF-α, GM-CSF, IL-5, IL-8, IL-10, IL-13, CCL2, CCL3, CCL4, CCL5, CXCL10	anti-tumor	Cytotoxic activity without prior antigen presentation, modulation of adaptive immune response
NKT cells	CD3$^+$, CD56$^+$, CD161$^+$, CD1a$^+$, CD16$^+$	IFN-γ, TNF-α, GM-CSF, TGF-β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A	anti-tumor	Cytotoxic activity, antigen-specific immunological memory
Tregs	CD4$^+$, CD25$^+$, FOXP3$^+$	IL-10, IL-35, TGF-β, VEGF	pro-tumor	Immunosuppression, promotion of angiogenesis
Platelets	CD41$^+$, CD42a$^+$, CD42b$^+$, CD61$^+$	ADP, ATP, calcium, 5-HT, CD63, LAMP1/2, GP-Ib, P-selectin, integrin αII-β3, fibrinogen, vitronectin, thrombospondin, fibronectin, VWF, MMPs, GLUT3	pro-tumor	Thrombosis, wound healing, maintaining of homeostasis, vasoconstriction, promotion of cell proliferation, immunoevasion by platelet aggregation
CAFs	α-SMA$^+$, FAP$^+$, FSP-1$^+$, CD33$^-$, absent cytokeratin	EGF, HGF, VEGF, CXCL12, CXCL14, CCL5, CCL7, IL-6, IL-17A, MMPs	pro-tumor	Stimulation of tumor growth, invasion, angiogenesis, metastasis, induction of chemo- and radio-resistance, ECM degradation

Reproduction from Peltanova et al. 2019[40]. Used under the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).
Abbreviations: *TAMs* tumor-associated macrophages, *TANs* tumor-associated neutrophils, *MCs* mast cells, *MDSCs* myeloid-derived suppressor cells, *NK* natural killer cells, *NKT* natural killer T cells, Tregs regulatory T cells, CAFs cancer-associated fibroblasts, *NET* neutrophil extracellular traps, *ECM* extracellular matrix

TILs

Tumor Infiltrating Lymphocytes (TILs) constitute a subpopulation of immunocompetent lymphocytic cells including T cells, B cells, and natural killer (NK) cells. Among these, a subset of CD3+ and CD4+ T helper type 1 (Th1) lymphocytes, cytotoxic CD3+ and CD8+ T cells (CTLs) and regulatory T lymphocytes CD4+, CD25+, Foxp3+ (Tregs) are most prominent in the TME. In recent years, many studies have explored the prognostic

significance of TILs in HNSCC. The overall conclusion is that tumors with substantial TIL infiltration in the TME are associated with better outcomes.[40,41]

In a healthy immune system, the PD-1 receptor, expressed on activated T cells, interacts with its ligands, PD-L1 and PD-L2, preventing excessive inflammatory or autoimmune responses. The modulation of the PD-1 pathway within the TME may contribute to potential immune evasion. Tumor cells expressing PD-L1 can reduce T-cell effector activity, dampening immune responses. Tumor-infiltrating T lymphocytes from the host mediate PD-L1 expression through the secretion of interferon-γ, illustrating the PD-1 pathway's dual function in preserving immune balance and facilitating immune escape in tumorigenesis.[42]

TAMs

Tumor Associated Macrophages (TAMs) are key immunosuppressive cells in the TME and play a critical role in promoting tumor progression, angiogenesis, and immune response suppression. TAMs support tumor immunity, cell growth, proliferation, local invasion, and distant metastasis. They modulate immune response through pathogen phagocytosis and antigen presentation.

TAMs can polarize towards two types of phenotypes: M1-like macrophages and M2-like macrophages. M1-like macrophages release pro-inflammatory cytokines and chemokines that stimulate the expression of PD-L1 on macrophages. They actively engage in the anti-tumor immune response by enhancing Th1 activity, suppressing proliferation, and exerting cytotoxic abilities. M2-like macrophages have anti-inflammatory properties. Their capacity to generate immune-inhibitory factors positions them in roles related to tissue remodeling, angiogenesis, and tumor progression.[41] Recent studies have affirmed that high levels TAMs within the TME are correlated with an unfavorable prognosis in HNSCC patients.[43,44]

An illustrative simplified image of these cell populations and their roles within the TME is presented in Figure 1.1.[39]

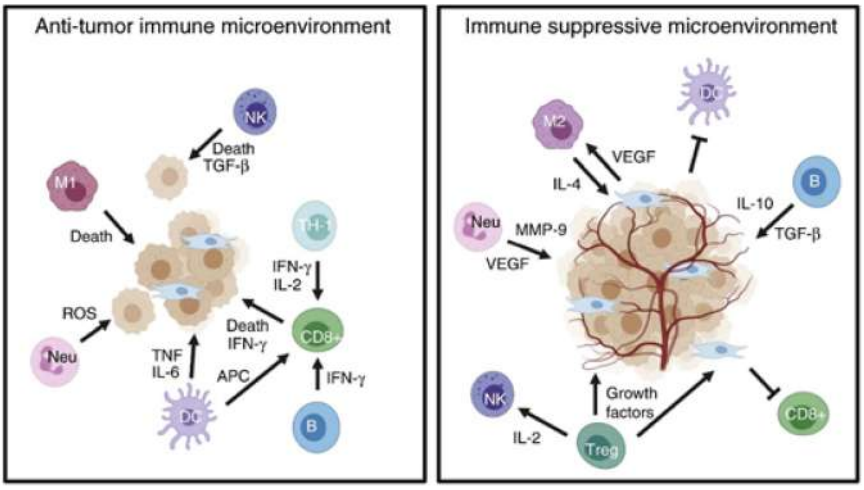

Figure 1.1. Influence of immune cells in the TME.
(Reproduced with permission from Anderson NM and Simon MC (2020) "The tumor microenvironment." *Current Biology*, 30(16): R921–R925. © 2020 Elsevier.)[39]

The composition and activity of these immune cells in the TME seem to play a crucial role in the development and progression of HNSCCs. Exploring the variations in the stromal composition of the HNSCC TME may provide valuable insights into the mechanisms underlying differential therapeutic responses.

Immunosenescence

One prominent aspect of the older population is a natural deterioration of the immune system with age, also called *immunosenescence*.[45] This gradual deterioration of immunity leads to diminished overall immunosurveillance, which is associated with inflammation and malignancies in the elderly population.[45,46] It is suggested that immunosenescence is based on multiple, distinct alterations in the antitumor immune response, as illustrated in Figure 1.2 (based on the figure of Daste et al).[46]

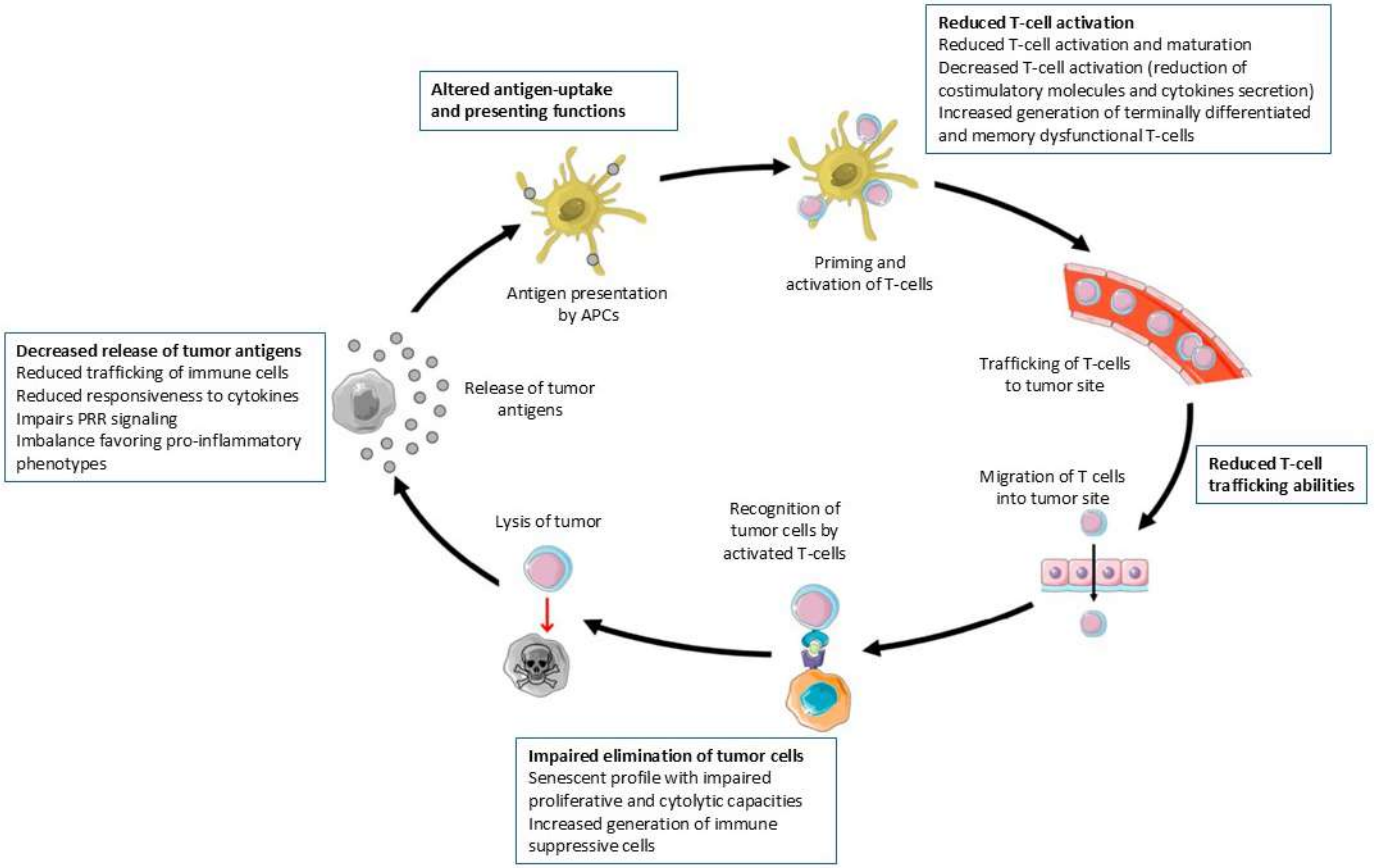

Figure 1.2. Immunosenescence could impair each step of the antitumor immune response. Reproduced from Daste et al. (2017)[46], Figure 1.2. Immune checkpoint inhibitors and elderly people: A review. European Journal of Cancer, 82, 155–166. © 2017 Elsevier. Reproduced with permission.[46]

Age-related changes in the hematopoietic stem cell (HSC) compartment contribute to decreased immune surveillance in the elderly, potentially promoting tumorigenesis. While the number of HSCs increases in older individuals, their shortened telomere length hinders their proliferation and generation of naive lymphocytes.[47] These changes also disrupt the balance among the various lineages derived from HSCs, leading to an overall reduction in the generation of lymphoid cells and a concurrent increase in the differentiation toward the myeloid lineage.[48] Furthermore, immunosenescence is associated with a decrease in the release of tumor antigens, thus impairing the initiation of antitumor immune responses.[46] Additional, diminished numbers of antigen-presenting cells (APCs) and changes in their ability to uptake and present antigens are linked to the ageing process, resulting in an overall dampened cancer immunosurveillance.[49]

Immunosenescence is also associated with impaired T-cell activation. An overall reduction in the production of naive T cells due to thymic involution leads to a reduced production of mature naive T cells.[50] Moreover, naive mature T cells migration from thymus to secondary lymphoid organs is thought to be reduced. [51] Furthermore, senescent T cells seem to be characterized by a reduced ability to eliminate tumor cells.[46] This reduction of the cytolytic functions of T lymphocytes eminently contributes to tumor growth.

Immunotherapy

A relatively new systemic therapy option that emerged in recent years in cancer treatment is immunotherapy. Immune checkpoint inhibitors that target the programmed cell death protein 1 (PD-1) receptor on T cells, such as Nivolumab and Pembrolizumab, showed remarkable results in patients diagnosed with advanced staged melanoma, lung- and renal cell carcinoma.[52] For HNSCC, the role of Anti-PD-1 therapy in the curative setting is being explored, but to date, the evidence is not convincing and appears to offer no additional benefit over existing therapies.[53,54] Additional immune checkpoint receptors, including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), are increasingly being analyzed for potential application in monotherapy or in combination with other immunotherapies or conventional cancer therapeutics.[55]

Regarding immunosenescence, aging appears to affect multiple stages of tumor recognition and elimination, potentially leading to a less effective anti-tumor immune response.[46] This raises concerns about the efficacy of immunotherapy, as an aging immune system may be less capable of mounting a robust response. Consequently, a better understanding of immunosenescence could assist in identifying appropriate

candidates for immunotherapy, especially since older and very elderly patients (>75) are often under-represented in clinical trials.[56] Notably, only one randomized controlled trial on targeted therapy specifically in older patients has been published.[57] Furthermore, the older patients included in clinical trials tend to be healthier than the general elderly population, often exhibiting better overall health status and organ function.

The rising life expectancy has resulted in a larger population of older individuals diagnosed with HNSCC, highlighting the challenge of developing effective treatments tailored to this group. This thesis delves into the unique aspects of HNSCC in the elderly and addresses several key inquiries. First, it aims to investigate potential variations in epidemiology and tumor biology in older patients compared to their younger counterparts. Second, it seeks to underscore the significance of biological age over chronological age as a predictor for treatment outcomes, particularly its influence on the TME. Finally, the landscape of the TME is further explored, with specific interests in its possible contribution in determining patient's prognosis. The goal is to provide insights that contribute to more effective and personalized approaches for HNSCC patients, with a particular focus on the older patient population.

Outline of this thesis

This thesis explores the epidemiological, biological, and immunological aspects of HNSCC, with a particular focus on older patients. It highlights the role of biological age and the tumor microenvironment (TME) in predicting outcomes, aiming to support more personalized and effective treatment strategies.

The second and third chapters of this thesis focus on age-related clinical aspects of HNSCC. *Chapter 2* analyzes trends in treatment patterns and survival over the past 20 years in older HNSCC patients, using data from the Netherlands Cancer Registry. *Chapter 3* addresses conflicting findings in the literature regarding age as a risk factor for distant metastases (DM) and investigates age alongside previously identified predictors of DM in HNSCC patients.

Chapter 4 presents a narrative review of age-specific oncogenic pathways in HNSCC and identifies a subcategory of patients with distinct pathophysiology: elderly (HPV-negative) HNSCC patients without a history of tobacco or alcohol consumption.

Chapter 5 explores the relationship between TME and both chronological and biological age, as well as the potential impact of these variations on survival outcomes in HNSCC patients. Chapters 6 and 7 further investigate the predictive and prognostic relevance of the TME. *Chapter 6* provides a comprehensive systematic review and meta-analysis of the prognostic role of tumor-associated macrophages (TAMs) in HNSCC. *Chapter 7* evaluates the added predictive value of the TME composition beyond conventional clinicopathological characteristics in predicting recurrence in OSCC.

Chapter 8 concludes with a summary of the main findings, a general discussion, and an elaboration on future perspectives.

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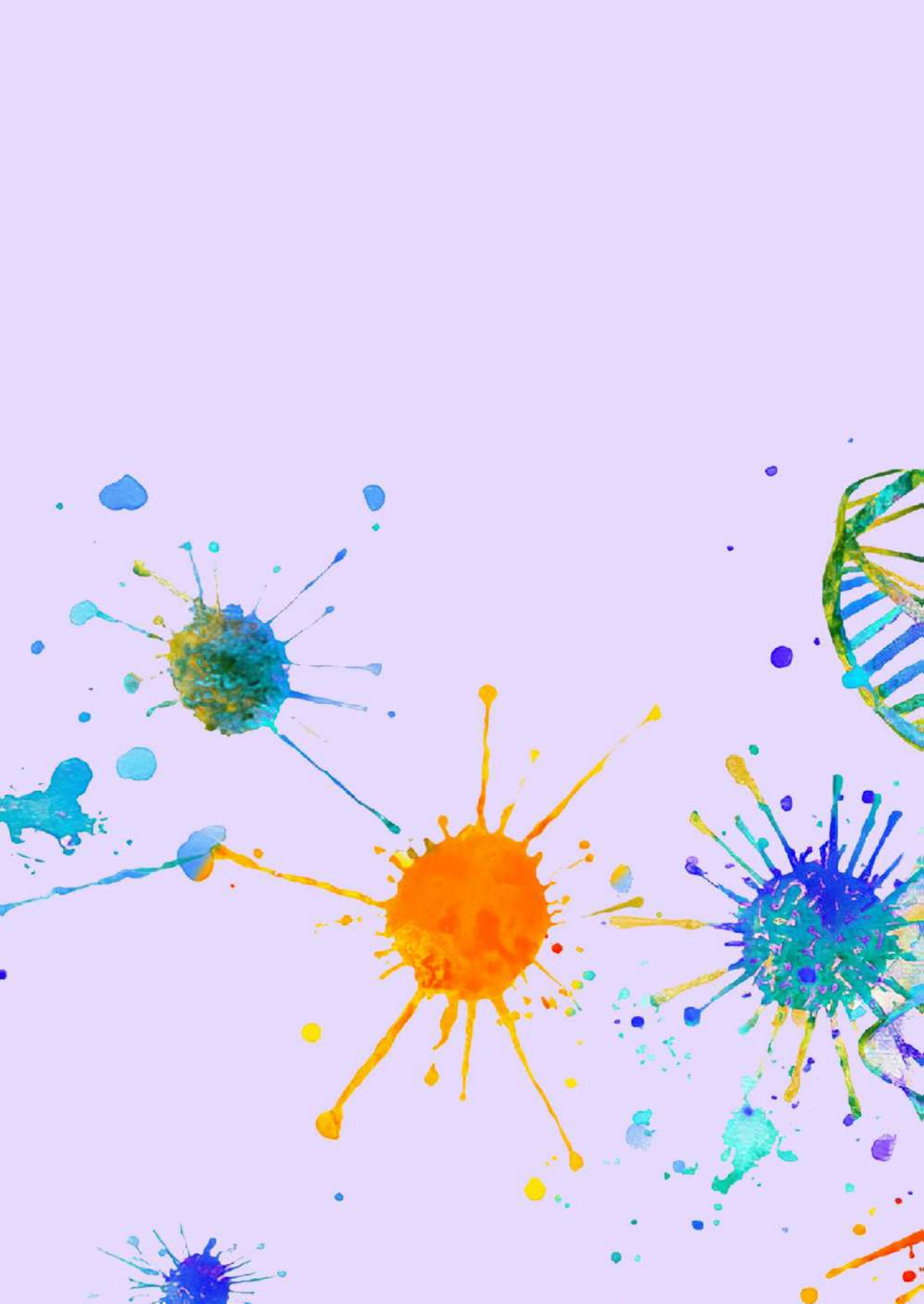

CHAPTER 2

To what extent has the last two decades seen significant progress in the management of older patients with head and neck cancer?

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Abstract

Introduction

Life expectancy is rising and therefore also the number of older patients with head and neck cancer. Different treatment regimens are often applied for older patients. The aim of this study is to investigate how treatment patterns and survival rates have changed over the past 20 years in older patients with head and neck squamous cell carcinoma (HNSCC).

Materials and methods

Patient and tumor characteristics, treatment and 5-year survival data from the Netherlands Cancer Registry of patients aged ≥60 years diagnosed with HNSCC in 1990-1995 and 2010-2015 were compared using chi-square test and relative survival analysis.

Results

Data of 14,114 patients were analyzed. Oral cavity cancer treatment did not change over time, while survival improved from 54% to 58% ($p = 0.03$). Oropharyngeal and hypopharyngeal cancer treatment shifted towards non-surgical, with survival improving from 31% to 51% ($p < 0.01$) and 26% to 34% ($p < 0.01$), respectively. Laryngeal cancer treatment changed towards surgery in stage I and non-surgical treatment in stage III and IV disease. Survival in laryngeal cancer stage I remained stable and favorable at a relative survival rate of around 90%. Survival non-significantly changed from 54% to 49% for stage III disease and from 37% to 33% for stage IV laryngeal cancer.

Conclusion

Relative survival increased for all head and neck cancer sites in older patients, except for laryngeal cancer. For oropharyngeal, hypopharyngeal and advanced laryngeal cancer, a shift towards non-surgical treatment modalities was observed.

Introduction

Over the past decades, life expectancy has been rising.[1] Cancer can be considered an age-related disease, either directly or through age-related factors.[2,3] More than half of head and neck cancer cases occur in patients aged 60 years or older,[4] and the proportion of these older patients is increasing.[4,5]

Treatment protocols have changed over time, becoming more (sub)site- and stage-specific. Treatment recommendations became more individualized due to a multidisciplinary approach.[6] Treatment of advanced head and neck cancer has changed towards non-surgical treatment modalities such as conformal and intensity-modulated radiotherapy and additional systemic therapy, such as Cetuximab, to reduce treatment burden and retain functionality.[7,8] Several studies show similar survival rates when applying concomitant chemoradiotherapy instead of surgical treatment in advanced stage laryngeal cancer to preserve laryngeal function.[9,10] Based on these studies, the organ preservation protocol was introduced. Treatment of early stage laryngeal cancer has also developed. Transoral laser surgery is nowadays widely used, since this technique has shown good results with reduced complications, good local control and respectable functional outcome.[11–15]

Earlier research identified differences in treatment patterns between younger and elderly patients, with older age being predictive of receiving non-standard treatment.[16,17] With the increase of age, head and neck squamous cell carcinoma (HNSCC) patients are less likely to undergo surgical, multimodality and tumor-directed treatment.[18] This despite the fact that several studies have demonstrated that elderly patients can tolerate more intensive treatment with similar response and survival outcomes.[2,19–22]

To our knowledge, it has not yet been investigated how treatment regimens and survival rates have changed over time in older HNSCC patients. The aim of our study is to compare changes between 1990-1995 and 2010-2015 in older HNSCC patients in (1) the numbers of patients, (2) treatment and (3) relative survival.

Materials and methods

Patients

Anonymous data were obtained from the Netherlands Cancer Registry (NCR). The most important signaling source for the NCR are pathology reports from the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA). Trained NCR personnel gather population-based data on patient, tumor and treatment characteristics directly from the medical records. The NCR was shown to encompass a high level of reliability and completeness.[23]

Patients were included in the study when they were aged 60 years and older with a newly diagnosed invasive squamous cell carcinoma (SCC) of the oral cavity, oropharynx, hypopharynx, or larynx, diagnosed in 1990-1995 or 2010- 2015. In case of multiple tumors in the head and neck region, only the first diagnosed tumor was included. Patients with distant metastasis were excluded. Included variables were gender, age, clinical stage, final stage, treatment regimen and vital status. This study was approved by the privacy review board of the NCR.

Age categories

In this study, a cut-off for patients aged 60 years and older was chosen. For detailed analysis of the role of age in head and neck cancer treatment and survival in older patients, patients were categorized into 5 age groups: 60-64 years, 65-69 years, 70-74 years, 75-79 years and ≥80 years.

Tumor staging

Tumors were staged according to the UICC TNM classification system, editions TNM_4 (1987), revised TNM_4 (1992) in the cohort of 1990-1995 and TNM_7 (2010) in the cohort of 2010-2015. Stage was categorized as stage I, II, III and IV.

Treatment categories

For treatment, we distinguished surgery, radiotherapy, chemotherapy or combinations of these modalities and no (tumor-directed) treatment. The proportionate share of surgery, radiotherapy and chemotherapy for specific sites was compared between the entire cohort of the two periods as well as for the specific age groups.

Statistical analysis

Baseline characteristics were compared between the two periods and presented as frequencies. Pearson's chi-square test was used to compare the distribution of variables for the two periods.

Survival rates were based on the number of years between the date of diagnosis and the date of death, emigration, or censoring date (January 31, 2019). Relative survival rates were calculated using the Ederer II method for relative survival.[24] In brief, this is the ratio of the observed survival rate compared with the expected survival rate (based on sex, age and calendar year number from Statistics Netherlands (CBS). Poisson regression modeling was used to calculate relative excess risk of dying[25] for period 2010-2015 compared to period 1990-1995.

P-values are presented to indicate whether a statistically significant difference between periods existed, a *p*-value < 0.05 was considered statistically significant.
Statistical analyses were performed using Stata/SE 14.2.

Results

Patient and tumor characteristics

In total, 14,114 patients were included in the analyses (Table 2.1). The number of all 60+ HNSCC patients was larger in the second period. In the second period, the number of oral cavity and hypopharyngeal SCC patients almost doubled (from 1,569 to 3,061 and 380 to 714, respectively). The number of oropharyngeal SCC more than doubled (from 719 to 1,919 patients), while the number of laryngeal SCC remained more or less consistent (2,772 in 1990-1995 and 2,989 in 2010-2015).

The population of the Netherlands grew from 14,892,574 to 16,574,989 inhabitants, and life expectancy has gradually increased from 77 years in 1990-1995 to 81-82 years in 2010-2015.[26] In our study, the male-to-female ratio changed from 3.2:1 to 2.1:1. This increase in the proportion of women was most pronounced in laryngeal SCC. The proportion of stage IV disease was larger in the second period and observed for all tumor sites.

Table 2.1. Patient characteristics per tumour site compared between 1990-1995 and 2010-2015.

	HNCSC- all sites			Oral cavity			Oropharynx			Hypopharynx			Larynx		
	1990-1995	2010-2015	P-value	1990-1995	2010-2015	P-value	1990-1995	2010-2015	P-value	1990-1995	2010-2015	P-value	1990-1995	2010-2015	P-value
Total	5440	8674		1569/5440	3061/8674		719/5440	1919/8674		380/5440	714/8674		2772/5440	2989/8674	
	(100%)	(100%)		(29%)	(35%)		(13%)	(22%)		(7%)	(8%)		(50%)	(34%)	
Gender			<0.01			0.05			0.02			0.90			<0.01
Man	4143	5884		868	1602		507	1264		303	567		2465	2451	
	(76%)	(68%)		(55%)	(52%)		(71%)	(66%)		(80%)	(79%)		(89%)	(82%)	
Woman	1298	2789		701	1459		212	655		77	147		307	529	
	(24%)	(32%)		(45%)	(48%)		(30%)	(34%)		(20%)	(21%)		(11%)	(18%)	
Age category*			<0.01			0.12			<0.01			<0.01			<0.01
60-64	1381	2388		347	739		221	679		108	234		705	736	
	(25%)	(28%)		(22%)	(24%)		(31%)	(35%)		(28%)	(33%)		(25%)	(25%)	
65-69	1381	2260		350	739		89	552		91	216		751	753	
	(25%)	(26%)		(22%)	(24%)		(26%)	(29%)		(24%)	(30%)		(27%)	(25%)	
70-74	1142	1517		303	523		157	338		79	122		603	534	
	(21%)	(17%)		(19%)	(17%)		(22%)	(18%)		(21%)	(17%)		(22%)	(18%)	
75-79	788	1192		231	444		90	187		60	74		407	487	
	(14%)	(14%)		(15%)	(15%)		(13%)	(10%)		(16%)	(10%)		(15%)	(16%)	
≥80	748	1317		338	616		62	163		42	68		306	470	
	(14%)	(15%)		(22%)	(20%)		(9%)	(9%)		(11%)	(10%)		(11%)	(16%)	
cTNM			<0.01			<0.01			<0.01			<0.01			<0.01
I	1552	2297		358	942		48	110		16	16		1130	1229	
	(29%)	(27%)		(23%)	(31%)		(7%)	(6%)		(4%)	(2%)		(41%)	(41%)	
II	1260	1601		413	674		73	255		41	69		733	603	
	(23%)	(19%)		(26%)	(22%)		(10%)	(13%)		(11%)	(10%)		(26%)	(20%)	
III	861	1252		269	277		174	310		77	109		341	556	
	(16%)	(14%)		(17%)	(9%)		(24%)	(16%)		(20%)	(15%)		(12%)	(19%)	
IV	1530	3385		413	1081		396	1226		228	515		493	563	
	(28%)	(39%)		(26%)	(35%)		(55%)	(64%)		(60%)	(72%)		(18%)	(19%)	
Unknown	237	139		116	87		28	18		18	5		75	29	
	(4%)	(2%)		(7%)	(3%)		(4%)	(1%)		(5%)	(1%)		(3%)	(1%)	

*Age at diagnosis.

Treatment modality

Surgical treatment vs. non-surgical treatment

Changes between the two periods regarding treatment and age are shown in Tables 2.2, 2.3 and 2.4. Treatment in oral cavity SCC was not different between the investigated periods, surgical treatment was still the cornerstone of the treatment with 85% in all age categories ($p = 0.89$), varying between 78% and 89% in 2010-2015 (Table 2.2a). The proportion of non-surgical treatment in oropharyngeal SCC was significantly larger in 2010-2015 (80%) than in 1990-1995 (56%), $p < 0.01$ (Table 2.2b). This larger proportion was observed in all age categories, except in those 80 years or older. The proportion of non-surgical treatment in hypopharyngeal SCC showed a similar pattern: the proportion equaled 81% in 2010-2015 and 55% in 1990-1995 and significantly increased in all age groups except those 80 years or older (stable at 77%), $p < 0.01$ (data not shown). The proportion of non-surgically treated laryngeal SCC patients significantly decreased from 77% in 1990-1995 to 63% in 2010-2015 $p < 0.01$ (Table 2.3). Table 2.3 shows changes in treatment modality by stage for laryngeal SCC. The proportion of non-surgical treatment decreased for stage I and II tumors and increased for stage III and IV tumors. In stage I, non-surgical treatment significantly decreased from 86% in 1990-1995 to 40% in 2010-2015, $p < 0.01$. In stage IV, the preferred treatment shifted from surgical treatment towards non-surgical treatment, demonstrated by a significant increase from 36% in 1990-1995 to 66% in 2010-2015 for non- surgical treatment ($p < 0.01$).

Table 2.2a. **Proportion of surgically[a] treated patients in oral cavity SCC by age category for 1990–1995 and 2010-2015.**

	Oral cavity SCC		
Age category[b]	**1990–1995 n (% of total)**	**2010–2015 n (% of total)**	**Δ%, (95% CI), *p*-value[c]**
60–64	289 (87%)	596 (86%)	−1%, (−6%; 4%), 0.66
65–69	292 (86%)	614 (87%)	1%, (−3%; 6%), 0.60
70–74	258 (89%)	431 (87%)	−3%, (−7%; 2%), 0.29
75–79	173 (82%)	361 (89%)	7%, (1%; 13%), 0.02
≥80	232 (80%)	391 (78%)	−3%, (−8%; 3%), 0.37
All 60+	1244 (85%)	2393 (85%)	0, (−2%;-2%), 0.89

[a] Surgically treated = primary surgical treatment with or without adjuvant treatment modalities (i.e. systemic therapy and/or radiotherapy); [b] Age at diagnosis; [c] $p < 0.05$ was considered statistically significant.

Table 2.2b. Proportion of non-surgical[a] treated patients in oropharyngeal SCC by age category for 1990–1995 and 2010–2015.

	Oropharyngeal SCC		
Age category[b]	1990–1995 n (% of total)	2010–2015 n (% of total)	Δ%, (95% CI), *p*-value[c]
60–64	100 (50%)	498 (79%)	29%, (21%; 36%), <0.01
65–69	99 (57%)	414 (81%)	25%, (17%; 32%), <0.01
70–74	83 (58%)	251 (80%)	22%, (13%; 31%), <0.01
75–79	41 (56%)	126 (79%)	23%, (11%; 36%), <0.01
≥80	33 (75%)	102 (85%)	10%, (−3%; 23%), 0.14
All 60+	356 (56%)	1394 (80%)	24%, (20%; 28%), <0.01

[a] Non-surgical treated = systemic therapy and/or radiotherapy, other; [b] Age at diagnosis; [c] $p < 0.05$ was considered statistically significant.

Table 2.3. Proportion of non-surgical[a] treated patients in laryngeal SCC by stage for 1990–1995 and 2010-2015.

	Laryngeal SCC		
Stage	1990–1995 n (% of total)	2010–2015 n (% of total)	Δ%, (95% CI), *p*-value[b]
I	949 (86%)	481 (40%)	−46%, (−50%;-42%), <0.01
II	669 (93%)	520 (88%)	−5%, (−8%;-2%), <0.01
III	230 (70%)	467 (89%)	18%, (13%; 24%), <0.01
IV	164 (36%)	326 (66%)	31%, (24%; 37%), <0.01
All stages	2035 (77%)	1799 (63%)	−13%, (−16%;-11%), <0.01

[a] Non-surgical treated = systemic therapy and/or radiotherapy, other; [b] $p < 0.05$ was considered statistically significant.

Chemotherapy

In Table 2.4, chemotherapy treatment in HNSCC in the specific age categories was compared between 1990-1995 and 2010-2015. The proportion of chemotherapy was significantly higher in 2010-2015 (13%) compared to 1990-1995 (4%) ($p < 0.01$). This higher proportion was exclusive for the younger age groups: the proportion significantly increased from 5% to 24% in the 60-64 age category ($p < 0.01$), from 5% to19% in 65-69 age category ($p < 0.01$), and from 3% to 5% in the 70-75 category ($p = 0.03$). No significant changes were observed in patients aged 75 years and older.

Table 2.4. Chemotherapy in different age categories for 1990–1995 and 2010–2015 in HNSCC.

	Chemotherapy		
Age category[a]	1990–1995 n (% of total)	2010–2015 n (% of total)	Δ%, (95% CI), *p*-value[b]
60–64	75 (5%)	574 (24%)	19%, (16%; 21%),<0.01
65–69	63 (5%)	420 (19%)	14%, (12%; 16%), <0.01
70–74	37 (3%)	75 (5%)	2%, (0%; 3%), 0.03
75–79	16 (2%)	17 (1%)	−1%, (−2%; 1%), 0.30
≥80	14 (2%)	13 (1%)	−1%, (−2%; 0%), 0.09
All 60+	205 (4%)	1099 (13%)	9%, (8%; 10%), <0.01

[a] Age at diagnosis; [b] $p < 0.05$ was considered statistically significant.

5-year relative survival

The 5-year relative survival rate for all HNSCC was comparable for both periods: 57% in 1990-1995 and 58% in 2010-2015 ($p = 0.40$, data not shown). Figure 2.1 displays differences in survival between the two periods per tumor site.

In oral cavity SCC (Figure 2.1), a significant increase in survival rates from 54% to 58% was observed in all 60+ patients ($p = 0.03$) with the largest increase observed in the 75-79 age category from 50% to 60% ($p < 0.01$).

Also in oropharyngeal SCC, a significant increase was observed in all 60+ patients (from 31% to 51%, $p < 0.01$) and in the 60-79 age categories (varying between 25-36% to 40-54%, $p < 0.01$, Figure 2.1).

Likewise, a significant increase was observed in all 60+ hypopharyngeal SCC patients (from 26% to 34%, $p < 0.01$), and in the 60-64 and 70-74 age categories (from 25% to 38%, $p = 0.02$; and 21% to 36%, $p = 0.04$, respectively; Figure 2.1).

For laryngeal SCC, the survival rate was comparable for the two periods (69% in 1990-1995 and 68% in 2010-2015; $p = 0.31$) (Figure 2.1). No notable differences among the different age categories were observed.

For laryngeal SCC, stage-specific analyses were additionally conducted and are shown in Figure 2.2. A stable survival rate of 90% was observed in stage I laryngeal SCC, $p = 0.88$, with minor insignificant differences between the periods in all age categories. In stage II, relative survival rate estimates were mostly (but never significantly) higher in the second period. Relative survival rates did not improve, with non-statically significant lower rates in 2010-2015 compared to 1990-1995 for stage III and stage IV tumors (54% to 49%, $p = 0.45$ and 37% to 33%, $p = 0.09$, respectively).

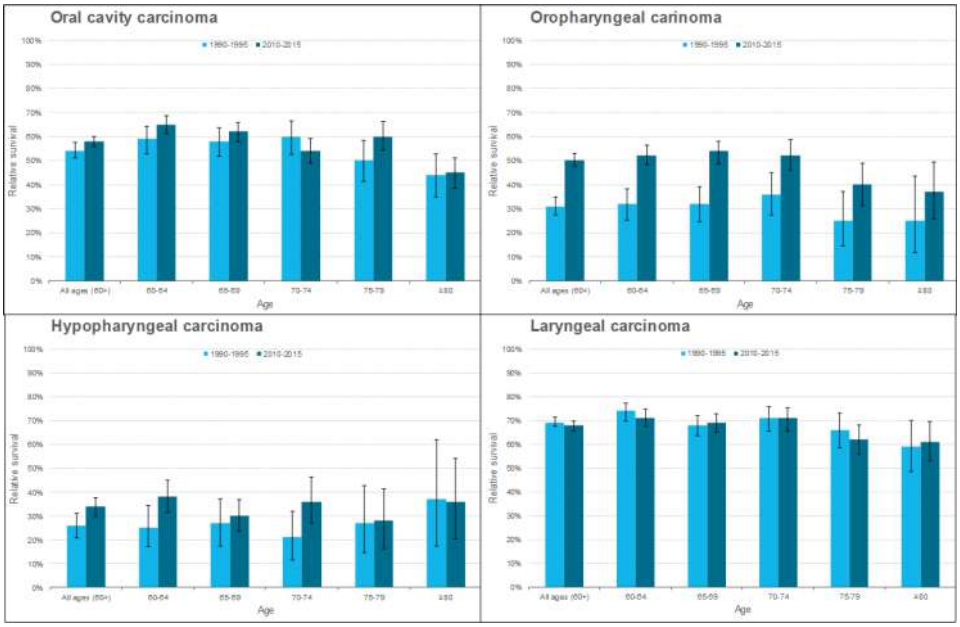

Figure 2.1. Five-year relative survival per age category per site HNSCC.

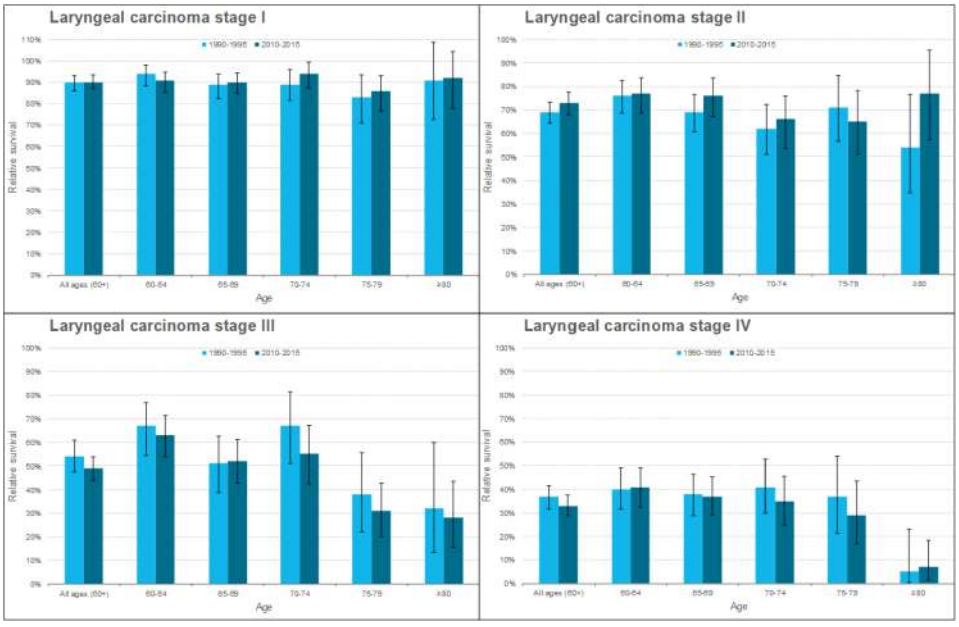

Figure 2.2. Five-year relative survival per age category in stage I-IV laryngeal SCC.

Discussion

In this study, we identified evident changes in the treatment regimens of older HNSCC patients between 1990-1995 and 2010-2015. Most notable is the shift in primary treatment of oropharyngeal SCC from surgical towards non-surgical treatment. This treatment paradigm shift was accompanied by significantly improved survival rates.
Furthermore, the proportion of non-surgical treatment in early stage laryngeal SCC was lower in 2010-2015 compared to 1990-1995, probably due to the introduction of transoral laser surgery. This change did not affect the favorable survival rates of these patients. However, the introduction of organ preserving protocols in advanced stage laryngeal SCC do not seem to have resulted in improved survival rates. Moreover, survival rates decreased in 2010-2015 compared to 1990-1995 albeit at an insignificant degree. No specific age-related changes regarding treatment regimen were observed between the two periods.

Our study showed an increase in the proportion of oropharyngeal, hypopharyngeal and oral cavity SCC. This increase of oropharyngeal SCC was also observed in other studies, most expressed in young, white patients, and thought to be related to HPV infection.[27–32] However, these studies described incidence trends that were established more than a decade ago. A more recent study found that also in older patients the incidence of (HPV-related) oropharyngeal cancer is increasing.[33] Increased incidence of HPV-related oropharyngeal cancer in older patients may also partly explain the increased survival in our cohort, since HPV-positive oropharyngeal cancers represent a unique disease entity with superior survival compared to HPV-negative oropharyngeal SCC.[33–35] Unfortunately, information on HPV status was not available for this study. Nevertheless, in a study of Rietbergen et al., a significant increase in the proportion of HPV-positive oropharyngeal SCC in the Netherlands was observed, from 5.1% in 1990 to 29.0% in 2010 (p = 0.001).[36]

Tobacco smoking and alcohol use are the most well known risk factors for oral cavity SCC.[37] Over the last decades an increase in the incidence of oral cavity SCC has been observed.[30,38,39] However, this increase has been reported to be most pronounced in young, non-smoking women, a subgroup that is not included in the current study.[30,38] In non-smoking oral SCC patients, tongue SCC is frequently seen in young female patients while gingival/buccal SCC affects elderly women.[40] The reason for the increase of oral cavity SCC in this population of young women remains unknown.[38,41] The higher proportion of oral cavity SCC in 2010-2015 might be explained by the ongoing effect of tobacco smoking in the 80s and 90s, which may have resulted in an increase in oral cavity SCC approximately 20 years later. In the Netherlands, the total percentage of

smokers (> 18 years) decreased from 35% in 1990-1995 to 25% in 2010-2015.[42] Unfortunately, data on this item was lacking in our study cohort for this time period. On the other hand, this effect would then also be expected in the group of laryngeal SCC patients, of which tobacco smoking is likewise a major risk factor.[43] In this study a lower proportion of laryngeal SCC was observed in 2010-2015 compared to 1990-1995 (in particular in men), which may actually reflect the impact of antismoking campaigns. This decrease in laryngeal SCC is also confirmed by other authors.[29,44] Unfortunately, information on tobacco usage and alcohol consumption was lacking in our cohort. Overall, the reason for the higher proportion of oral cavity SCC in the latter period in our cohort remains elusive.
The higher proportion of stage IV disease in 2010-2015 compared to 1990-1995 was seen in all primary HNSCC sites. This higher proportion may be explained by improved diagnostic imaging, resulting in the ability to detect smaller metastases and better possibilities to visualize bone invasion. However, since this was not the scope of our study, we cannot substantiate this statement.

Since the 1990s, the management of head and neck cancers has improved with the use of concurrent chemoradiation.[9,10,45,46] Radiotherapy techniques, including three-dimensional conformal radiotherapy (3D-CRT), intensity-modulated radiotherapy (IMRT), and proton radiotherapy, have been developed. With these techniques the conformal degree of target areas is increased and the radiation dose and toxicity of surrounding organs is therefore reduced.[47,48] Also the introduction of transoral laser surgery has contributed to reduced morbidity rates and fewer complications, resulting in an improvement of quality of life.[49,50] Similarly, advances in the overall care of the patients, including individual nutritional support, have resulted in the improvement of quality of life and reduced complication rates.[51] These developments have undoubtedly also improved the survival rates of HNSCC patients. The findings for this time period are mirrored in similar studies in other European countries.[52] The most striking increase in survival in our study was seen in oropharyngeal SCC patients, which was also observed in several other studies.[33,35] The rise in incidence of HPV-related oropharyngeal cancer, linked to better prognosis, is one of the logical explanations to the improved survival rates.[35] However, the treatment shift towards non-surgical treatment and development of non-surgical treatment techniques are possibly also responsible for these improved survival rates in oropharyngeal SCC.[46]
Laryngeal SCC survival rates were consistent between 1990-1995 and 2010-2015. Worldwide, this lack of improvement of survival in laryngeal SCC is confirmed.[44,53-55] The shift towards organ-preservation treatment is assumed to be one of the reasons for this lack of improvement.[55–57] The decrease in primary surgical treatment and increase in

(chemo-)radiotherapy in laryngeal SCC found in our study is confirmed by Timmermans et al. in 2016,[57] and this is no surprise since the Dutch guidelines for laryngeal SCC were adjusted after the publication of a consensus document by the Dutch Head and Neck Society.[58] In our study, survival mainly decreased in patients older than 70 years with stage III and IV disease, although this decrease was not statistically significant. These patients have been excluded from adjuvant chemotherapy since the 2009 adjustment to the guidelines based on the study of Pignon et al.[45] In this study, chemotherapy was more often part of the treatment in the latter period in 'younger' patients, while low percentages and no significant increase was observed in patients > 75 years. A recent publication has shown that treatment with chemoradiation for HNSCC is also feasible and effective for the older patient population.[59] Therefore, this may be a topic for reconsideration. Likewise, Moye et al. confirmed that older patients receiving stage-appropriate treatment for early and late stage cancer had oncologic outcomes equivalent to those of their younger counterparts, and suggest that advanced age alone should not preclude patients from receiving stage-appropriate therapy.[60] Consequently, treatment decisions should be made according to a patients' general condition and comorbidities, and not rely purely on chronological age.

Despite the unimproved survival rates following the shift towards organ-preservation treatment protocols, some positive effects have been observed following amendments in laryngeal SCC treatment protocols. For stage I disease, survival rates have remained favorable since the introduction of minimal invasive transoral laser surgery.[61] This technique offers the potential for organ preservation with less functional morbidity than open surgery, as well as less toxicity, a shorter treatment schedule, and decreased expenses compared to radiotherapy. Furthermore, transoral laser surgery can be performed repeatedly in case of recurrent disease or a second or third primary laryngeal carcinoma, while radiotherapy can essentially be applied only once. Minimal invasive transoral laser surgery has a comparable voice and quality of life outcomes in early laryngeal cancer compared to radiotherapy. [62,63]

Strengths of our study are the inclusion of a representative cohort from the population-based Netherlands Cancer registry (NCR) with a large number of patients. Furthermore, we had detailed data on specific head and neck cancer sites, rather than analysis for head and neck cancer in general. There are also limitations that warrant discussion. First, there is no general consensus regarding the definition of elderly, and the cut-off of age in "elderly" patients highly differs among studies. Research suggests that HNSCC patients may have higher biological age due to their unhealthy lifestyle, compared with patients with other solid malignancies.[64] Additionally, the guidelines for chemotherapy for patients ≥ 70 changed 2009. To evaluate a possible effect not only in patients ≥70, also

patients 60-69 were included. Based on those arguments, a cut-off value of HNSCC patients aged 60 years and older was chosen. Second, in the NCR, data regarding tobacco use and alcohol consumption, comorbidity, functional outcome, quality of life and specific tumor characteristics such HPV tumor status were not available for the investigated time period. Additional information on these items would have been relevant for the complete picture of our findings.

Conclusion

In most of the primary tumor sites of older HNSCC patients, survival improved over time with an exception of laryngeal SCC. This phenomenon was observed in parallel with a larger proportion of non-surgical treatment modalities for oropharyngeal, hypopharyngeal and advanced laryngeal SCC in 2010-2015 compared to 1990-1995.

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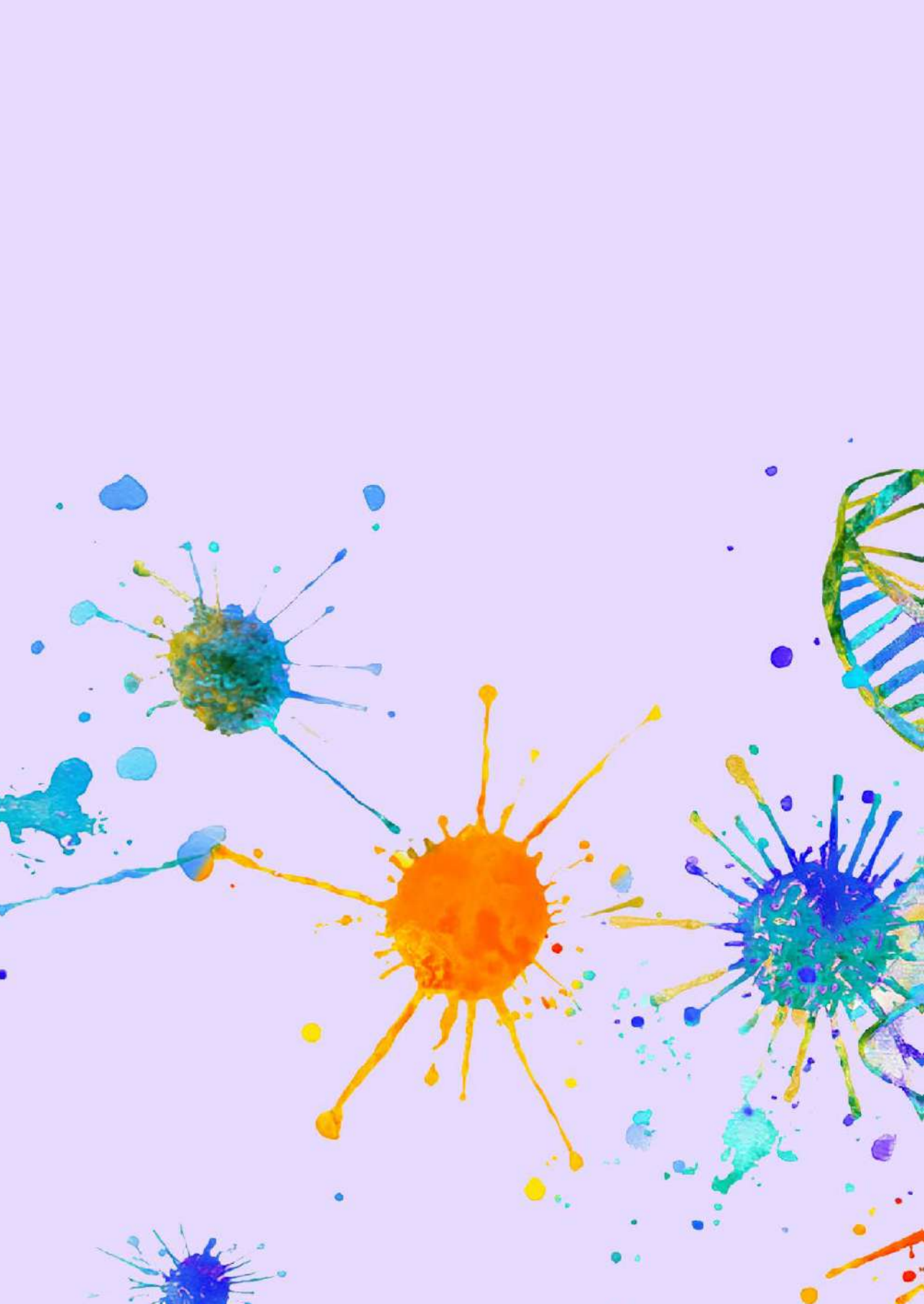

CHAPTER 3

Predictors for distant metastasis in head and neck cancer, with emphasis on age

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Abstract

Purpose

Distant metastasis (DM) in patients with head and neck squamous cell carcinoma (HNSCC) is uncommon, but strongly deteriorates prognosis. Controversy exists regarding age as a predictor for the presence and development of DM. The aim of this study was to investigate age and other predictors for DM in HNSCC patients.

Methods

From 1413 patients diagnosed with a primary HNSCC between 1999 and 2010 in a tertiary referral centre, patient, disease and pathological characteristics were extracted from patient files. Uni- and multivariable Cox regression analyses were performed to identify risk factors for DM as primary outcome.

Results

DM occurred in 131 (9.3%) patients, of which 27 (1.9%) were diagnosed simultaneously with the primary tumor, 27 (1.9%) were diagnosed synchronous, and 77 (5.4%) were diagnosed metachronous. The most common site of DM was lung (51.1%), followed by bone (19.1%) and liver (11.5%). Multivariable analysis identified male gender (HR = 1.95, 95% CI 1.23-3.10) hypopharyngeal tumors (HR = 3.28, 95% CI 1.75-6.14), advanced T-stage (HR = 1.61, 95% CI 1.09-2.38), poor differentiation grade (HR = 2.49, 95% CI 1.07-5.78), regional lymph node metastasis (HR = 5.35, 95% CI 3.25-8.79) and extranodal extension of regional lymph nodes metastasis (HR = 3.06, 95% CI 1.39-6.72) as independent prognostic factors for the presence or development of DM. No relation with age was found.

Conclusion

Age is not related to the presence or development of DM. This study emphasizes the importance of screening for DM, especially in males, patients with hypopharyngeal tumors, advanced T-stage, histopathological poor differentiation grade, regional lymph node metastasis and extranodal extension.

Introduction

In patients with head and neck squamous cell carcinoma (HNSCC), the incidence of distant metastasis (DM) varies between 3% and 52%.[1–9] When patients present with DM, the most affected sites are the lungs, followed by bone and liver.[5,10,11] In the last decades, detection of DM improved with the development of imaging techniques (e.g. fluorodeoxyglucose positron emission tomography (FDG-PET) scan), leading to higher detection rates of late DM.[3,12,13] The development of DM in HNSCC patients results in an infaust prognosis in most of the cases. Patients with DM receive palliative treatment and unfortunately around 90% decease within 12 months.[2] Patients that are diagnosed with DM prior to treatment are withheld from intensive curative treatment. Nevertheless, approximately 11% patients undergo treatment with curative intent and are shortly afterwards diagnosed with DM.[14] In retrospect, these patients are unnecessarily treated with major consequences for quality of life and healthcare costs.[5] On the other hand, routine screening for DM in all HNSCC patients does not seem rational, because of low incidence of DM in HNSCC patients.

It has been noted that different predictive factors could influence the development of DM, such as advanced T- and N-classification, specific tumor site, poor differentiation grade, extranodal extension and human papillomavirus (HPV) negative oropharyngeal SCC.[1,2,4,7,8,10,12,15,16] These predictive factors are globally recognized and adapted in the AJCC/UICC TNM staging system.

Decision making in elderly patients is very complex and decision regret is high in HNSCC patients who receive intensive therapy.[17] Consequently, the question arises whether older age is also a prognostic factor for DM. However, age as a risk factor for DM is less well studied, and results are contradictory.[1,2,4,7–9] Three studies identified age to be a predictive factor, however it remains unclear whether older[1] or younger[4,9] patients are at higher risk for developing DM. Therefore, we aimed to identify age and other predictive factors for the development of DM by the analysis of a large cohort of HNSCC patients.

Patients and methods

Ethical considerations

The study has been registered in the Research Register of the University Medical Center Groningen (UMCG). No approval of the Medical Ethical Committee was needed because of the retrospective nature of the study, in accordance with Dutch Medical Research Law legislation.

Patients

This retrospective study includes a cohort of HNSCC patients diagnosed at the UMCG, a tertiary referral head and neck oncology center in the Netherlands. Data were obtained from the Netherlands Cancer Registry (NCR), managed by the Netherlands Comprehensive Cancer Organization (IKNL). Included patients were 18 years or older, diagnosed with primary oral cavity, oropharyngeal, hypopharyngeal and laryngeal squamous cell carcinoma between 1999 and 2010. Patients with cutaneous squamous cell carcinoma and other tumor types than squamous cell carcinoma of any site were excluded. Patients presenting with multiple or second primary HNSCC were excluded, because of the uncertainty from which site a DM originated. The in- and exclusion of patients for this study are shown in Figure 3.1.

Patients with >T1 tumors were standardly screened for regional and intrathoracic metastasis during the primary diagnostic work-up in our hospital by (earlier chest radiography) computed tomography (CT) or magnetic resonance imaging (MRI). Additional MRI, CT, and/or PET were performed in case of clinical suspicion of DM or as part of staging when locoregional failure is diagnosed. Histological confirmation was not standardly performed but only conducted to distinguish between DM and second primary tumors, and was only performed in cases in which further diagnostics had clinical relevance.

Variables

Data from patient medical files were extracted and included patient characteristics (age, gender, oncological medical history and comorbidities), tumor characteristics (date of diagnosis, tumor site, TNM classification according to the AJCC 7th edition and date of DM) and detailed histopathological information (differentiation grade, perineural growth, angioinvasion and extranodal extension of lymph node metastasis).

Comorbidity was scored using 'The Adult Comorbidity Evaluation 27.[18] Perineural growth, angioinvasion and extranodal extension were only analyzed in surgically treated patients, because nonsurgical cases were lacking complete histopathological information.

Depending of the time of discovery of the DM, DM was classified as: simultaneous (discovered with the primary tumor), synchronous (within the first 6 months after diagnosis), and metachronous (after 6 months of initial diagnosis).

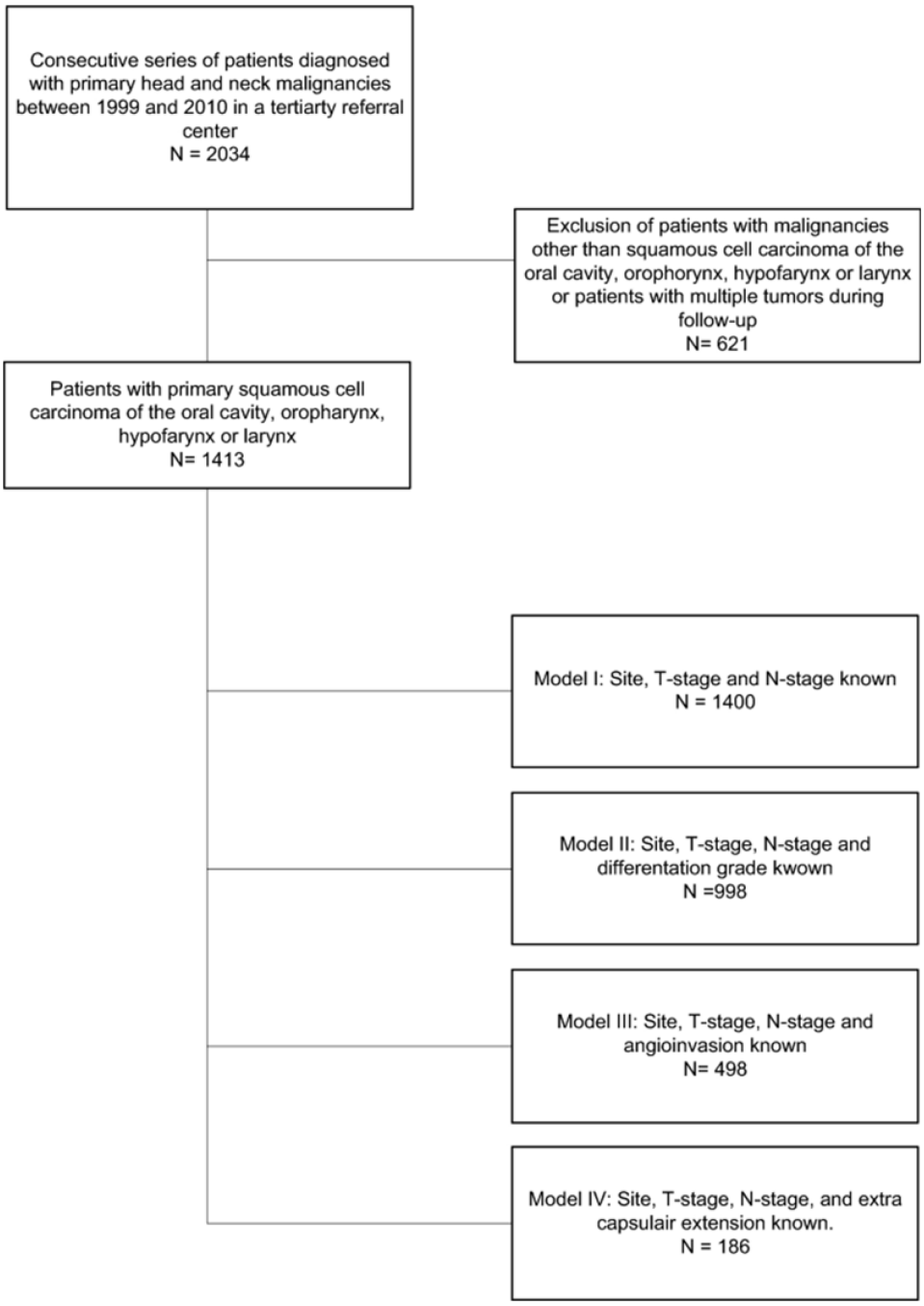

Figure 3.1. Flow-chart in-/exclusion criteria.

Statistics

Descriptive statistics were used for patient, disease and detailed pathological characteristics. These factors were also stratified by DM during follow-up. To identify potential factors that might be associated with the occurrence of DM, univariable as well as multivariable Cox regression analyses were performed after checking whether the proportional hazards assumption was met by evaluating log minus log plots. For both univariable and multivariable analyses, T-stage was grouped into early stage and advanced stage disease. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were reported; a 95% CI that did not include 1 or a *p* value below 0.05 was considered statistically significant.

Significant variables in univariable analyses were used for multivariable analysis. Because the multivariable model would include a substantially reduced number of cases when adding all selected variables in one model, four models with subgroups were generated. Model I included all patients whose age, gender, primary tumor site, T- and N-stage were known (n = 1399). Model II included patients of which the histological differentiation grade was also known (n = 997). Model III included patients of which angioinvasion was also known while differentiation grade was left out (n = 473). Model IV also included extranodal extension, while N-stage, differentiation grade and angioinvasion were left out (n = 186). Variables included in the final model were selected using a backwards step regression.

For the analysis on the relationship between DM and age, we divided patients in different age categories; <39, 40-49, 50-59, 60-69, 70-79 and ≥80 years.

Kaplan-Meier curves were created to determine patients with and without DM for significant factors according to the multivariable models.

Statistical analyses were performed using IBM SPSS Statistics 23 (IBM, Chicago, IL).

Results

A total of 1413 patients were included in the study, as shown in Figure 3.1. Table 3.1 shows the patients and disease characteristics. In 131 patients (9.3%) DM developed, of which 27 were diagnosed simultaneously with the primary tumor, 27 were diagnosed synchronous and 77 were diagnosed metachronous. In these cases, the mean time between diagnosis and DM was 20.5 months (SD: 20.8; range 0–116 months, data not shown). Mean age of patients with DM was younger (59.6 years) compared to the patients with no DM (62.6 years). Patients were predominantly male (73.3%). Although the most common primary tumor site was the larynx, most DM originated from the

oropharynx (38.9%), followed by the hypopharynx (25.2%), larynx (24.4%) and oral cavity (11.5%). Most primary tumors were diagnosed as a T1 tumor, which had the lowest DM incidence rate (7.6%). Early stage tumors (both T1 and T2) had lower DM incidence rate than advanced stage tumors (both T3 and T4), 5.9 versus 14.7% respectively. Regional lymph node metastasis occurred in 41.1% of all patients. In most patients with DM, also lymph node metastases were detected (77.1%). Of the patients without lymph node metastasis (N0), 3.4% developed DM vs. 17.9% with positive regional lymph nodes. DM was found in 7.1% of well differentiated tumors, while 54.5% originated from tumors with moderate differentiation and 38.4% with poor differentiation. In the DM group, 20.7% of the tumors showed perineural growth, versus 18.3% of the tumors in the group of patients without DM. Angioinvasion was observed in almost one third (30.8%) of the surgically treated patients who developed DM, versus 11.9% without DM. In surgically treated regional lymph nodes (n=221), extranodal extension was more common in the DM group (70.0 versus 46.8%).

The most common site of DM was lung (51.1%), followed by bone (19.1%) and liver (11.5%) (data not shown).

For age analysis, categories were applied as described earlier. The number of DM was highest in the 50–59 year old patient population (n = 55; 12%), followed by the 60–69 (n = 42; 11%), 40–49 (n = 15; 10%), 70–79 (n = 16; 6%), ≥80 (n = 2; 2%) and ≤39 (n = 1; 3%) (p = 0.003), shown in Figure 3.2. Age as either a continuous or categorical variable was not a significant predictor for DM in the multivariable model (Table 3.3), of which the results of the continuous variable are presented in Table 3.3.

Table 3.2 shows the results of the univariable analysis of potential factors influencing the presence or development of DM. Age, male gender, oropharyngeal and hypopharyngeal tumor site, advanced T-stage, positive N status, moderate and poor differentiation grade, the presence of angioinvasion and extranodal extension were identified as significant risk factors associated with DM. Comorbidities, larynx tumors and perineural growth were not identified as significant risk factors for DM.

Table 3.1 Patient and disease characteristics, overall and stratified by DM at diagnosis or during follow-up.

	All (%)	DM (%)	No DM (%)
	1413 (100)	131 (9.3)	1282 (90.7)
Age, mean (SD)	62.3 (11.8)	59.6 (9.0)	62.6 (12.0)
Gender			
Male	1036 (73.3)	109 (83.2)	927 (72.3)
Female	377 (26.7)	22 (16.8)	355 (27.7)
Comorbidity (ACE-27)			
None	451 (32.0)	47 (35.9)	404 (31.6)
Mild	412 (29.2)	39 (29.8)	373 (29.1)
Moderate	346 (24.5)	29 (22.1)	317 (24.8)
Severe	202 (14.3)	16 (12.2)	186 (14.5)
Unknown	2	0	2
Site			
Oral cavity	313 (22.2)	15 (11.5)	298 (23.2)
Oropharynx	422 (29.9)	51 (38.9)	371 (28.9)
Hypopharynx	137 (9.7)	33 (25.2)	104 (8.1)
Larynx	541 (38.3)	32 (24.4)	509 (39.7)
T-stage			
T1	430 (30.6)	10 (7.6)	420 (32.9)
T2	428 (30.4)	41 (31.3)	387 (30.3)
T3	194 (13.8)	23 (17.6)	171 (13.4)
T4	350 (24.9)	57 (43.5)	293 (23.0)
Tx	5 (0.4)	0	5 (0.4)
unknown	6	0	6
N-stage			
N0	843 (59.9)	29 (22.3)	814 (63.7)
N1	146 (10.4)	14 (10.8)	132 (10.3)
N2	356 (25.3)	68 (52.3)	288 (22.5)
N3	63 (4.5)	19 (14.6)	44 (3.4)
Unknown	5	1	4
M-stage[a]			
M0	1380 (98.1)	104 (79.4)	1276 (100.0)
M1	27 (1.9)	27 (20.6)	0
unknown	6	0	6
Differentiation grade			
Good	195 (19.4)	7 (7.1)	188 (20.8)
Moderate	636 (63.3)	54 (54.5)	582 (64.2)
Poor	174 (17.3)	38 (38.4)	136 (15.0)
Unknown	408	32	376
Perineural growth[b]			
Yes	75 (18.5)	6 (20.7)	69 (18.3)
No	331 (81.5)	23 (79.3)	308 (81.7)
Unknown	241	14	227
Angioinvasion[b]			
Yes	51 (13.1)	8 (30.8)	43 (11.9)
No	337 (86.9)	18 (69.2)	319 (88.1)
Unknown	259	17	242
Extranodal extension[c]			
Yes	95 (50.5)	21 (70.0)	74 (46.8)
No	93 (49.5)	9 (30.0)	84 (53.2)
Unknown	33	7	26

[a] M-stage at diagnosis of the primary tumor; [b] Only surgically treated cases were included (n = 647); [c]Only cases with surgically treated cervical nodes were included (n = 221).

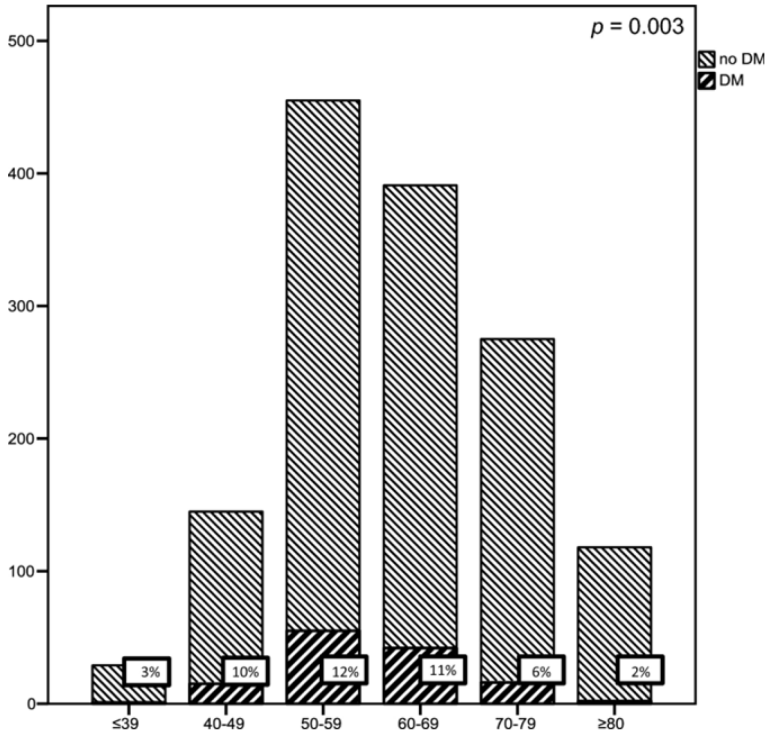

Figure 3.2. Percentage of DM per age category.

Significant factors determined in univariable analysis were tested in a multivariable model, as shown in Table 3.3. Male gender, hypopharynx tumor site, advanced T-stage, positive N status, poor differentiation grade and extranodal extension were found to be independent predictive factors in multivariable analysis. Male patients had a higher chance to develop DM compared to women, with a hazard ratio (HR) of 1.95, $p = 0.005$. Hypopharyngeal tumors had a higher chance to develop DM with a HR of 3.28, $p = 0.001$. Advanced T-stage was also a significant predictor; with a HR of 1.61, $p = 0.017$. Regional lymph node metastasis was an independent predictor with a HR of 5.35, $p < 0.001$. Poor differentiation grade was an independent predictor of developing DM, with a HR of 2.49 $p = 0.015$. Extranodal extension was an independent predictor with a HR of 3.06, $p = 0.006$. Due to a decrease in included cases in the different models, certain variables such gender, site, and T-stage lost significance in the different models.

Significant variables in multivariable analysis are also presented in Kaplan-Meier curves in Figure 3.3A-F. All variables were also significant in the Kaplan-Meier curves.

Table 3.2. Univariable analysis of potential factors related to DM at diagnosis or during follow-up.

Variable	HR	(95% CI)	*p* value
Age	**0.98**	**(0.97-1.00)**	**0.038**
Gender			**0.006**
Male	1		
Female	**0.53**	**(0.33-0.83)**	
Comorbidities			0.895
None	1		
Mild	0.93	(0.61-1.43)	
Moderate	0.88	(0.55-1.40)	
Severe	0.82	(0.46-1.45)	
Site			**<0.001**
Oral cavity	1		
Larynx	1.15	(0.62-2.12)	0.665
Oropharynx	**2.86**	**(1.61-5.08)**	**<0.001**
Hypopharynx	**8.28**	**(4.48-15.28)**	**<0.001**
T-stage[a]			**<0.001**
Early stage	1		
Advanced Stage	**3.85**	**(2.70-5.48)**	
N-stage			**<0.001**
N0	1		
N+	**8.18**	**(5.40-12.39)**	
Differentiation grade			**<0.001**
Good (Grade I)	1		
Moderate (Grade II)	**2.64**	**(1.20-5.80)**	**0.016**
Poor (Grade III)	**7.73**	**(3.45-17.33)**	**<0.001**
Perineural growth[b]			0.400
No	1		
Yes	1.47	(0.60-3.62)	
Angioinvasion[b]			**0.002**
No	1		
Yes	**3.80**	**(1.64-8.77)**	
Extranodal extension[c]			**0.005**
No	1		
Yes	**3.10**	**(1.14-6.79)**	

Bold numbers represent significant values ($p < 0.05$). [a] Early stage represents T1 and T2 tumors, advanced stage represents T3 and T4 tumors; [b] Only surgically treated cases were included (n = 647); [c] Only cases with surgically treated cervical nodes were included (n = 221).

Table 3.3. Multivariable analysis of potential factors related to DM at diagnosis or during follow-up.

	Model I N = 1399			Model II N = 997			Model III N = 473			Model IV N = 186		
Variable	HR (95% CI)		*p* value	HR (95% CI)		*p* value	HR (95% CI)		*p* value	HR (95% CI)		*p* value
Age	1.00	(0.98-1.02)	0.788	1.00	(0.98-1.02)	0.894	1.01	(0.98-1.04)	0.561	1.01	(0.97-1.04)	0.726
Gender			**0.005**			**0.016**			0.071			**0.006**
Female	1			1			1			**1**		
Male	**1.95**	**(1.23-3.10)**		**1.96**	**(1.14-3.38)**		2.30	(0.93-5.67)		**7.61**	**(1.80-32.14)**	
Site			**0.001**			**0.010**			0.056			0.429
Oral cavity	1			1			1			1		
Larynx	1.48	(0.80-2.76)		1.80	(0.90-3.57)		1.51	(0.54-4.17)		1.24	(0.32-4.81)	
Oropharynx	1.86	(1.04-3.34)		1.71	(0.83-3.55)		1.20	(0.49-2.99)		1.33	(0.53-3.34)	
Hypopharynx	**3.28**	**(1.75-6.14)**		**3.26**	**(1.57-6.75)**		**3.58**	**(1.31-9.78)**	**0.013**	2.45	(0.82-7.28)	
T-stage*			**0.017**			**0.013**			0.193	1	(0.42-1.97)	0.990
Early stage	1			1			1			0.98		
Advanced stage	**1.61**	**(1.09-2.38)**		**1.77**	**(1.13-2.77)**		1.50	(0.70-3.22)				
N-stage			**<0.001**			**<0.001**			**<0.001**			
N0	1			1			1					
N+	**5.35**	**(3.25-8.79)**		**4.68**	**(2.58-8.48)**		**4.30**	**(1.97-8.37)**				
Differentiation grade						**0.015**						
Good (Grade I)				1								
Moderate (Grade II)				1.42	(0.64-3.17)							
Poor (Grade III)				**2.49**	**(1.07-5.78)**							
Angioinvasion§									0.219			
No							1					
Yes							1.64	(0.74-3.66)				
Extranodal extension¶												**0.006**
No										1		
Yes										**3.06**	**(1.39-6.72)**	

Bold numbers represent significant values (p<0.05). A (multivariable) backwards Cox regression analysis was performed to produce 4 different models to predict distant metastasis, based on statistically significant variables obtained in univariate analysis. Model I included patients with complete information on the variables age, gender, primary tumor site, T- and N-stage (n=1399). Model II included the patients of model I of whom histological differentiation grade was also known (n=997). Model III included patients of model II of whom angioinvasion was also known (n=473). Model IV included patients of model I who also of whom histopathological capsular extension of cervical lymph nodes was also known (n=186). *Early stage represents T1 and T2 tumors, advanced stage represents T3 and T4 tumors; §Only surgically treated cases were included (n=647); ¶Only cases with surgically treated positive cervical nodes were included (n=221).

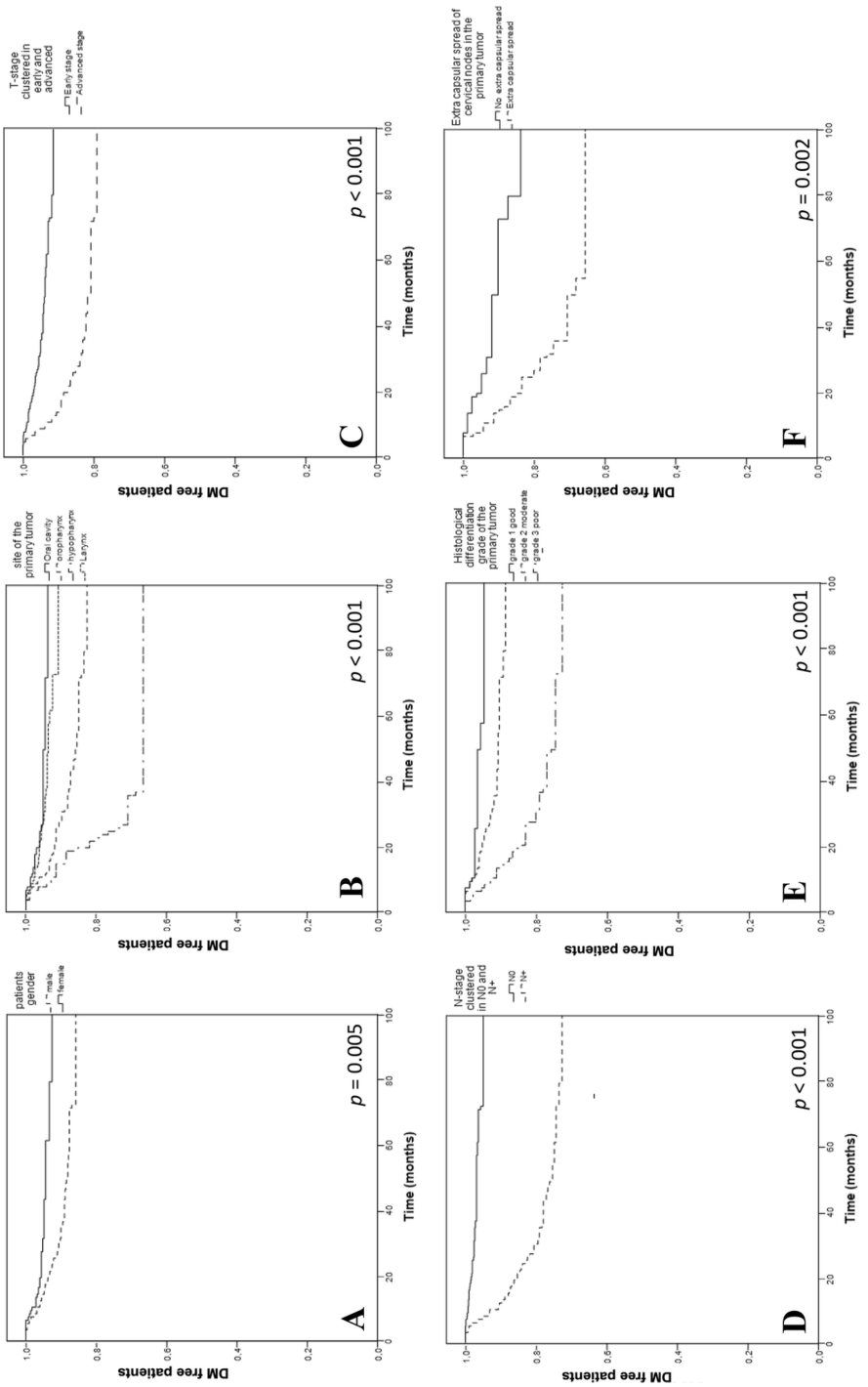

Figure 3.2

a–f Kaplan-Meier curves of predictors of DM in HNSCC. Kaplan–Meier curves showing time to metastasis sorted on gender (a), the primary tumor site (b), T-stage, early: T1-2 vs. advanced: T3-4 (c), N-stage, N0 vs. N+ (d), histological differentiation grade (e) and extra capsular spread in cervical nodes (f)

Discussion

In the present study, analyzing a consecutive series in one of the largest homogenous published study cohort on this subject, younger age was associated with higher occurrence of DM in univariable analysis, however no relation between age and DM could be observed in multivariable analysis. We confirmed earlier identified independent predictive factors for the development of DM in HNSCC patients: male gender, location of the primary tumor at the hypopharynx, advanced T-stage, regional lymph node metastasis, poor differentiation grade and extranodal extension of regional lymph node metastasis.

In our study, 9% of patients developed DM. However, the reported incidence of DM in HNSCC varies widely in the published literature, between 3% and 52%.[1–9] This is mainly due to different study populations and study designs. The timing of DM diagnosis plays a crucial role in these differences. Some papers study DM at the time of diagnosing the primary tumor, others during complete follow-up and others at autopsy.[12] In our study 20.6% of the DM were discovered at the same time as the diagnosis of the primary tumor.

Age by itself was significantly related to DM in univariable analysis. However, in the four tested models of the multivariable analysis as well as in additional analysis in which patients were divided per age category and related with DM, no significant relation was found between age and DM. In a large study, including over 27,000 patients, older age was identified as an independent significant risk factor for DM, as well as primary site, nodal status, tumor size, and race.[1] However, the authors could not explain these findings and simply state that it is unclear why older patients are at high risk for DM. In contrast, in another large cohort of almost 2,000 HNSCC patients, younger age (< 45 years) was found to be significantly associated with the risk of DM.[4] Hypopharyngeal localization, advanced T stage and/or N stage tumor, high histologic grade, and locoregional control were also related to the development of DM. This paper also could not clarify these age related findings. The authors presume that the role of younger age is of limited importance. In a more recently published study, also younger age at diagnosis was also discovered a risk factor for developing pleural metastasis.[9] Also in this study, the authors do not give an explanation for this finding, concluding none of the above mentioned studies can interpret their age-related findings. In concordance with our results, few other studies also did not find an association between age and DM.[7,8] A retrospective study analyzing 130 advanced stage HNSCC identified clinically palpable neck disease (N1-3), histological evidence of metastatic nodal disease, extranodal

extension, and three or more positive lymph nodes as predictors for developing DM.[7] Age, gender, primary site, history of radiation therapy, perineural invasion and tumor grade were not associated with a higher risk for DM. In another study, development of DM was not related with age, while N stage, T stage, and pre-treatment maximum standardized uptake value of the lymph node were strongly associated.[8] In a larger study including over 1,200 HNSCC patients, age was also not related to the frequency of DM.[2] None of these studies give an explanation for their age related findings. Discrepancies in age related findings between this study and the above mentioned studies could partly be explained by differences in study design. Some studies include a cross-sectional study design, measuring DM only at diagnosis, while others performed a longitudinal study including both DM found at diagnosis as well as during follow-up after treatment. This explains the differences in reported incidence of DM, which might also impact other results, such as age-related findings. Furthermore, the method of detection of DM is different among the above discussed studies. In most studies, description of the diagnostic process (like for example the applied imaging modalities) is lacking. Even within a study, work-up might vary due to differences in work-up between high-risk and lower-risk patients, which can also affect results. Differences in the epidemiological profile of patients among studies may also play a role. Our study population contains all HNSCC in a between 1999 and 2010, while others included only advanced staged HNSCC patient, for instance specifically patients that underwent intensity-modulated radiotherapy or only patients with pleural metastasis. All these factors might have consequences for age analysis. Finally, limited sample size and thus the low power of many currently available studies may also partly explain conflicting results. However, also studies with large statistical power found opposite results. All in all, the explanation for these different results concerning age and DM remains unclear.

In a recently published study, CART analysis (classification and regression tree) was used to assess the impact of age on the survival of patients with HNSCC. Age was a significant prognostic factor in predicting 5-year disease-specific survival, based on the uni- and multivariate analyses. However, in their CART model, the authors found that age plays only a minor role in HNSCC survival. This method revealed that the impact of age varied for different patient groups according to the presence or absence of other prognosticators. This was different from our results; however, comparison between these studies is difficult as the cited study did not specifically investigated DM.[19]
We identified the presence of regional lymph node metastasis as the strongest independent predictor for the presence of DM. The important role of advanced N-stage as a predictor of DM development has already been described in numerous studies and is now also supported by our findings.[1,4,7,8,10,12,20] Two of these studies also found

extranodal extension an independent predictor of DM, in concordance with our results.[7,21] While in one other study, extranodal extension was not a significant predictive factor for DM.[22]

Hypopharyngeal tumors seem to give the highest risk of DM.[1,2,4,10,20,23] In line with these findings, in our cohort patients with hypopharyngeal tumors had more than three times as high chance to develop DM as their counterparts with oral cancer.

In this study, advanced tumor stage and poor differentiation grade were also found to be a significant independent predictor of DM. These factors were also described as high risk factors for DM in a review on DM in HNSCC.[12]

Male gender was also found to be an independent predictor of the development of DM. Others also investigated the relation between gender and the development of DM, most of those studies didn't find male gender to be a significant predictive factor.[1,2,5,8,9] In other tumors this trend was also observed, for instance in malignant melanoma[24]; however, the explanation for this finding in HNSCC remains unclear.

Also the effect of angioinvasion and perineural growth on the development of DM was studied. Interestingly, neither angioinvasion nor perineural growth were significant independent predictors for DM, which is confirmed by two other studies.[7,10] This finding is remarkable, as perineural growth is considered to be a robust prognostic factor in cancer. However, it has found to be associated with an increased risk of local recurrence and regional metastasis rather than with DM.[25,26]

Overall, the most frequent locations for DM in the present study were found to be the lungs (51.1%), followed by the bones (19.1%) and the liver (11.5%). In line with our findings, this pattern is also described in other studies.[5,10,11] In a large study, in which 832 patients with HNSCC were autopsied, most metastasis were found in the lungs (80%), followed by mediastinal nodes (34%), liver (31%) and bone (31%), indicating that a clinical diagnosis of DM might be less precise than a pathological diagnosis.[23] Because of the very poor prognosis of patients with DM, in case of clinical suspicion, further diagnostics are often omitted as it has no clinical relevance.
In this study, DM only occurred in the first 120 months of follow-up. Typical Kaplan Meier curves of DM in HNSCC show a rapid increase between months 0 and 8, a slow increase between months 8 and 24, and a plateau between months 24 and 84,5 indicating the absence of late distant metastasis.[10]

This study included a very large series of consecutive cases and well-documented records. Because of the retrospective nature of this cohort study, missing data was expected due to incomplete case records. However, our study population was large and adequate to perform multivariable analyses. Despite, some risk factors described in literature (e.g. large lymph nodes and bilateral and low neck level involvement)[2,21,22,27,28] could not be included in our analysis due to high number of missing data in the retrospective database. In addition, we did not have information on HPV status of the oropharyngeal tumors. Though, this would be interesting to analyse, as HPV-related oropharyngeal cancer is known to be associated with higher stage at diagnosis and more favourable outcomes.[16]

Due to the improved locoregional control and the increasing number of HNSCC survivors in the last decades, data on DM development gained more importance, recently.[12] On the other hand, recognizing patients with high chance of developing DM is essential, as these patients need to be screened before they undergo intensive treatment while costly imaging modalities can be spared in their counterparts who do not likely develop DM. According to the present study, regional lymph node metastasis is the strongest predictor for DM in HNSCC patients. However, more factors should be considered as indicators for screening, such as male gender, hypopharyngeal tumors, advanced T-stage, poor differentiation grade, regional lymph node metastasis and extranodal extension. Based on our study, a large, representative and homogenous cohort, age does not play a role in the development of DM. Therefore, in all patients with high risk of DM development, extensive screening should be considered, irrespective of patients' age. Screening should include the whole body, as metastasis may occur in the lungs, but also in the liver, the bones or in multiple organs. For this reason, the most appropriate method seems to be FDG-PET/CT. However, the method of screening was not the subject of this study.

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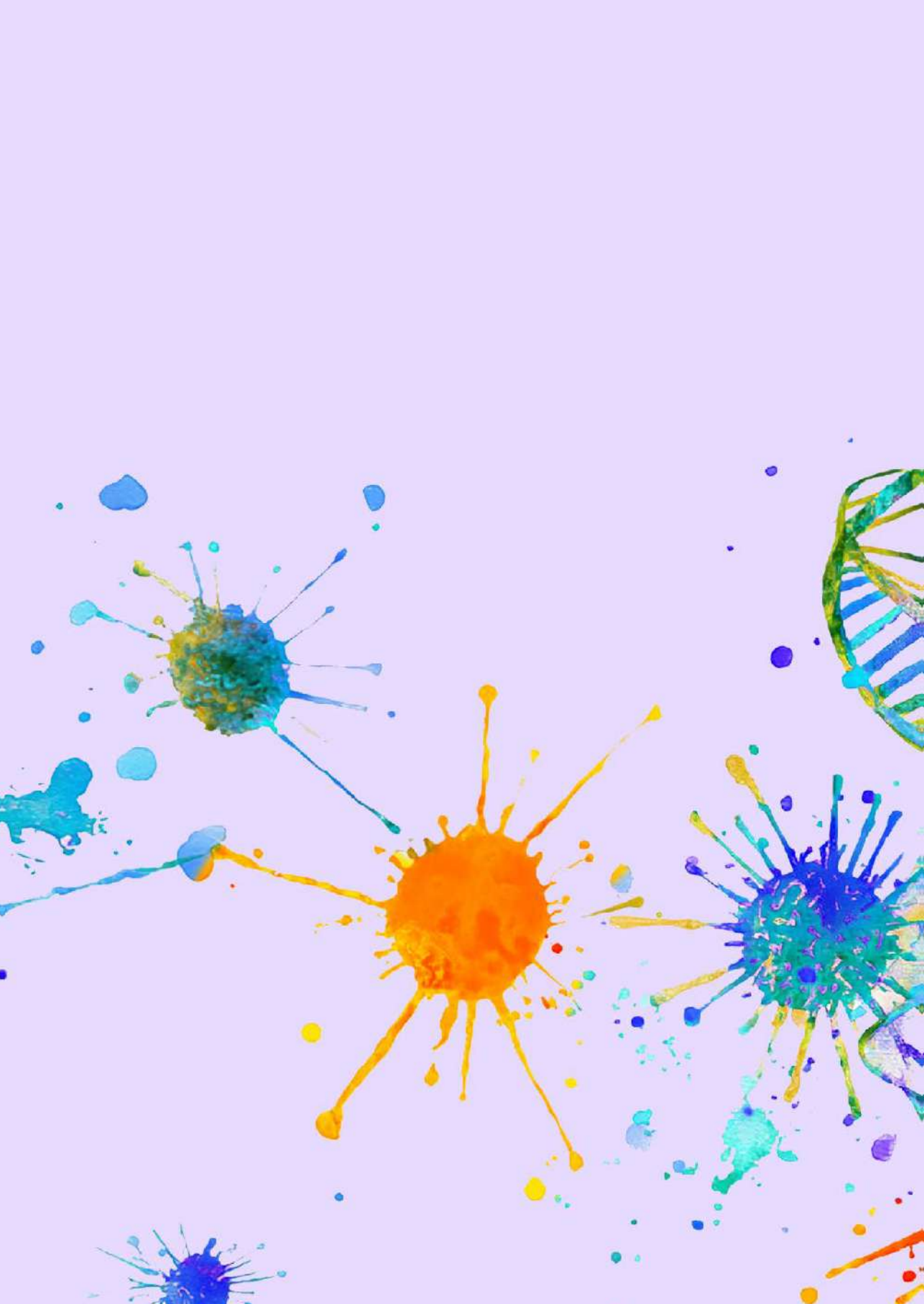

CHAPTER 4

Age-specific oncogenic pathways in head and neck squamous cell carcinoma – are elderly a different subcategory?

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Abstract

Background

In recent clinical practice, an increasing number of elderly patients suffering from head and neck squamous cell carcinoma (HNSCC) of unknown pathophysiology is observed. The majority of HNSCC patients can roughly be divided into three subcategories. First, a small group of young patients who present with variants of genomic aberrations and inheritable diseases like Fanconi anemia. Second, an increasing population of HPV-related HNSCCs that are regarded as genomic stable tumors with a more favorable prognosis. Though HPV-related tumors used to be more common among younger males, a notable rise in the elderly population is observed. The third subcategory, that of HPV-negative tumors, has been shown to be more heterogeneous with involvement of a variety of oncogenic pathways related to lifestyle factors like smoking and alcohol consumption, often seen in middle-aged males. Some of these pathways could be related to age, such as TP53 alterations, EGFR activation, apoptotic pathway alterations and field cancerization.

Conclusions

In this narrative review, we provide an overview of established and newly discovered age-specific pathophysiological mechanisms underlying HNSCC. We propose a fourth subcategory of patients with a suspected different pathophysiology: elderly (HPV-negative) HNSCC patients without a history of tobacco and alcohol consumption. In this subcategory, carcinogenesis seems to be a multi-step process based on genomic instability, immunosenescence, cell cycle disruption and telomere shortening. To conclude, we discuss suggestions for future research to fill the knowledge gap about age-dependent HNSCC carcinogenesis.

Introduction

Head and neck cancer is the sixth most common type of cancer worldwide, with approximately 600,000 new diagnoses and 250,000 deaths annually.[1,2]. Most head and neck cancers comprise head and neck squamous cell carcinomas (HNSCCs), derived from the mucosal epithelium in the oral cavity, pharynx or larynx. HNSCCs commonly require aggressive multimodality treatment, consisting of surgery, radiotherapy and systemic treatment. Importantly, the prognosis of HNSCC not only depends on tumor stage, treatment modality and patient-specific factors, but also on the underlying pathophysiology, which varies among patients of different ages. When considering the *younger population* of HNSCC patients, inherited diseases such as Fanconi anemia (FA), Dyskeratosis Congenita (DC) and Bloom's syndrome are acknowledged risk factors for developing HNSCC.[3] Familial oral squamous cell carcinoma (OSCC) and nasopharyngeal carcinoma are also more common in younger patients.[4] Furthermore, Human Papilloma Virus (HPV)-related tumors are predominately observed in young males.[5] This group of HNSCCs appears to be a distinct entity with a stable genome and relatively better treatment response.[6-9] The patterns noted above differ from the *middle-aged* patients with HNSCC. This latter category typically encompasses male patients with traditional risk factors such as excessive tobacco and alcohol consumption.[10] Tumors in this group of patients are mostly HPV-negative, and the pathophysiology of the tumors is assumed to be based on a complex multi-step process, rather than on a single molecular event/pathway.[11] Finally, ageing has contributed to an increased incidence of HPV-negative HNSCCs in *the elderly population*, resulting in 25 to 30% of patients being over the age of 70.[12,13] In this relatively new but expanding group of patients, the aetiology and pathophysiology remain largely unknown. In other tumor types, ageing has been found to be associated with genomic instability, telomere attrition, epigenetic changes, proteostasis, nutrient sensing and metabolism, as well as cellular senescence and stem cell function.[14] Based on the age-related distribution of risk factors as described above, the absence of well-known aetiological factors for HNSCC (i.e., tobacco and alcohol consumption) in elderly patients suggests the involvement of different oncogenic pathway(s). This could subsequently influence treatment response and prognosis.[15] Unravelling potential differences in oncogenic pathways in this specific subcategory of elderly patients with HNSCC could be helpful to facilitate treatment decisions and possibly generate age-specific treatment strategies. As yet, however, the literature is scarce on this issue.

The aim of this review was to describe the distinct age-specific characteristics of HNSCC. We will provide an overview of the literature on potential age-specific oncogenic pathways in the four suggested subcategories of HNSCC patients, being (i) inherited

HNSCC, (ii) HPV-related HNSCC, (iii) HPV-negative HNSCC in young and middle-aged patients and (iv) elderly patients not exposed to tobacco and/or alcohol. Distinction of the latter group of elderly HNSCC patients is a novelty and may have clinical consequences, leading to different treatment policies for this group, which will be discussed in the final chapter. Given the broad scope and aim of this review, an open literature search rather than a systematic literature search was performed in Pubmed/Medline. The description of our search strategy is summarized in Appendix 4.1.

Inherited HNSCC

Inherited cancers occur due to hereditary genetic mutations, and account for 5-10% of all cancer cases.[4] These cancer types often affect younger patients.[4] An overview of the most well-known inherited head and neck cancer syndromes is listed below and shown in Figure 4.1.

Familial Oral Squamous Cell Carcinoma (OSCC) is an autosomal dominant disorder.[4] Impairment in regulator proteins such as overexpression of MDM2[16] and deletion of the CDKN2 locus and loss of ADP-Ribosylation Factor 1 (ARF) affecting p53 degradation and p53 response to DNA damage are found in familial HNSCC.[4] Germline TP16 mutations, associated with increased p16/CDKN2A protein levels are particularly linked to OSCCs.[16]

*Fanconi anemia (*FA) results from chromosomal instability due to biallelic mutations in one of the 17 known FA genes (FANCA to FANCS), inherited in an autosomal recessive or X-linked recessive pattern.[17] Germline mutations in genes of cellular DNA repair pathways are assumed to facilitate accumulation of mutations during HNSCC development.[18] Mutations in these FA genes may also contribute to the development of HNSCC in general.[18] Several studies have investigated the role of sporadic genetic variants of FA in the development of HNSCC in non-Fanconi patients, i.e., downregulation,[19] loss of heterozygosity[18] and increased mutational loads[20] of multiple FA genes. Increased mutation of FANCD2, FANCE, and lower expression of Glutathione S-Transferase P1 (GSTP1), FANCA and FANCG proteins has been reported to be particularly common among younger patients.[20,21]

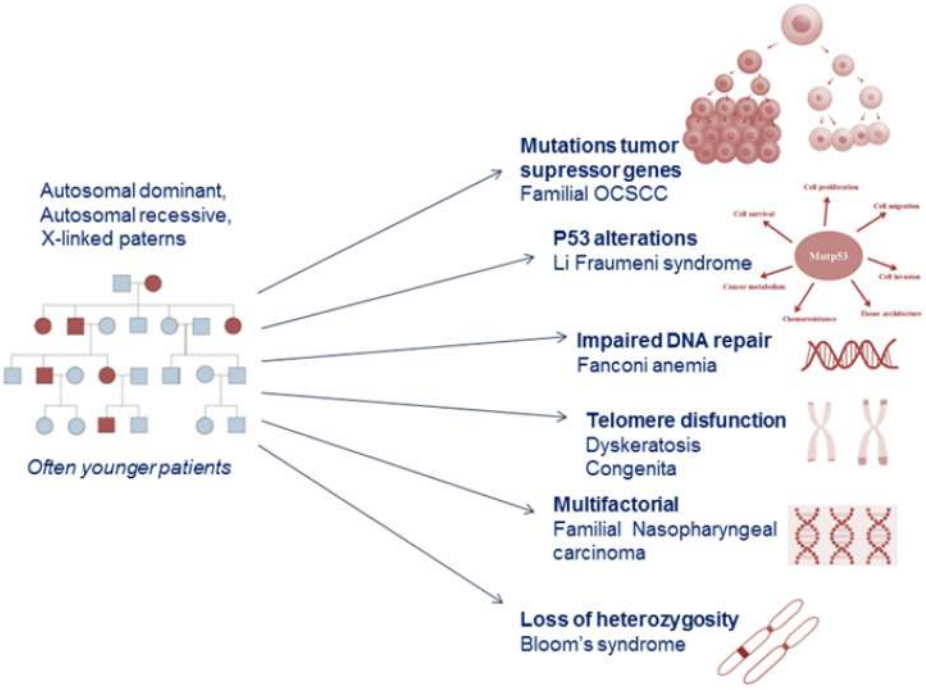

Figure 4.1 Inherited head and neck cancer syndromes. Autosomal dominant, autosomal recessive and X-linked inheritance patterns have been described for inherited HNSCC, affecting mostly young patients. In familial OCSCC a series of mutations in tumor suppresser genes has been described.[4,16] In Li Fraumeni syndrome, germline mutations in TP53 have been found to be responsible for an increased cancer risk.[28] Germline mutations in Fanconi anemia deregulate cellular DNA repair pathways, resulting in HNSCC.[18] In Dyskeratosis Congenita, mutations in telomere maintenance genes cause telomere shortening leading to an increased cancer risk.[4,23] A high rate of loss-of-heterozygosity resulting in chromosomal instability is responsible for an increased cancer risk in patients with Bloom's syndrome.[24,26] The increased risk for familial nasopharyngeal carcinoma is thought to be multifactorial, including genetic, ethnic and environmental factors, as well as Epstein-Barr virus infection.[22]

The etiology of *the Familial Nasopharyngeal Carcinoma* (fNPC) is thought to be multifactorial, being based on variations in ethnicity, genetics, environmental factors and infection with Epstein-Barr virus (EBV).[22] fNPC has a pronounced geographic distribution.[4] Thus far, several susceptibility loci or genes and polymorphisms in immune-related cytokines and surface proteins on immune cells have been found to be associated with fNP, but the results are not concordant and, therefore, preclude firm conclusions.[22]

Dyskeratosis Congenita follows various inheritance patterns: X-linked, autosomal dominant or autosomal recessive.[4] In Dyskeratosis Congenita-related cancer, mutations in 10 genes have been described (including *DKC1, TERC, TERT, TINF2, NOP10, NHP2, TCAB1, C16orf57, RTEL1*).[4,23] Mutations in nine of these genes affect telomere maintenance, leading to excessively short telomeres and, consequently, cancer development.[23]

Bloom's syndrome is an autosomal recessive disorder characterized by early predisposition to multiple cancers, including HNSCC.[24,25] Loss-of-function mutations of the BLM gene cause chromosome instability. A resulting fourfold higher rate of mutations and a 50-fold higher rate of loss of heterozygosity are likely to be responsible for the increased cancer risk.[24,26]

Li Fraumeni syndrome is an autosomal dominant disorder.[27] Individuals with this syndrome often harbor germline mutations in the p53 tumor suppressor gene,[28] resulting in a 50% increased risk of developing cancer by the age of 30 and a 90% increased risk by the age of 70.[28,29] Also missense mutations located primarily in exons 4–9, harboring hot spot codons 205–248, have been associated with Li Fraumeni syndrome.[16]

Based on these observations, it appears that inherited HNSCC syndromes share pathways involved in (dis)functional DNA damage repair systems and surveillance of genetic stability. The affected patients are essentially of younger age.[30] It remains unclear why they have a predilection for squamous cell carcinoma, but it is worth noting that the affected genes are similar to those seen in non-inherited HNSCC, and include p53, p16 and FANCA-M.[3]

Age-related pathways in HPV-related HNSCC

The prevalence of HPV-related HNSCC, mostly oropharyngeal squamous cell carcinoma (OPSCC), is described as between 36% and 46%.[31,32] Previous studies showed that most patients with HPV-related OPSCC are males between 45 and 60 years of age,[5,33] have fewer comorbidities, report less tobacco exposure and higher numbers of sexual partners compared to traditional HNSCC patients.[5,34-37] However, a recent study showed that the incidence of (HPV-related) oropharyngeal cancer is also increasing in the older population.[38] HPV-related tumors have fewer genomic aberrations than HPV-negative tumors,[9] suggesting that these tumors have a relatively stable genome compared to

HPV-negative tumors. Infection with HPV into the host cellular genome and expression of the E6 and E7 oncoproteins result in degradation of p53 and functional inactivation of the Retinoblastoma (Rb) protein[39] (Figure 4.2). E7-driven inactivation of Rb leads to p16 overexpression, as Rb normally represses p16 transcription.[6] The p16 protein decelerates cell cycle progression from the G1 phase to the S phase by binding to cyclin dependent kinase (CDK)4 or 6, thereby preventing the formation of a catalytically active cyclin D–CDK4/CDK6 complex to release E2F through phosphorylation of the Rb protein. This liberates E2F1 from its bound state in the cytoplasm and allows it to enter the nucleus. Once in the nucleus, E2F1 promotes the transcription of target genes that are essential for transition from the G1 to S phase.[40-42] Hereby, cells are released from their growth inhibitory effects, resulting in abnormal cell cycling and growth.

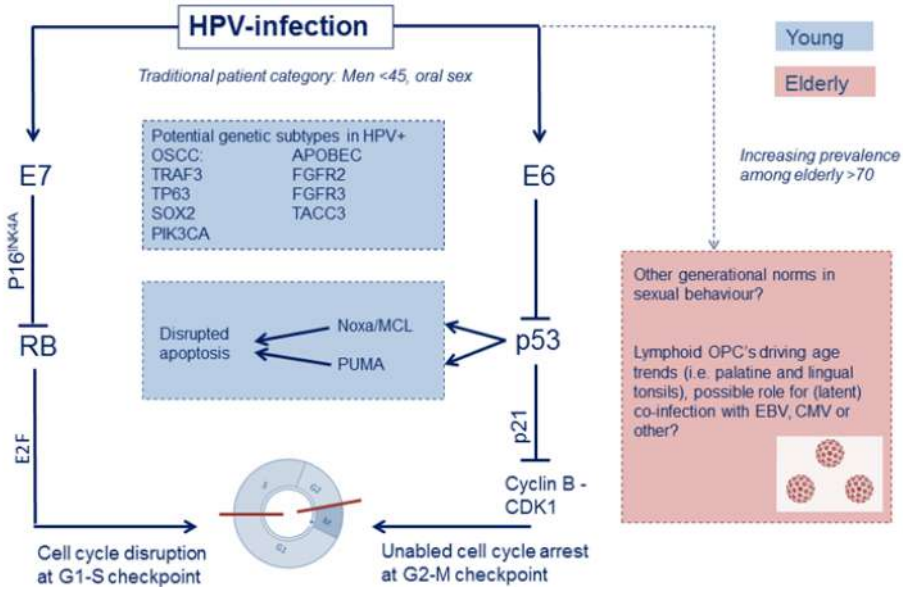

Figure 4.2 Role of ageing in HPV-driven HNSCC. HPV-driven HNSCCs are located at the oropharyngeal subsite (tonsils, base of the tongue), mostly in male patients<45 years of age with oral sexual contacts. In this category, induction of carcinogenesis through up-regulation of E6 and E7 has been reported, leading to cell cycle arrest failure and cell cycle disruption, respectively.[39,40,41,42] In addition, some genetic subtypes have been formulated in younger patients that may increase the risk to develop HPV-driven OSCC (blue dotted squares).[43,44,45,46] Disrupted apoptosis through Noxa/MCL and PUMA has been suggested as a possible pathway for carcinogenesis in younger HPV+patients (blue dotted squares).[47,48,49] In addition, an increasing prevalence of HPV-driven HNSCC has been observed in elderly patients over the age of 70 (red dotted square). Although the pathophysiology underlying this latter category is as yet unclear, it has been suggested that other generational norms in sexual behavior and co-infection with other viruses such as EBV could play a role.[5,50,51]

When it comes to ageing, certain genetic changes also increase the risk for developing HPV-related tumors, particularly in younger patients (Figure 4.2). HPV-related tumors exhibit specific deletions of the chromosomal regions 14q32 and 9q, which contain tumor necrosis factor receptor associated factor 3 (TRAF3), focal amplification of E2F1 and, in contrast to HPV-negative HNSCC, lack of deletions in the 9q21.3 region containing the CDNK2A gene.[43,44] Also, amplification of the chromosome 3q26-28 region containing tumor protein 63 (TP63), sex determining region Y-box 2 (SOX2) and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) have been observed in HPV-related tumours.[39] PIK3CA, the gene that encodes the p110 alpha subunit of Phosphoinositide-3-kinase (PI3K), is the most frequently altered oncogene in HNSCC overall, with a possible enrichment in HPV-related tumours.[45] The apolipoprotein B mRNA-editing enzyme catalytic subunit (APOBEC) has emerged as a potential mutagenic factor in HPV-related tumors, resulting from high cytosine deaminase activity.[44] Besides this, mutations in Fibroblast Growth Factor Receptor (FGFR) 2 and 3 have also been identified in HPV-related tumours,[46] as well as rare FGFR3-Transforming Acidic Coiled-coil-Containing 3 (TACC3) fusions.[43] Mutation rate differences were not found to be associated with HPV status.[39,44]

In younger patients, another possible pathway in HPV-related tumors is that of disrupted apoptosis and/or uncontrolled cellular growth (Figure 4.2). Apoptosis (programmed cell death) can be activated via two routes, i.e., by intracellular mitochondrial signaling (intrinsic pathway) or receptor mediated signaling (extrinsic pathway).[47] Genetic variants of Phorbol 12-myristate 13-acetate induced protein 1 (PMAIP1 gene, also known as Noxa), myeloid cell leukemia 1 (MCL1)[48] and p53 upregulated modulator of apoptosis protein (PUMA)[49] seem to be involved in the apoptotic cascade of HPV-related HNSCC. Noxa is a pro-apoptotic protein, while MCL1 is an anti-apoptotic protein; both are regulated by p53. Based on these mechanisms, the balance between Noxa and MCL1 is influencing the *intrinsic* apoptotic pathway. Puma, another pro-apoptotic protein, influences the *intrinsic* apoptotic pathway via E6-mediated p53 degradation.[49] Both intrinsic apoptotic pathways seem to be related to younger patients.[48,49]

Over the past years the age of diagnosis for HPV-related oropharyngeal squamous cell carcinoma (OPSCC) has increased rapidly, with a simultaneous rise in the proportion of HPV-related OPSCCs among all age groups – especially in the elderly population.[50] Elderly patients with HPV-related OPSCC have an inferior survival rate compared to younger HPV-related OPSCC patients.[50] In a study of Rettig et al.,[50] the authors further specified the characteristics of the elderly cohort with HPV-related OPSCC and noted significantly higher comorbidity scores in elderly patients compared to younger patients.

Moreover, elderly patients tended to present with higher T-stages and less likely to be treated by surgery. Furthermore, the 70+population received palliative treatment more often than the sub-cohort of patients under 50 years. All these factors may contribute to inferior survival outcomes in the elderly. Another explanation may be co-infection with EBV or other viruses that are associated with the effect of increasing cultural acceptability of certain sexual behaviours.[5,50] Viral co-infection with EBV in particular could enhance invasive phenotypes of HPV-related OPSCC by a delay in epithelial differentiation and the establishment of EBV latency.[51] However, results on viral co-infection and HPV-related tumors remain conflicting, since some researchers claim that this is mainly the case in OSCC rather than in OPSCC,[52] while others could not find subsite-related correlations nor age-related differences.[53]

Overall, HPV-related HNSCCs show a different biological behavior with a more favorable prognosis compared to HPV-negative tumours.[6] The survival advantage of HPV-positivity is, however, attenuated in older age groups.[50]

Age-related pathways in HPV-negative HNSCC

According to the Surveillance, Epidemiology, and End Results (SEER) Cancer Statistics data, the peak incidence of head and neck cancer is between 55-65 years.[10] Tobacco and alcohol abuse are the most well-known etiological factors with a synergistic effect in the development of HNSCC,[54] particularly in this 'middle aged' patient category.[15] These types of head and neck tumors are remarkably heterogeneous, as highlighted by various RNA and DNA profiling studies.[55] Several potential pathophysiological pathways have been described for this third sub-category of HNSCC patients, and are listed below and summarized in Figure 4.3.

The process of *field cancerization* can be explained by a mechanism in which multiple cell groups undergo neoplastic transformation due to stress resulting from regional carcinogenic activity (i.e., smoking and/or excessive alcohol usage). Presumably, a critical genetic alteration in a single cell attains a growth advantage over its neighboring cells. At some point after transformation, cells harboring these early genetic alterations migrate to colonize contiguous tracts of mucosa, accumulate other alterations, acquire additional growth advantages, and ultimately transform into aggressive subclones.[56,57] At the molecular level in dysplasia, loss of heterozygosity at chromosomes 3p, 9p and 17p reflect these early carcinogenic steps (Figure 4.3).[15] In addition, p53-mutated clonal units represent early oncogenic changes in the mucosa.[15,56] A significant increase in both

numerical and structural chromosome abnormalities has been found to be associated with an increased age.[58,59] The size of precancerous fields has also been found to be larger in older patients compared to younger patients.[60] Consequently, older patients (>50 years) appear to have an increased recurrence risk after surgical removal of head and neck tumors compared to younger patients.[60]

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Tobacco and alcohol consumption are primarily associated with alterations in the tumor suppressor gene TP53,[61-63] reported to be affected in 53–80% of HNSCCs.[11,15,64] During the cell cycle, p53 induces p21, a CDK inhibitor that arrests the cell cycle.[65] The presence of a TP53 mutation has been found to be a negative pro gnostic factor for HNSCC in various studies.[11,15,64,66,67] Over the last decades, the relation between TP53 mutations and age has extensively been studied.[67-71] Since populations and methodologies vary widely among these studies it is difficult to compare the results. In most studies no relation with age was found,[67,70-72] but two studies found an increase in TP53 mutations in younger HNSCC patients compared to older patients.[68,69]

Glutathione S-transferase (GST) is an important enzyme in the *detoxification* of cells from carcinogenic substrates, such as tobacco components, that can cause DNA damage. Besides the strong correlation with smoking, a GST polymorphism has also been found to be a potential risk factor for HNSCC[73] and, more specifically, for LSCC in young adults.[74] In patients with a GSTM1 and GSTT1 null genotype, the entire gene is absent, resulting in complete loss of functional activity of the respective enzymes.[75] The

GSTT1 null genotype seems to be related to a higher age of onset in patients with OSCC.[76]

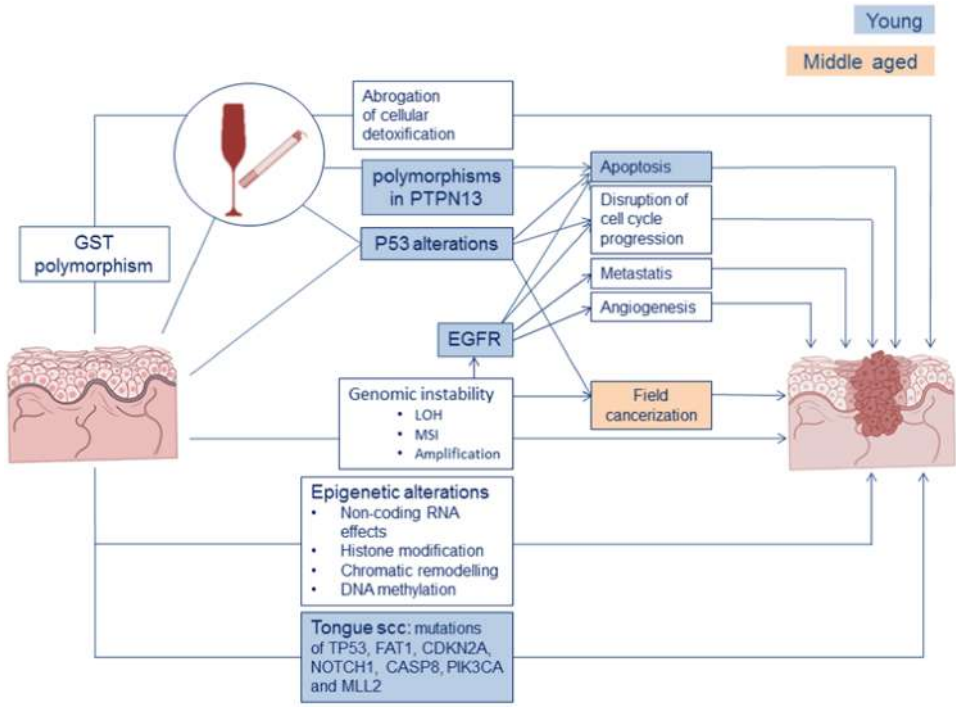

Figure 4.3 Age-related pathways in HPV-negative HNSCC. Tobacco and alcohol consumption have been linked with various pathways leading to HNSCC. Mutations in TP53, associated with tobacco and alcohol consumption, are responsible for cell cycle de-regulation and apoptosis. A high p53 expression is more frequently observed in young patients with HNSCC and tongue SCC.[70,71] Also higher expression of EGFR, causing (in)activation of various pathways that influence cell proliferation, apoptosis, metastasis and angiogenesis, has been found in younger patients.[78,79] Loss of heterozygosity at chromosomes 3p, 9p and 17p and TP53-mutated clonal units represent early oncogenic changes in the mucosa, described as field cancerization, which is related to middle-aged patients.[59] Conflicting results concerning age and GST polymorphisms, involved in the cellular detoxification pathway, have been reported.[73,74,76] For angiogenesis, young and elderly OSCC patients showed similar results.[83,141] MSI is characterized by expansion or contraction of short tandem repeats, and indicates genomic instability. In normal human somatic cells, MSI increases linearly with age. It has also been associated with head and neck tumors, although the age-specific results in HNSCC vary between studies.[86,90,91] Epigenetic alterations represent heritable changes that affect gene expression without changing the DNA sequence. Hypermethylation is more commonly seen in tumors of younger female patients, particularly at the anterior tongue.[95] The most frequently mutated genes in young patients with tongue SCC have been reported to be TP53, CDKN2A, NOTCH1, CASP8, FAT1, PIK3CA and MLL2.[98]

Amplification of *Epidermal Growth Factor Receptor* (EGFR) plays an important role in the development of HPV-negative HNSCC and may act as a driver.[43] As a result, specific signaling functions of EGFR may be disturbed, causing (in)activation of different

pathways that affect cell proliferation, apoptosis, invasion, angiogenesis and metastasis. The exact contribution of the EGFR pathway in HNSCC is still elusive. Numerous studies have investigated the prognostic value of EGFR expression in head and neck cancer, concluding that elevated EGFR levels are associated with a reduced survival.[77] Also, higher EGFR expression seems to be relate d to younger age.[78,79]

Enhanced *angiogenesis* is suggested to be involved in tumor growth, advanced clinical stage, metastasis, and to be associated with a worse prognosis.[80,81] The angiogenesis profile does not seem to differ between young and elderly HNSCC patients,[82-84] or to be correlated with age at all in HNSCC patients.[80] Distinct mutational clusters in regions that regulate angiogenesis have been identified in very old patients (aged 81 to 87) compared to young HNSCC patients (aged 19 to 40).[85] No differences among gene mutation patterns were seen, rather an accumulation of mutations in the elderly group. The role of the tumor microenvironment (TME) harboring stimulating neovascularization factors has been discussed widely over the last decades. To our knowledge, the impact of ageing on specific parts of the TME involved in angiogenesis has not yet been described, and this gap in the literature is mentioned in some studies (e.g. ref. 84).
Loss of heterozygosity (LOH) has also been associated with the development of HNSCC. Most studies on this topic focus on correlations between LOH and prognosis, which is described for regions on chromosome arms 3p, 8p, 9p, 14q, 17p and 18q.[86,87] No relation between LOH and age in HNSCC has been found so far.[88,89] Also, no consensus has yet been reached on the relation between *microsatellite instability* (MSI) and age, i.e., the results are contradictory.[86,90,91] MSI is characterized by expansion or contraction in the length of short tandem repeats and, just like LOH, it indicates genomic instability. In normal human somatic cells, MSI increases linearly with age, but it has also been associated with head and neck tumours.[92]

Epigenetic alterations such as DNA methylation, histone modification, chromatin remodeling and non-coding RNA effects have been associated with HNSCC,[93] which suggests that they may play a role in driving HNSCC development.[94] As yet, contradictions exist regarding the relation between p16 methylation and age in HNSCC.[45,95]

The incidence of *oral tongue squamous cell carcinoma* (OTSCC) in young patients without exposure to conventional risk factors is increasing worldwide.[96] The most frequently mutated genes in OTSCCs in young patients are TP53, CDKN2A, NOTCH1, CASP8, FAT1, PIK3CA and MLL2,[69,97] comparable to those affected in elderly patients.[98] Recently, two novel driver genes, ATXN1 and CDC42EP, have been added to this list of

frequently mutated genes in OTSCC.[97] Early-onset OTSCC patients seem to have fewer (non-silent) mutations than older OTSCC patients, when adjusted for tobacco use.[59,97]

To conclude, it seems that in HPV-negative tumors cells can accommodate various genetic alterations that lead to the same clinical disease state. Several pathways can be distinguished and, therefore, this type of HNSCC appears to be heterogeneous. TP53, EGFR and apoptotic pathway alterations seem to be age-dependent and to more frequently affect younger patients. The mutational burden seems to be lower in younger OTSCC patients, while field cancerization seems more pronounced in the 'classic' middle-aged patient category. For both angiogenesis and genomic instability pathways, no relation with age has been found. Conflicting results regarding age in relation with the GST-pathway and epigenetic alterations have been reported. Further exploration of these pathways could be important for the development of molecular targeted therapies, regardless of patient age.

Pathophysiology of HNSCC in elderly patients

In patients over the age of 70, the most affected tumor sites comprise the larynx, oropharynx and oral cavity.[99] In contrast to the middle-aged population —which is either ex posed to extensive tobacco and alcohol usage or HPV-infection – the underlying cause and tumor biology amongst the elderly remain largely unknown. This knowledge gap may result in inferior treatment outcomes in the elderly. Currently, elderly patients receive comparable treatment to younger patients, including radiation therapy and/or surgery. However, based on a meta-analysis by Pignon et al.,[100] it has become clear that patients over the age of 70 do not receive adjuvant chemotherapy due to the absence of survival benefit. A better insight into the pathophysiology of HNSCC could form a basis for clinical studies on novel, age-specific treatment strategies in the elderly. Unfortunately, studies regarding this topic are scarce.

It is well-known that the risk of developing cancer increases with age: by the time an individual passes the age of 70, the chance of developing any type of invasive cancer has risen to 33%, compared to approximately 6% of individuals below the age of 60.[101] This suggests an overlap in molecular biology of ageing with carcinogenesis. Overlapping mechanisms between ageing and carcinogenesis of various tumors have been described regarding telomere shortening, genomic instability, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence and stem cell exhaustion.[14,102] However, only very few basic and translational studies focusing on the

pathophysiology of HNSCC in elderly patients as a separate group have been reported. These are discussed below and summarized in Figure 4.4.

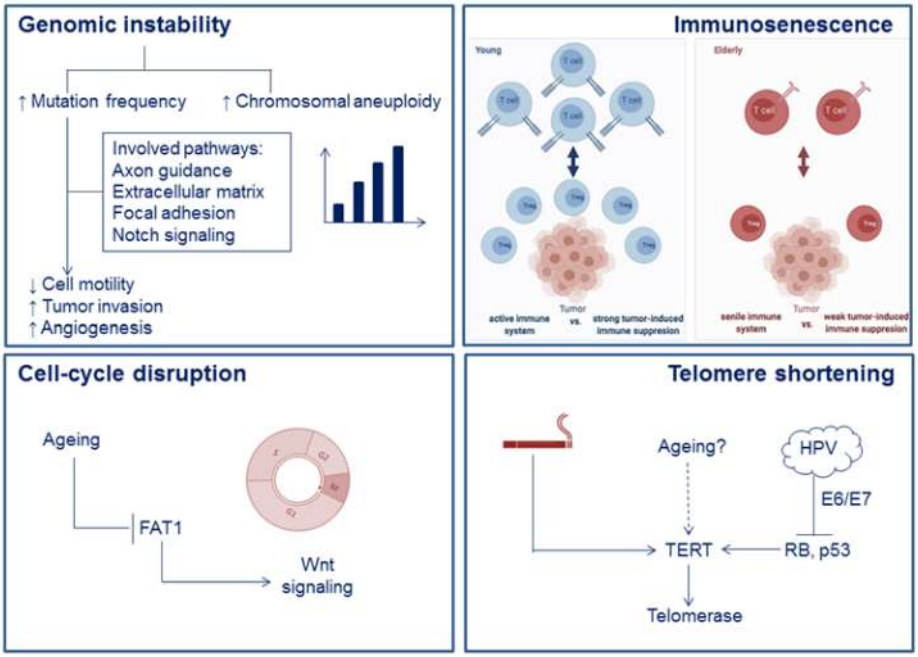

Figure 4.4 Possible pathophysiological mechanisms of HNSCC in elderly patients. Overlapping mechanisms between ageing and carcinogenesis have been described regarding telomere shortening, genomic instability, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence and stem cell exhaustion.[14,102] However, only four mechanisms have been described in HNSCC in elderly patients as a separate group. The first, genomic instability, seems more frequent in elderly patients causing impaired cell mobility and increased tumor invasion and angiogenesis.[85] Second, elderly patients developing HNSCC are thought to have deteriorated or weaker immune responses (immunosenescence) compared to younger patients with an active immune system. As suggested by Schuler et al., young subjects may have more CD8+T cells expressing mainly CCR7 and CD73, while old subjects may have less CD8+T cells expressing more PD-1. Young patients have an active immune system with a strong tumor-induced immune suppression with many Tregs, while old patients have a senile immune system with a weak immune suppression and less Tregs.[128] Third, ageing is believed to cause inactivation of the FAT1 tumor suppressor which, subsequently, leads to activation of the Wnt signaling pathway causing cell cycle disruption.[8,125] Many studies have investigated the role of telomere shortening and telomerase reverse transcriptase (TERT) in HNSCC. Both smoking and HPV infection have been shown to induce TERT activity. Remarkably, this has not yet been proven for ageing in HNSCC, while telomere shortening is believed to be one of the driving factors of ageing.[107,109,120]

Telomere shortening

Shortening of telomere length is a well-known phenomenon associated with ageing.[103] The lifetime of a cell is ultimately limited by the length of its telomeres, which shorten after every cell division until all telomeres become critically short and the cell becomes

senescent. Senescence prevents infinite cell divisions with a concurrent accumulation of mutations, thereby also reducing the risk of cancer development. This process is counteracted by telomerase reverse transcriptase (TERT), an enzyme whose activity can result in prolonged or even unlimited cell divisions.[104]

Carcinogenesis in HNSCC has also been associated with a shortened telomere length and subsequent TERT overexpression.[103,105,106] Aberrant telomere shortening has been observed in pre-cancerous lesions of HNSCC and has been suggested to be a marker for field cancerization.[107-109] Increased expression of TERT has been found to be highly specific for malignant LSCC,[110] but has also been reported in other HNSCC sub-sites.[106] Considering the molecular pathways underlying TERT overexpression, one can distinguish HPV-related OPSCC from non-HPV related HNSCC. In non-HPV related HNSCC, overexpression is often the result of mutations in the TERT gene promoter.[111-113] This phenomenon has been linked to life-style factors such as smoking, and results in a poor overall survival of LSCC patients.[114-116] In HPV-related OPSCC, the high-risk oncoproteins E6 and E7 are thought to be responsible for inactivation of Rb, p53 and subsequent increased expression of TERT. Nevertheless, in vitro studies with TERT inhibitors revealed alternative lengthening of telomeres in keratinocytes,[117] suggesting that telomere homeostasis is maintained by various pathways.[118]

Although ageing leads to telomere shortening, TERT overexpression in HNSCC strongly correlates with tumor aggressiveness (poor differentiation grade, increased risk for metastasis and poor response to treatment), rather than with patient age.[107,119,120] In one study, age and telomere length were investigated in HNSCC, but no correlation was found in neither cancer tissue nor in surrounding mucosa.[107] The authors speculated that patients with short telomeres found in mucosa surrounding the tumor had a higher risk of mucosal failure based on a study performed on colorectal carcinoma in which telomere length in non-cancerous cells was inversely correlated with age.[121]

Patterns of genetic variants

In HNSCC a comprehensive genetic analysis on mutational load and mutational patterns during ageing has been performed.[85] In this study, somatic single nucleotide polymorphisms (SNP's) in 203 selected HPV-negative and TP53-negative patients whose genetic data were available via The Cancer Genome Atlas (TCGA) were investigated. In concordance with other studies on ageing, the investigators found that mutation frequency rather than mutation spectrum differed between young and old patients with HNSCC. By analyzing genetic clusters of 'very old' patients (defined as 81–87 years old, n = 11), four pathways were found to be enriched compared to those of young patients

(defined as 19–40 years old, n = 11), being the 'axon-guidance' pathway, the 'extracellular matrix receptor interaction' pathway, the 'focal adhesion' pathway and the 'notch-signaling' pathway. Although this was one of the few studies to compare genetic mutations between young and old HNSCC patients, the sample size was very limited. Also, the tumor sub-sites varied, the majority being oral cavity and larynx. Despite these limitations, the study points to possible pathways involved in HNSCC development in the ageing population and suggests further investigations on this topic.

Chromosomal aneuploidy, or the phenomenon of aberrant chromosome numbers in cells, is not only associated with syndromic disorders, but is also a common feature in tumor cells. The biological severity generally correlates with the size of the chromosome anomaly and related gene copy number changes.[122] Furthermore, the International Workshop on Genotoxicity Testing (IWGT) workgroup report stated that the frequency of aneuploidy increases with age, but is not associated with smoking or gender. This is in concordance with another study that investigated ageing and aneuploidy in 220 OSCC patients, in which in non-smokers the association of patient age with DNA aneuploid OPMDs/OSCCs was significantly higher compared to those with DNA diploid OPMDs/OSCCs.[123] The mechanism of aneuploidy further underlines the importance of genomic instability involved both in carcinogenesis and ageing, but whether this applies to other sub-sites of HNSCC and possible treatment regimens remains a subject for further investigation.

Disruption of apoptosis and cell cycle progression

Protocadherin FAT1 (FAT1) is a tumour suppressor gene located on chromosome 4q35.2 and is mutated in 23% of HNSCC cases and lost or deleted in 8% of them.[11] Inactivation of this gene promotes carcinogenesis by inducing the WNT signalling pathway.[124] WNT signalling plays a key role in cell orientation and cell fate and thereby in stem cell maintenance. In two studies, patient age was generally higher among individuals with a FAT1 mutation.[69,125] One study showed a lower mutation frequency of FAT1 among younger patients (in this study defined as<45 years) compared to older patients (≥45 years) with SCC of the oral tongue.[69] Interestingly, despite the mutation frequency of TP53 usually being associated with cigarette smoking, this study found that the mutation rate was higher in young patients who were all non-smokers. In another study, the authors suggest that FAT1 could be a potential prognostic marker in HNSCC patients based on an association between lower expression of FAT1 and improved survival in HPV-negative HNSCCs.[125] This study showed a higher mean age of individuals with the FAT1 mutation. Therefore, the actual effect of FAT1 expression is speculative and seems to play a more important role in elderly patients than in younger patients.

Immune cell signaling

Immunosenescence, defined as gradual deterioration of the immune system associated with age, is described as an important factor contributing to carcinogenesis in elderly patients.[126] Tumor infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) involved in the innate immune system are key components of the tumor microenvironment and drive tumor progression.[127] Changes in the total number of circulating innate immune cells or in the relative percentage of different subpopulations have been reported in elderly patients. With the introduction of new immunotherapeutic agents in the treatment of head and neck cancer (e.g. Nivolumab, Pembrolizumab), immunosenescence mechanisms may have direct clinical implications. In a recently published study, differences in T-cell subgroups and their expression profile with increasing age were investigated in healthy subjects consisting of young (40–69 years; n = 17) and elderly (70-90 years; n = 20) HNSCC patients. The authors found a lower concentration of TILs in peripheral blood samples of the elderly patients, suggesting that in elderly tumor patients the immune system is impaired and the tumor-induced immune escape is less pronounced.[128] This concept is illustrated in Figure 4.4, which contains an adapted version of the figure reported by Schuler et al.

Programmed death-ligand 1 (PD-L1) is becoming increasingly important as a biomarker and therapeutic target in HNSCC.[129] PD-1 is variably expressed by head and neck tumor cells, and immunotherapies that block inhibitory immune cell signaling have demonstrated clinical efficacy in advanced head and neck cancers.[130,131] During carcinogenesis, anti-tumor activity by the immune system is suppressed by upregulation of PD-L1 on tumor cells, which binds to PD-1 on T-cells. Conflicting results on PD-L1 levels in HNSCC and age have been reported. In one study, higher PD-L1 expression on tumor cells in HNSCC patients≤45 years has been described.[37] An association was noted between a) higher PD-L1 levels, b) higher numbers of Inducible T-cell Co-Stimulator (ICOS)-positive TILs and c) a higher ratio of FOXP3+Tregs and ICOS+TILs relative to effector CD8+T-cells and younger patients.[37] In contrast, in a large meta-analysis on the prognostic and clinicopathological significance of PD-L1 overexpression in OSCC, no significant association was found between PD-L1 overexpression and age (>56,>60,>65 years).[132] Conversely, two other studies found an association between elevated levels of PD-L1 in HNSCC tissues[133] and peripheral blood lymphocytes[128] and older patients. PD-L1 expression was observed in 80% of patients and was significantly associated with old age (≥65 years).[133] In the second study, peripheral blood lymphocytes were obtained from HNSCC patients (n=33, 47–90 years) and healthy volunteers (n = 48, 21–84 years). A higher PD-L1 expression was found to be associated with increased age.[128] In both studies, PD-L1 positivity[37] and high PD-L1 expression (≥50%)[133] seemed to be prognostic factors for a poor survival. Another study found that young female OSCC patients< 45 years with increased membranous PD-L1 positivity showed a decreased risk of recurrence and an improved survival.[134]

In summary, PD-L1 expression seems to play a more important role in patient prognosis than age. However, results on immunosenescence are conflicting and consensus on the effects of immune checkpoint molecules on the prognosis of HNSCC has not yet been reached. Detailed knowledge on age-related alterations of the immune system is necessary in order to offer an adequate treatment option for this growing group of HNSCC patients and must be further investigated.

Impact of ageing on the biology of tumors other than HNSCC; DNA methylation and epigenetic clocks

Many overlapping mechanisms involving ageing and carcinogenesis have not been studied in HNSCC. A particular mechanism of interest is DNA methylation. In DNA methylation, methyl groups are covalently linked to cytosines. When such methylation occurs in a gene promoter, it can result in repression of gene transcription. In carcinogenesis, DNA methylation has been associated with inactivation of tumor suppressor genes.[135] Interestingly, DNA-methylation as a marker for "epigenetic aging" has been shown to correlate well with biological aging.[136] The first "epigenetic clock" theory originates from Horvath et al.[137] and has inspired others to identify methylation biomarkers and drifts in blood that can predict aging.[138] Based on application of these methods, investigators were able to predict a biological age acceleration in colorectal carcinoma that was associated with disease onset and/or death.[139] Other subsites showing epigenetic drift include hematopoietic stem cells and skin cells.[138] Unfortunately, as mentioned previously, DNA methylation has so far only been investigated in younger patients with HNSCC leading to contradictory results.

Based on the mechanisms described above, we conclude that in elderly HNSCC patients accumulation of mutations is the most relevant driving force.[85] Carcinogenesis seems to represent a multi-step process rather than a single hit mutation in this specific group of patients. Further research, especially in the field of immunosenescence, holds promise for the identification of potential prognostic biomarkers and the development of novel therapeutic strategies.

Conclusions, recommendations for future research and possible clinical applications

The aim of this review was to provide an overview of potential age-specific molecular pathways in HNSCC. By doing so, we introduced a possible new biological entity: non-intoxication driven and non-HPV related HNSCC of the elderly. Age-related pathophysiology in inherited HNSCC, HPV-related HNSCC and HPV-negative HNSCC has been outlined. The number of studies investigating the molecular background of HNSCC in general is overwhelming. Our search for age-related carcinogenesis in particular was, however, hampered by the fact that most studies do not focus on ageing as a central

question, resulting in poorly defined age categories with divergent cut-off values, and a subsequent lack of specific comparisons among age categories. In addition, many results on age-specific molecular mechanisms seem to be ambiguous.

Due to the lack of molecular studies in elderly patients in particular, identification of possible pathways required deduction of findings in studies from other tumor types. These studies suggest a higher degree of accumulating genetic mutations acquired with ageing, which was also cautiously confirmed in HNSCC.[85] Furthermore, immunosenescence is intensely studied, but consensus on this subject has not yet been reached, while studies on mechanisms like loss of proteostasis or differences in epigenetics and cellular senescence in HNSCC still need to be initiated.

Because of the lack of studies and the heterogeneous character of HNSCC in elderly patients, it seems impossible to point out one specific targeted therapy for this patient category. An attractive strategy to identify molecular pathways and potential therapeutic targets, however, is the use of multi-omics profiling technologies. Multi-omics profiling or integrative omics is a comprehesive molecular approach in which multiple molecular features, such as the genome, proteome, phosphoproteome, transcriptome, epigenome, metabolome and microbiome, are measured as comprehensive as the analytical technology allows. In contrast to single-omic analyses, multi-omic analysis integrates molecular data layers in multiple steps based on molecular information (genomics to proteomics and metabolomics) in order to find a causal relationship between molecular alterations, for instance at pre- and post-translational levels. Therefore, a multi-omic approach is thought to provide a more complete tumor biology picture.

A recent study by Huang et al. used this principle to identify subgroups of HNSCC patients that could respond to targeted therapy.[140] Based on multi-omic data in 110 patients with HPV-negative tumors, the investigators could distinguish three clusters of (epi)genetic, proteomic and phospho-proteomic profiles that could potentially respond to treatment with CDK4/6 inhibitors, anti-EGFR antibodies or immunotherapy. Each cluster contains its own set of markers and it would be interesting to use these markers in the analysis of HPV-negative elderly patients to see in which molecular cluster they fit, and to estimate whether they could benefit from one of the suggested targeted therapies. Once future multi-omic analysis techniques become less costly and available for clinical practice, individualized multi-omics may become the diagnostic approach for HNSCC patients in all age-categories, not only the elderly, to provide tailor-made treatment options.

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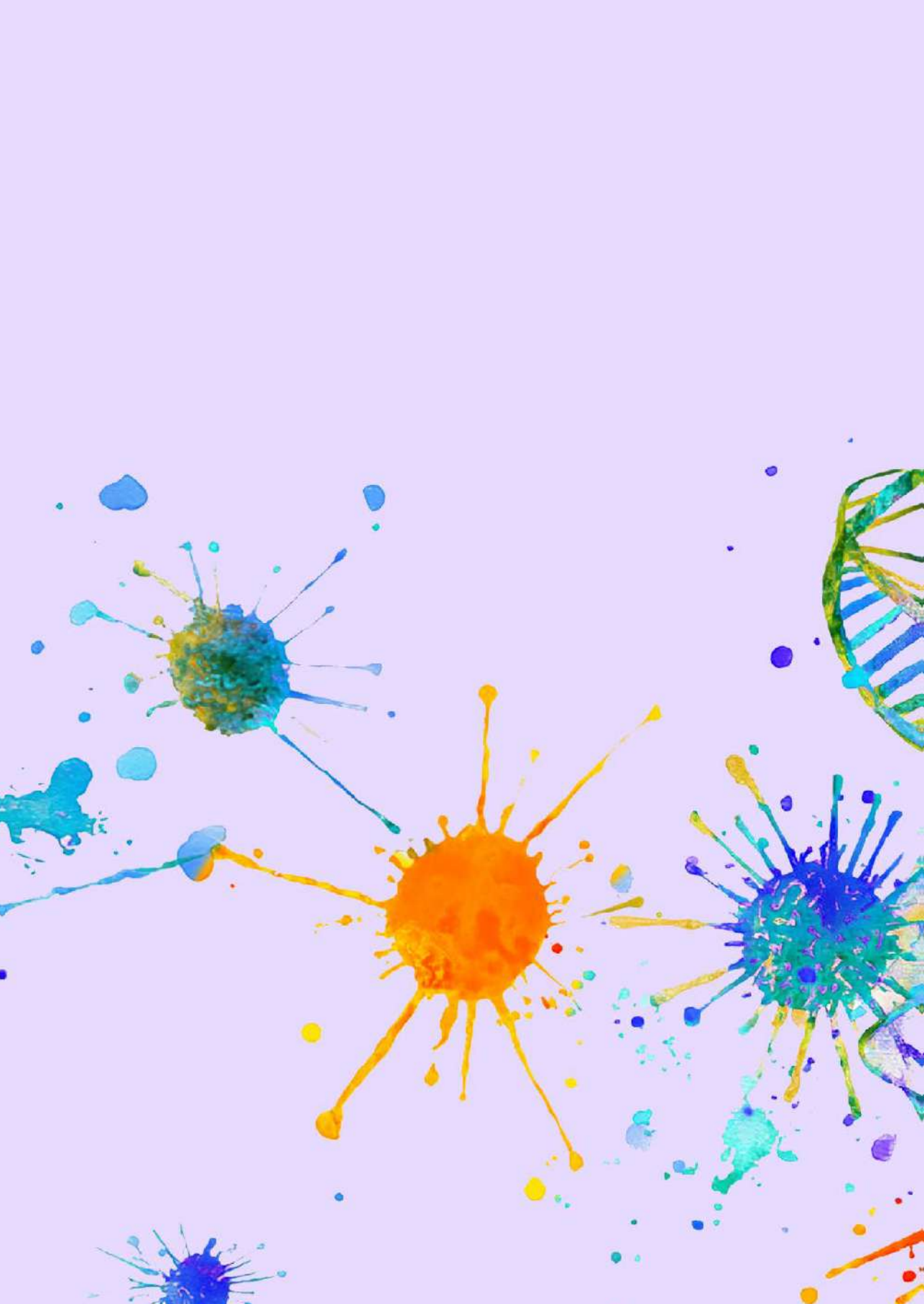

CHAPTER 5

Association of tumor microenvironment with biological and chronological age in head and neck cancer

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Abstract

There is often a mismatch between the chronological and biological age of head and neck squamous cell carcinoma (HNSCC) patients. Treatment is based on chronological age, while biological age seems to be a better prognosticator for treatment toleration. This study investigated whether tumor characteristics are associated with chronological and biological age. The relation with survival was also assessed. Prospectively collected data from 164 newly diagnosed HNSCC patients enrolled in the OncoLifeS database were analyzed. Biological age was assessed by a multidomain geriatric assessment. Several immunological markers were tested by immunohistochemistry on tissue microarray sections from the tumor. Disease-free survival (DFS), adjusted for chronological- and biological age, was assessed by univariable and bivariable analyses. In biologically old patients, a lower infiltration of CD163+ macrophages (p = 0.036) as well as CD4+ (p = 0.019) and CD8+ (p = 0.026) lymphocytes was found in the tumor microenvironment. Chronological older patients showed significantly lower PD-L1 combined positive scores (p = 0.030). Advanced tumor stage and perineural growth were related to a worse DFS. None of the immunological markers showed a significant association with DFS. Biological age might have a stronger influence on tumor microenvironment than chronological age. These findings should initiate clinical studies investigating the response to specific treatment regimens (e.g., immunotherapy) according to the biological age.

Introduction

Head and neck squamous cell carcinoma (HNSCC) may occur at all ages, however, the peak incidence is around the fifth and sixth decade.[1] Typically, these middle-aged patients are heavy smokers and drinkers or have HPV-related oropharyngeal cancer. However, patients that develop HNSCC at an older age are usually less exposed to these risk factors. It is known that age itself is a risk factor for cancer,[2] and there is overlap between the genetic pathways and biochemical processes that play a role in both aging and carcinogenesis, like telomere shortening and epigenetic alterations.[3] Aging of the immune system (immunosenescence), is suggested to be an important factor in carcinogenesis in older patients.[4]

Biological age, also known as frailty, describes a condition of increased susceptibility to adverse effects after a stressor event due to a progressive decline in physiologic reserves of multiple organ systems.[5] Due to their unhealthy lifestyle, HNSCC patients are often frailer, or biologically older, even compared to patients with other solid malignancies.[6] Moreover, in HNSCC patients there is often a mismatch between chronological and biological age. Biological age is presumably better related to tumor biology than chronological age. Furthermore, biological age has been shown to be a better predictor of treatment tolerance in oncological surgery than chronological age.[7]

Currently, older HNSCC patients receive comparable treatment regimens as younger patients, including radiotherapy or surgery. One of the important differences is withholding chemotherapy in patients above 70. This policy is based on a meta-analysis of Pignon et al., which showed no survival benefit when adding chemotherapy to treatment in older HNSCC patients.[8] However, in this meta-analysis, older patients were strongly underrepresented (357 older patients of the 17000 analyzed cases), non-cancer related deaths were also included and only chronological age, but not biological age was determined.

A better insight into tumor biology of HNSCC could form the basis of clinical studies on novel, age-specific treatment strategies in older patients. A recently published review proposes age-related HNSCC as a new entity besides the known (1) inherited, (2) HPV-related and (3) traditional, substance abuse-associated pathways HNSCC.[9] It is suggested that the pathophysiology of age-related HNSCCs is based on genomic instability, cell cycle disruption, telomere shortening and immunosenescence, leading to mutations.

Immunosenescence, defined as gradual deterioration of the immune system associated with age, could be of importance when considering patients for immunotherapy and immune checkpoint inhibitor therapy. However, a recent meta-analysis showed that chronological age-associated impairments of the immune system did not affect the efficacy of immune checkpoint inhibitor therapy.[10] Therefore, exploring biological age-related tumor characteristics could form new insights on this topic.

Thus far, only limited literature on the relation between tumor biology and chronological age has been published.[10–13] However, the relation between these characteristics and biological age has not been investigated yet. Therefore, the aim of the present study was to determine whether tumor characteristics, including immunological tumor markers, are associated with biological age. Furthermore, we aimed to determine the relation between these tumor characteristics and survival.

Materials and methods

Study design

The patients included in this study were enrolled in OncoLifeS, a prospective oncological data biobank at the University Medical Center Groningen (UMCG) (Netherlands Trial Register registration number NL7839).[14] OncoLifeS was approved by the local Medical Ethical Committee and this study was approved by the OncoLifeS scientific board. All patients signed informed consent before inclusion.

For this study, consecutive patients with HNSCC, diagnosed between 2014 and 2016 in the UMCG were included. Patients aged 18 years and older presenting with a newly diagnosed invasive squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx were included. Patients with recurrent disease or multiple tumors in the head and neck region were excluded. Patients with HPV-related tumors (P16-positive tumors) were excluded, due to the small number of HPV-related tumors in this cohort (n = 20) and their distinctive biology.

Data collection

Patient, tumor and treatment characteristics were obtained from the OncoLifeS database. Patient characteristics included age, gender, comorbidities and outcomes of geriatric assessment. Tumor characteristics included tumor site and stage (according to the TNM classification UICC 8th edition). Detailed histopathological information included

differentiation grade, tumor diameter, depth of invasion (doi), lymph-angioinvasion, tumor invasive growth pattern, bone/cartilage invasion, perineural growth.

Immunohistochemistry (IHC)

To construct tissue microarrays (TMAs), representative regions of the tumor were marked on the H&E-stained slides by an experienced head and neck pathologist. Three cores of 6 µm diameter were taken from each donor block and transferred into a recipient paraffin block using the Manual Tissue Arrayer (Beecher Instruments, Silver Spring, MD, USA).[15]

For the staining of the immunohistochemical markers, 3µm sections were sectioned from the TMA. Sections were stained on a Ventana BenchMark Ultra immunestainer for CD4 (clone SP35, Ventana Medical Systems, Inc., Tucson, AZ, USA), CD8 (clone C8/144B, Dako), CD20 (clone SP33, Ventana Medical Systems, Inc., Tucson, AZ, USA), CD57 (clone NK-1, Ventana Medical Systems, Inc., Tucson, AZ, USA), CD68 (clone KP-1, Ventana Medical Systems, Inc., Tucson, AZ, USA), CD163 (clone MRQ-26, Ventana Medical Systems, Inc., Tucson, AZ, USA), Ki67 (clone 30-9, Ventana Medical Systems, Inc., Tucson, AZ, USA) and Pan Keratin (anti-PAN Keratin, clones CKAE1, CKAE3 and PCK26, Ventana). Visualization was performed according to the manufacturer's protocol by using UltraView DAB. For all antibodies, antigen retrieval was performed using Cell Conditioning 1 (Ventana Medical Systems, Inc., Tucson, AZ, USA). The CD-8 antibody was diluted 1:20; all other antibodies were pre-diluted by the manufacturer. Sections were stained for PD-L1 (clone 22C3, Dako) on a Dako Autostainer Link 48 following the manufacturer's protocol by using EnVision FLEX visualization system.

Analysis of IHC

IHC stains were analyzed by using Digital Image Analysis (DIA). The stained slides were digitized using a Philips Ultra-Fast Scanner 1.6 (Philips, Eindhoven, the Netherlands). Digital slides were stored on a central image server and loaded into the DIA platform Visiopharm Integrator System (VIS) (Visiopharm, Hørsholm, Denmark).

For each CD4, CD8, CD20, CD57, CD68, CD163 and FOXP3 IHC, an individual algorithm was developed to detect and count respectively the amount of T-helper cells, Cytotoxic T-cells, B-cells, Natural Killer Cells, Macrophages, infiltrating M2 Macrophages and Regulatory T-cells of each core. The mean amount of positive cells was calculated from the three cores of each case. Cases were excluded from further analysis if ≥2cores were missing or did not contain tumor cells.

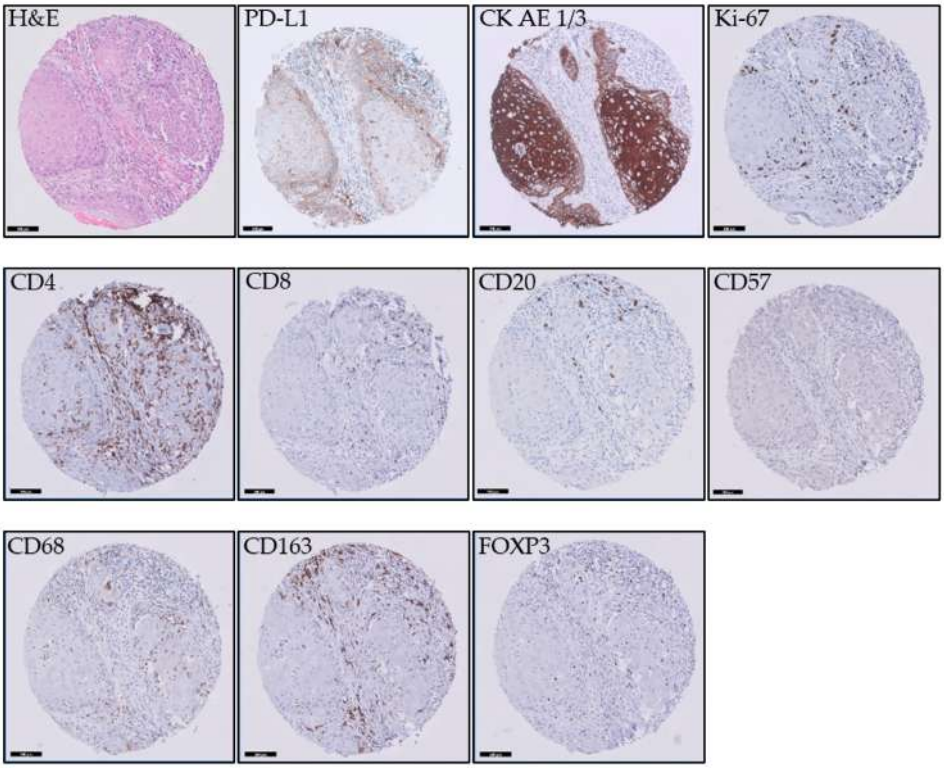

Figure 5.1. **Examples of immunohistochemical staining of the different markers.** The scale bar is 100 µm.

Ki67 was used to determine the proliferation index. A virtual double-staining (VDS) technique was developed for this study to align cores stained for CK-EA1/3 and Ki67. Visual verification of the alignment was performed for all cores, and alignment was optimized manually if needed. Cores were excluded from further analysis if alignment failed. After alignment two algorithms were used. The first algorithm was set to use the cytokeratin-stained area as the tumor classifier on the Ki67-stained core. Within the core, the complete tumor area was annotated. If present, saliva glands, dysplasia and tissue or staining artifacts were excluded.

Ki67 positivity was then analyzed with nuclear classification algorithm, which detect nuclei by morphological form and size and classifies these as positive or negative based on pixel color and intensity. Ki67 proliferation index was calculated by dividing the number of Ki67 positive cells by the total number of positive and negative cells within the area, classified as tumor by VDS. To compensate for intratumoral heterogeneity, the

mean proliferation index was then calculated from the three cores of each case. Cases were excluded from further analysis if ≥2 cores were missing or not contained tumor cells.

For analysis of PD-L1 a Combined Positive Score (CPS) of positive tumor cells and positive lymphocytes was used. A VDS technique was developed to align cores stained for CK-EA 1/3 and PD-L1 in the same way as with the Ki67 staining. The same tumor classifier was used as for the Ki67 analysis. PD-L1 positivity was analyzed with an algorithm which detected nuclei by morphological form and size and classify these as positive or negative based on diaminobenzidine (DAB) staining of linear structures corresponding to membrane fragments on the tumor cells and nuclear staining in the lymphocytic infiltrate. The CPS was calculated by dividing the number of positive tumor cells and the number of positive lymphocytes by the total number of tumor cells. To compensate for intratumoral heterogeneity, mean CPS was calculated from the three cores for each case. Cases were excluded from further analysis if ≥2 cores were missing or not contained tumor cells.

Assessment of biological age

The biological age of the patients was assessed by a multidomain geriatric screening before treatment. Three domains: physical, functional, and psychological were formulated for geriatric assessment, based on the study by Bras et al..[7] In short, the physical domain was based on the Adult Comorbidity Evaluation (ACE-27) and Malnutrition Universal Screening Tool (MUST). The functional domain was based on the Activities of Daily Living (Katz-ADL), Instrumental Activities of Daily Living (IADL) and Timed Up & Go (TUG). In OncoLifeS, mobility was added to the ADL questionnaire and financial information was not available in the IADL questionnaire. The psychological domain was based on the Mini Mental State Examination (MMSE) and Geriatric Depression Scale (GDS-15). An overview of these domains and cut-off points of the geriatric tests is shown in Table 5.1. Patients were considered compromised for a domain if one of the geriatric tests within the domain was impaired. When a patient was impaired in ≥2 domains (based on the accumulation of deficits theory in aging people[16]), the patient was considered biologically old. If information in one of the domains was missing or incomplete, the case was excluded from geriatric analysis.

Table 5.1. Geriatric screening.

Domain	Questionnaires/assessments	Abbreviation	Cut-off value
Physical	Adult comorbidity evaluation	ACE-27	None, mild, moderate, and severe
	Malnutrition Universal screening	MUST	≥1 medium to high risk of malnutrition
Functional	The Katz Activities of Daily living*	ADL	<1 considered impairment
	Instrumental Activities of Daily living**	IADL	Female: ≤6 considered impairment Male: ≤3 considered impairment
	Timed Up and Go	TUG	≥20 impaired mobility
Psychological	Mini Mental State examination	MMSE	≤24 considered impaired cognition
	Geriatric Depression Scale 15	GDS-15	≥6 relates to presence of depression

* Included information on the mobility of the patients; **Excluded financial information because not available.

To investigate the relation between tumor characteristics and chronological and biological age Chi-square test, Fisher's exact test and Mann-Whitney U test were performed. To determine the relation between immunological markers and chronological and biological age, logistic regression analysis was performed by providing odds ratios (ORs), 95% confidence intervals (95% CIs), and p-values. The immunological markers were categorized as < median and ≥ median.
For survival analysis, tumor stage was categorized in early-stage tumors (stage I and II) and advanced stage tumors (stage III and IV). Univariable and bivariable analysis were performed using the log-rank (Mantel-Cox) test and the Cox proportional hazard models. In bivariable analysis, prognostic factors were corrected for chronological and biological age. For the Cox proportional hazard models, the variables tumor diameter, depth of invasion and age were converted into categorical variables (tumor diameter cut-offs ≤20 mm, ≥ 21 mm and < 40 mm, ≥ 40 mm, depth of invasion cut-offs ≤4mm and > 4 mm, age cut-offs < 65 years and ≥ 65 years). Disease-free survival (DFS) was measured from the first consultation at the UMCG to the day of last follow-up or recurrence.

A p-value of < 0.05 was considered statistically significant. All statistical procedures were performed with SPSS Statistics 25.0 software (IBM, Armonk, New York, NY, USA).

Results

A total of 164 patients with a median follow-up time of 36 months were eligible for inclusion. Patient, tumor and treatment characteristics are presented in Table 5.2.

Table 5.2. Baseline characteristics.

Characteristics	N (%)
Total	*n* = 164
Chronological Age	
Mean (± SD)	67.02 (10.49)
Median (p25-p75)	66.53 (41-93)
Biological Age*	
Biologically young	107 (67.9%)
Biologically old	52 (32.7%)
Gender	
Male	113 (68.9%)
Female	51 (31.1%)
Tumor site	
Oral cavity	62 (37.8%)
Larynx	61 (37.2%)
Oropharynx	31 (18.9%)
Hypopharynx	10 (6.1%)
Stage	
I	36 (22%)
II	29 (17.7%)
III	25 (15.2%)
IV	74 (45.1%)
Primary Treatment**	
Surgery	83 (54.6%)
Radiotherapy	47 (30.9%)
Chemoradiotherapy	22 (14.5%)

*Missing data n = 5; **Missing data n = 12.

Tumor characteristics

Tumor characteristics were compared between chronologically and biologically young and old patients and are shown in Table 5.3. In five patients, geriatric information was not complete and therefore they were excluded from the geriatric analysis. None of the tumor characteristics were significantly associated with either chronological or biological age.

Immunological markers

Table 5.4 shows the relation between immunological markers and chronological and biological age. Lower PD-L1 expression was related with higher chronological age (p = 0.030). Expression of CD163, CD68, FOXP3, CD4, CD8, CD20 and CD57 showed no significant difference between chronologically young and older patients.

Lower CD163, CD4 and CD8 expression was related to higher biological age (p = 0.036, p = 0.019, p = 0.026, respectively). Expression of CD68, FOXP3, CD20, CD57 and PD-L1 showed no significant difference between biologically young and older patients. Also shown in Figure 5.2.

Table 5.3. Tumor characteristics in chronological and biological young vs. old patients with HNSCC.

		Chronological Age			Biological Age		
	Total *n* = 164 (100%)	Young <65 y *n* = 72 (43.9%)	Old ≥65 y *n* = 92 (56.1%)	*p*-Value	Young *n* = 107 (67.9%)	Old *n* = 52 (32.7%)	*p*-Value
Tumor diameter (mm) median (±range)	25 (0–70) [a]	21 (1–70) [b]	27 (0–65) [c]	0.138 ¶	23 (0–70) [d]	27 (1–60) [e]	0.462 ¶
Depth of invasion (mm) median (±range)	6 (1–55) [f]	6 (1–30) [g]	6 (0–55) [h]	0.465 ¶	6 (1–30) [i]	7 (1–55) [j]	0.580 ¶
Differentiation grade	*n* = 151	*n* = 65	*n* = 86		*n* = 101	*n* = 46	
Well differentiated	30(19.9%)	14 (21.5%)	16 (18.6%)		21 (20.8%)	8 (17.4%)	
Moderately	115 (76.2%)	47 (72.3%)	68 (79.1%)	0.456 ¥	75 (74.3%)	37 (80.9%)	0.735 ¥
Poorly	6 (4%)	4 (6.2%)	2 (2.3%)		5 (5%)	1 (2.2%)	
Growth pattern	*n* = 81	*n* = 33	*n* = 48		*n* = 52	*n* = 25	
Infiltrative	59 (72.8%)	25 (75.8%)	34 (71.4%)	0.800	39 (75%)	16 (64%)	0.420
Pushing	22 (27.2%)	8 (24.2%)	14 (28.6%)		13 (25%)	9 (36%)	
Perineural growth	*n* = 128	*n* = 56	*n* = 72		*n* = 84	*n* = 41	
Yes	17 (13.3%)	7 (12.5%)	10 (13.9%)	1	11 (13.1%)	6 (14.6%)	1
No	111 (86.7%)	49 (87.5%)	62 (86.1%)		72 (86.9%)	35 (85.4%)	
Lymphangio-invasion	*n* = 130	*n* = 56	*n* = 70		*n* = 83	*n* = 42	
Yes	15 (11.7%)	5 (8.9%)	10 (13.9%)	0.423	8 (9.6%)	7 (16.7%)	0.382 ¥
No	113 (88.3%)	51 (91.1%)	62 (86.1%)		75 (90.4%)	35 (83.3%)	
Bone/cartilage invasion	*n* = 9	*n* = 2	*n* = 7		*n* = 5	*n* = 4	
Yes	4 (44.4%)	0	4 (57.1%)	0.444 ¥	3 (60%)	1 (25%)	0.524 ¥
No	5 (55.6%)	2 (100%)	3 (42.9%)		2 (40%)	3 (75%)	
Proliferation index	*n* = 73	*n* = 31	*n* = 42		*n* = 50	*n* = 22	
<median	41 (56.2%)	14 (45.2%)	27 (64.3%)	0.104	26 (52%)	15 (68.2%)	0.201
≥median	32 (43.8%)	17 (54.8%)	15 (35.7%)		24 (48%)	7 (31.8%)	

p-values were estimated with the chi-square test. ¥ Fisher's exact test performed instead of Chi-square test. ¶ *p*-values determined with the Mann-Whitney test. Tumor diameter and depth of invasion data was available only in a limited number of patient. Tumor diameter: [a] n = 104, [b] n = 43 [c] n = 61 [d] n = 68 [e] n = 34. Depth of invasion: [f] n = 61 [g] n = 24 [h] n = 37 [i] n = 41 [j] n = 20.

Table 5.4. Logistic regression analysis of the relation between immunological markers and chronological and biological age in patients with HNSCC.

		Chronological Age			Biological Age		
	Staining	*n* = 167	Odds Ratio (95%CI)	*p*-value	*n* = 162	Odds Ratio (95%CI)	*p*-value
Macrophages	CD163						
	<median	44 (53%)	Ref		43 (52.4%)	Ref	
	≥median	39 (47%)	0.667 (0.277–1.607)	0.366	39 (47.6%)	0.335 (0.121–0.929)	**0.036**
	CD68						
	<median	43 (52.4%)	Ref		43 (53.1%)	Ref	
	≥median	39 (47.6%)	0.940 (0.388–2.274)	0.891	38 (46.9%)	0.579 (0.218–1.537)	0.273
T-cells	FOXP3						
	<median	46 (54.8%)	Ref		45 (54.2%)	Ref	
	≥median	38 (45.2%)	0.724 (0.302–1.739)	0.470	38 (45.8%)	1.003 (0.387–2.599)	0.995
	CD4						
	<median	44 (53.7%)	Ref		44 (54.3%)	Ref	
	≥median	38 (46.3%)	0.467 (0.190–1.146)	0.096	37 (45.7%)	0.280 (0.097–0.808)	**0.019**
	CD8						
	<median	45 (54.9%)	Ref		45 (55.6%)	Ref	
	≥median	37 (45.1%)	0.797 (0.328–1.934)	0.616	36 (44.4%)	0.300 (0.104–0.866)	**0.026**
B-cells	CD20						
	<median	46 (22.4%)	Ref		46 (56.1%)	Ref	
	≥median	37 (44.6%)	0.458 (0.188–1.118)	0.087	36 (43.9%)	0.412 (0.149–1.141)	0.088
NK-cells	CD57						
	<median	40 (48.8%)	Ref		40 (49.4%)	Ref	
	≥median	42 (51.2%)	0.980 (0.405–2.371)	0.965	41 (50.6%)	0.762 (0.293–1.982)	0.577
Immune	PD-L1						
checkpoint	<1%	24 (29.6%)	Ref		24 (30%)	Ref	
inhibition	≥1%	57 (70.4%	0.292 (0.096–0.891)	**0.030**	56 (70%)	0.732 (0.260–2.059)	0.554

Biological age and chronological age were the independent factors in this analysis. *p*-values <0.05 are marked in bold.

Table 5.5. Univariable and bivariable analyses of prognostic factors for DFS in patients with HNSCC.

	Univariable Analyses		Bivariable Analyses			
			Chronological Age		Biological Age	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Chronological age						
<65	Ref					
≥65	0.871 (0.465–1.63350)	0.864				
Biological age						
Young	Ref					
Old	1.860 (0.949–3.646)	0.071				
Gender						
Male	Ref		Ref		Ref	
Female	1.333 (0.693–2.565)	0.390	1.329 (0.691–2.558)	0.394	1.416 (0.717–2.796)	0.317
Location						
Oral cavity	Ref		Ref		Ref	
Larynx	0.685 (0.324–1.448)	0.322	0.675 (0.319–1.431)	0.306	0.594 (0.269–1.312)	0.198
Oropharynx	1.119 (0.479–2.616)	0.794	1.100 (0.470–2.578)	0.826	1.101 (0.452–2.685)	0.832
Hypopharynx	1.230 (0.358–4.225)	0.743	1.243 (0.362–4.275)	0.730	1.386 (0.401–4.794)	0.606
Tumor stage						
Early stage (I–II)	Ref		Ref		Ref	
Advanced stage (III–IV)	3.293 (1.512–7.170)	**0.003**	3.290 (1.511–7.165)	**0.003**	3.690 (1.614–8.439)	**0.002**
Treatment						
Surgery	Ref		Ref		Ref	
Radiotherapy	0.978 (0.486–1.966)	0.950	0.985 (0.490–1.982)	0.967	0.838 (0.397–1.771)	0.644
Chemoradiation	0.715 (0.247–2.066)	0.535	0.674 (0.228–1.997)	0.477	0.732 (0.251–2.131)	0.567
Tumor diameter						
≤2 cm	Ref		Ref		Ref	
>2 cm–≤4 cm	2.193 (0.862–5.577)	0.099	2.516 (0.959–6.600)	0.061	2.228 (0.874–5.681)	0.093
≥4 cm	1.790 (0.601–5.332)	0.296	1.887 (0.631–5.641)	0.256	1.354 (0.427–4.294)	0.607
Depth of invasion						
≤4 mm	Ref		Ref		Ref	
>4 mm	2.664 (0.866–8.193)	0.087	2.916 (0.941–9.036)	0.064	3.017 (0.971–9.375)	0.056

Table 5.5. (continued).

	Univariable Analyses		Bivariable Analyses			
			Chronological Age		Biological Age	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Differentiation grade						
Well differentiated	Ref		Ref		Ref	
Moderately differentiated	1.278 (0.561–2.912)	0.559	1.298 (0.569–2.960)	0.535	1.387 (0.574–3.352)	0.468
Poorly differentiated	0.598 (0.074–4.860)	0.630	0.571 (0.070–4.655)	0.601	0.721 (0.087–5.991)	0.762
Growth pattern						
Pushing	Ref		Ref		Ref	
Infiltrative	1.981 (0.680–5.771)	0.210	2.016 (0.689–5.892)	0.200	1.934 (0.657–5.691)	0.231
Perineural growth						
No	Ref		Ref		Ref	
Yes	2.816 (1.264–6.277)	**0.011**	2.944 (1.309–6.626)	**0.009**	3.048 (1.341–6.929)	**0.008**
Lymphangio-invasion						
No	Ref		Ref		Ref	
Yes	0.474 (0.195–1.150)	0.099	0.437 (0.177–1.080)	0.073	0.462 (0.186–1.145)	0.095
Bone/cartilage invasion						
No	Ref		Ref		Ref	
Yes	0.275 (0.030–2.504)	0.252	0.348 (0.031–3.937)	0.394	0.299 (0.0.31–2.862)	0.295
Proliferation index						
<median	Ref		Ref		Ref	
≥median	1.695 (0.702–4.094)	0.241	1.605 (0.652–3.952)	0.304	2.498 (0.988–6.313)	0.053

Results showing hazard ratios (HRs) and confidence intervals 95% (CI). In bivariable analysis, prognostic factors are corrected for chronological and biological age. *p*-values < 0.05 are marked in bold.

Table 5.6. Univariable and bivariable analyses of molecular tumor markers for DFS in patients with HNSCC.

		Univariable		Bivariable			
				Chronological Age		Biological Age	
	Immunomarker	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
	Chronological age						
	<65	Ref					
	≥65	0.871 (0.465–1.634)	0.864				
	Biological age						
	Young	Ref					
	Old	1.860 (0.949–3.646)	0.071				
Macrophages	CD163						
	<median	Ref		Ref		Ref	
	≥median	1.416 (0.612–3.277)	0.417	1.391 (0.600–3.224)	0.441	1.934 (0.797–4.693)	0.145
	CD68						
	<median	Ref		Ref		Ref	
	≥median	1.400 (0.605–3.242)	0.432	1.347 (0.580–3.130)	0.488	2.030 (0.836–4.927)	0.118
T-cells	FOXP3						
	<median	Ref		Ref		Ref	
	≥median	0.932 (0.398–2.182)	0.872	0.901 (0.384–2.113)	0.810	0.968 (0.413—2.270)	0.940
	CD4						
	<median	Ref		Ref		Ref	
	≥median	0.559 (0.234–1.333)	0.190	0.514 (0.213–1.237)	0.137	0.691 (0.284–1.683)	0.416
	CD8						
	<median	Ref		Ref		Ref	
	≥median	1.150 (0.498–2.653)	0.743	1.145 (0.496–2.643)	0.751	1.547 (0.649–3.686)	0.325
B-cells	CD20						
	<median	Ref		Ref		Ref	
	≥median	0.993 (0.429–2.298)	0.986	0.930 (0.398–2.174)	0.866	1.191 (0.509–2.786)	0.688
NK-cells	CD57						
	<median	Ref		Ref		Ref	
	≥median	0.497 (0.209–1.187)	0.115	0.499 (0.209–1.190)	0.117	0.516 (0.216–1.231)	0.136
Immune checkpoint inhibitor	PD-L1						
	<1%	Ref		Ref		Ref	
	≥1%	1.263 (0.494–3.229)	0.626	0.837 (0.419–2.929)	0.837	1.519 (0.587–3.929)	0.389

Results showing hazard ratios (HRs) and confidence intervals 95% (CI). In bivariable analysis, prognostic factors are corrected for chronological and biological age

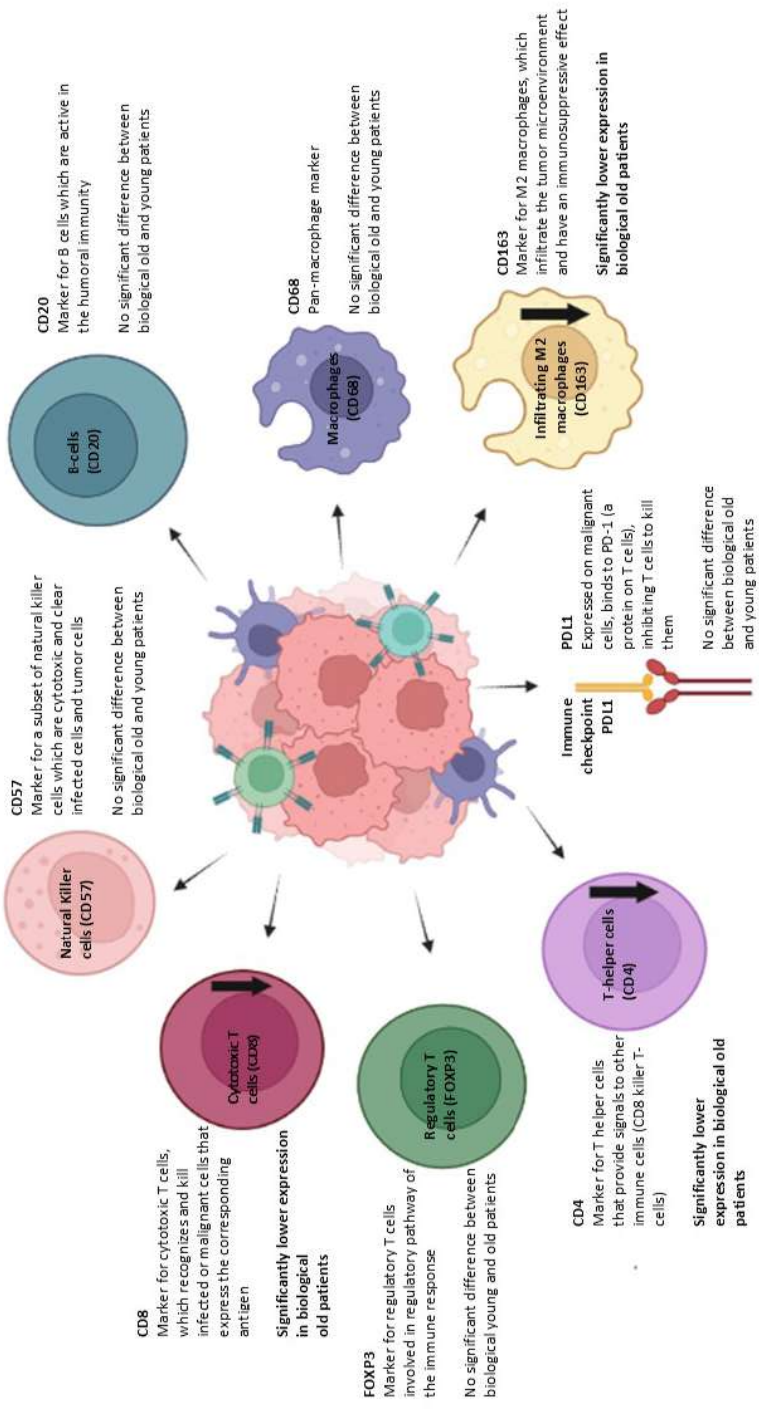

Figure 5.2. The influence of biological age on immunological markers in the tumor microenvironment. The down arrows showing lower CD4, CD8, and CD163 expression in biologically older patients.

Discussion

In this study we aimed to determine whether tumor characteristics, including immunological tumor markers, are associated with both the chronological and biological age of the patient. Furthermore, we aimed to determine the relation between these tumor characteristics and survival.

To our knowledge, this is the first study revealing a relation between biological age and tumor characteristics, including immunological markers. No age-related differences were found for histological characteristics, regarding both biological and chronological age. However, age-specific differences were found in the immunological markers, when age was assessed by biological age. In the tumor microenvironment (TME) of HNSCC in biologically old patients, lower numbers of CD163+ macrophages, CD4+ and CD8+ lymphocytes were found. On the other hand, chronologically older patients showed significantly lower PD-L1 combined positivity scores. This implies a potential decline in tumor immune evasion in the elderly. However, it remains unclear what the exact differences are between the mechanism of chronological and biological aging. None of the immunological markers were related to survival. However, as expected, advanced tumor stage and perineural growth were related to worse disease-free survival.

Measuring biological age can be problematic, as various measuring tools can be used to define biological age. “Comprehensive Geriatric Assessment” (CGA) is considered as the gold standard for biological age assessment. CGA is a multidimensional, time-consuming diagnostic process, performed by a geriatrician. In the clinical setting, referring all HNSCC patients to a geriatrician for performing CGA in is not feasible. Therefore, short frailty screeners have been developed to decide which patient would benefit from a CGA. However, these cannot be used to determine biological age, and their predictive value seems to be limited[17]. Determining deficits on various geriatric domains seems to be the most reliable method for screening,[7,16,18,19] therefore, it was applied in the present study.

Immunosenescence has been described as an important factor contributing to carcinogenesis in older patients[4,20]. Tumor infiltrating lymphocytes (TILs) and tumor associated macrophages (TAMs) involved in the innate immune system are key components in the TME, playing an important role in cancer biology (Figure 5.1). Jeske et al.[20] also studied the TME of HNSCC patients, comparing CD4+ and CD8+ TILs and regulatory T cells of chronologically young and older patients measured by flow cytometry. The effect of aging on the immune system was measured in peripheral blood

lymphocytes (PBL) of healthy volunteers, and compared to PBL and TILs of young and older HNSCC patients. In this study, lower frequencies and total numbers of CD8+ cytotoxic T-cells (responsible for eradication of tumor cells) were found in older HNSCC patients. In contrast, our study found lower CD4+ and CD8+ lymphocyte infiltration only in biologically older HNSCC patients and not in chronologically older patients.

Another study investigated the effect of immunosenescence on TAMs in oral squamous cell carcinoma (OSCC).[21] Immunohistochemistry for CD68 (a pan macrophage marker) and CD163 (a specific M2 macrophage marker, M2 macrophages are thought to facilitate tumor growth) was comparable between patients < 40 years, 40-65 years and > 65 years. All groups showed similar clinicopathological and immunohistochemical findings. The authors conclude that the similar TAM profiles in their study suggests the influence of other mechanisms, instead of immunosenescence, in young and older OSCC patients. This conclusion may be true, if only chronological age is investigated; however, we did show a significant decrease of M2 macrophages with increasing biological age, which suggests that M2 macrophages may be a contributing factor in changes in immunosenescence with increasing biological age.

Programmed Death Ligand 1 (PD-L1) is a transmembrane protein that can bind to lymphocytes. Expression of PD-L1 by tumor and/or immune cells has been linked to immune checkpoint inhibition. However, conflicting results on PD-L1 levels in HNSCC and age are reported.[20,22,23] In a recently published systematic review and meta-analysis investigating the role of PD-L1 in OSCC, no significant association between PD-L1 overexpression and age (>56, >60, >65) was found.[22] However, in two other studies performed in HNSCC patients, higher PD1 and PD-L1 expression was associated with older age[20,23]. In contrast, Ryu et al.[24] compared molecular alteration and tumor immunity in young (< 45 years) and old (≥45 years) HNSCC patients and found that PD-L1 positivity was more frequent in the younger aged group (p = 0.01), similar to the results in this study. However, only chronological age was assessed. Based on these findings, the PD-L1 expression seems to play an important role in predicting response to immunotherapy, irrespective of patients age.[22,23,25]

Stimulating the immune system opened new possibilities in cancer treatment. Since 2016, immune checkpoint inhibitors are approved for the treatment of recurrent or metastatic HNSCC.[26] The results of our study, showing lower concentrations of CD8+ T lymphocytes in biologically older patients, proposes that treatment with immune checkpoint inhibitors may be less effective in this specific population. To better select

patients who may benefit from the treatment with these novel therapeutic agents influence of biological age should be determined.

This study has some limitations. Both biopsies and surgical specimens were included in this study, resulting in low numbers of cases for specific features, like bone invasion, grow pattern and lymph-angioinvasion when investigated on the biopsy material. Therefore, the analysis of these characteristics may not be reliable. Furthermore, the pathological tumor characteristics were analyzed retrospectively and matched with the clinical database, which also may have its impact on a relatively high number of missing data. Last, this study was performed on a limited sample size. To strengthen the results, these data should be validated on a larger scale.

Conclusions

The number of older HNSCC patients is continuously growing and there is often mismatch between biological and chronological age. Therefore, knowledge of biologically age-related alterations of the immune system is necessary to offer adequate treatment options for this specific group of patients. This study shows that biological age might have a stronger influence on the composition of the tumor microenvironment than chronological age. However, the exact changes in the tumor microenvironment in biological versus chronological old head and neck cancer patients needs to be further investigated. Furthermore, our findings should also initiate clinical studies, investigating the response to specific treatment regiments (e.g., immunotherapy) according to the biological age of the patients.

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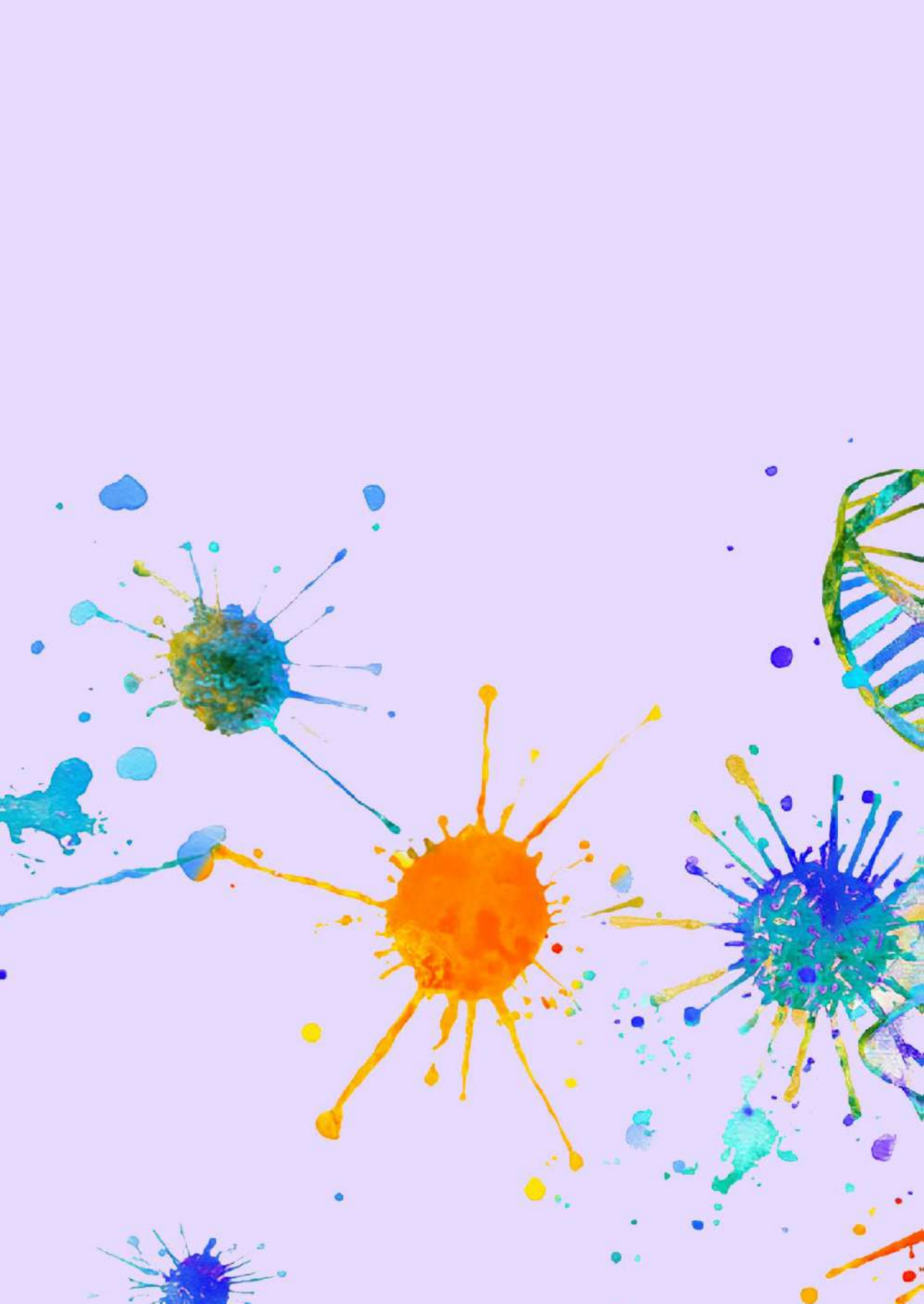

CHAPTER 6

The prognostic role of tumor associated macrophages in squamous cell carcinoma of the head and neck: A systematic review and meta-analysis

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Abstract

Background

Head and neck squamous cell carcinoma (HNSCC) is an immunogenic cancer type, and tumor associated macrophages (TAMs) are a major component of the tumor microenvironment (TME).

Methods

In this systematic review and meta-analysis, studies assessing tumor infiltration with CD68+, iNOS+, HLA-DR+, CD11b+, CD163+, CD206+, and CD204+ TAMs were included, and correlation to survival hazard was studied.

Results

A low number of CD68+ TAMs correlated to better overall survival (OS) in multivariate analysis (HR 1.36 95%CI (1.07-1.72) $p = 0.01$). CD68+ TAMs did not correlate to disease free survival (DFS), disease specific survival (DSS), progression free survival (PFS), or recurrence free survival (RFS). A low number of CD163+ TAMs correlated to better OS in uni- and multivariate analysis (resp. HR 2.65 95%CI (1.57-4.46) $p = 0.01$ and HR 2.42 95%CI (1.72-3.41) $p < 0.001$). A low number of CD163+ TAMs also correlated to better DFS and PFS, whereas a low number of CD204+ TAMs only correlated to PFS.

Conclusion

While IHC analysis of pan macrophage marker CD68 and M2-like marker CD163 both show prognostic utility in OS, CD163 is a stronger prognosticator, as indicated by multivariate meta-analysis. CD163+ TAMs also correlate to DFS and PFS; outcomes that are more relevant to patients, thus showing promising results for future clinical implementation.

Introduction

Head and neck cancer is the sixth most common cancer worldwide, with approximately 890,000 new cases and 450.000 deaths reported in 2020.[1] More than 90% of head and neck cancers are head and neck squamous cell carcinomas (HNSCC), derived from the mucosal epithelium of the oral cavity, pharynx, or larynx.[2,3] Main risk factors for HNSCC include smoking and tobacco use, as well as Human Papilloma Virus (HPV) specifically for oropharyngeal cancer. For patients diagnosed with early stage HNSCC (stage I or II), the prognosis is relatively favorable, with 5-year overall survival (OS) rates between 70-90% after treatment with surgery or radiotherapy. However, around 60% of HNSCC patients are diagnosed with locally advanced stage disease (stage III or IV). The 5-year overall survival rate of locally advanced stage disease is poor (less than 50%) due to an increased risk of recurrence and/or distant metastasis.[4]

Prognostic biomarkers predict the natural course of disease and thus, identify the likelihood of patient survival, irrespective of treatment. They serve several purposes in clinical setting, like predicting the risk of poor outcome in an individual, which could aid in managing patient expectations and guiding treatment decisions.[5] Classical cancer-related prognostic factors such as histological tumor type, tumor site, tumor size, lymph node involvement, distant metastasis, and HPV status are leading in the clinical management process of HNSCC patients.[6] The current standard of care is based on risk stratification according to the tumor-node-metastasis (TNM) classification (eight edition) and HPV status. However, the clinical outcome of patients diagnosed with the same TNM-stage is variable.[7] This underlines a need for further refinement of the TNM staging system to predict the clinical behavior of HNSCC better.

The tumor microenvironment (TME) plays a pivotal role in cancer progression and harbors various immune cells, such as macrophages, natural killer cells, and lymphocytes. Although not yet used in clinical practice, extensive studying of the TME in recent years has produced some promising prognostic biomarkers, such as tumor infiltrating lymphocytes (TILs).[7,8] Identifying additional prognostic biomarkers within the TME, with respect to the TNM classification, could refine HNSCC risk stratification, and aid in treatment decision making.

Macrophages are of innate origin and are classically divided into two phenotypes: type 1 macrophages (M1) and type 2 macrophages (M2). Classically activated macrophages, or the M1 macrophages, produce proinflammatory cytokines such as IL-12, IL-23, IFN-Y, TNF-a and show strong antimicrobial resistance through phagocytosis and activation of

inducible nitric oxide (iNOS). Alternatively, activated macrophages, or the M2 macrophages, produce anti-inflammatory cytokines such as IL-4, IL-10, and IL-13, and they play a central role in antiparasitic immunity, angiogenesis, tissue remodeling and allergic diseases.[9] It is important to state that the discrimination between M1 and M2 macrophages does not consider that macrophage subtypes exist on a broad spectrum and are polarized by the resident microenvironment. The most frequently used immunohistochemical marker for macrophages is CD68, a pan macrophage marker that does not differentiate between subtypes. M1 macrophages could be detected by staining iNOS (inducible nitric oxide synthase), a toxic cytoplasmic enzyme excreted by M1 macrophages, or by surface markers like CD11b and HLA-DR. M2 macrophages could be detected by CD163, a scavenger receptor for the hemoglobin-haptoglobin complex. They can also be detected by CD204, macrophage scavenger receptor 1, or CD206, a mannose receptor of type C lectin.[10,11]

The TME abundantly accommodates a specific class of macrophages: tumor associated macrophages (TAMs).[11,12] TAMs consist of two major subpopulations, the M1-like macrophages that are potent effector cells in the killing of tumor cells, and the M2-like macrophages which stimulate tumor growth and progression through several mechanisms. TAMs that reside within or near the tumor generally show characteristics of the M2 subtype.[13] Pro-tumorigenic M2-polarized TAMs are strongly involved in angiogenesis, which in turn plays an important role in inducing tumor growth and metastasis.[14] Reasoning from the biological function of TAMs within the TME, we hypothesize that M1-like TAMs are correlated with better survival, whereas M2-like TAMs are correlated with worse survival, and they could therefore function as prognostic biomarkers.[15]

This systematic review and meta-analysis aims to provide insight into the prognostic value of macrophage subsets found in the TME, and to thereby contribute to deliberate decision making in clinical practice.

Methods

Search strategy

An extensive systematic search was conducted on the 27th of June 2022 in two databases: PubMed/Medline and EMBASE. As search terms, synonyms of the term 'head and neck squamous cell carcinoma' and a variety of tumor associated macrophage

markers were used. The search also included a filter for prognostic studies. The full search strategy is shown in Supplementary Table S6.1.

In- and exclusion criteria

The selection of studies was conducted by title and abstract screening, followed by full-text reading of the selected articles. The title/abstract screening and full-text reading were conducted by researchers SKB and MvdK, and discrepancies were resolved by discussion. Studies were eligible for inclusion if they assessed the prognostic value of CD68+, iNOS+, HLA-DR+, CD11b+, CD163+, CD206+ and/or CD204+ macrophages in patients with HNSCC by a time-to-event analysis, described as overall survival (OS), disease free survival (DFS), disease specific survival (DSS), progression free survival (PFS), recurrence free survival (RFS) or locoregional control (LRC). See Supplementary Table S6.2 for the definition of the survival terms. Nasopharyngeal carcinomas were excluded due to distinct pathogenesis. Macrophages had to be evaluated by immunohistochemistry (IHC) and/or immunofluorescence (IF) techniques. Only original articles published in English were included. Animal studies, case reports, reviews, meta-analyses, conference abstracts, or repetitive studies were excluded.

Data extraction

From the studies selected by full-text screening, the following data were extracted: author's last name, year of publication, biomarkers, sample size, tumor subsite, HPV status, treatment modalities, staining and scoring methods, confounders, hazard ratios (HR) for outcome, confidence intervals, and p-values. Between studies, different definitions regarding tumor compartment were used. Most studies assessed macrophages in the intra-tumoral compartment, meaning the tumor epithelium (TE) or tumor stroma (TS) within the tumor mass. Two studies[16,17] assessed macrophages in the intra-tumoral compartment (IT), which included both TE and TS, and in the peritumoral compartment (PT), which only included stroma on the outskirts of the whole tumor. In this study, HRs for survival were separately extracted for tumor epithelium (TE) and tumor stroma (TS) in the intra-tumoral compartment. These data were entered in a standardized form creating a synopsis of all relevant articles.

Outcome

This study focused on macrophage markers and the correlation with survival (OS, DFS, DSS, PFS, RFS, and LRC) using meta-analysis. The Cochrane handbook advises pooling univariate and multivariate HRs for survival separately because of their different statistical interpretation. Studies were excluded from the meta-analysis if HRs were

missing. Data from studies that used IHC were pooled separately from studies that used IF to limit potential heterogeneity.

Critical appraisal

The Quality in Prognosis studies (QUIPS) was used to assess the risk of bias, as described by Hayden et al (2006).[18] This tool comprises six items: study participation, attrition, prognostic factor measurement, outcome measurement, confounding and statistical analysis, and reporting. For each of these items, the risk of bias was scored as low, moderate, or high by two independent researchers (SKB, MvdK). Discrepancies between the researchers were resolved by discussion, after which, the official QUIPS score was determined.

Quality of evidence

The quality of the evidence summarized in this study was rated by GRADE (Grading of Recommendations, Assessment, Development and Evaluations).[19]

Statistical analysis

Concordance between the QUIPS score of the reviewers was measured by calculating a Weighted Kappa. In the meta-analysis, HRs for survival were defined by low macrophages versus high macrophages. If the study mentioned HRs as high macrophages versus low macrophages, the reciprocal was used. Statistical heterogeneity was assessed using the I^2 statistics. The meta-analysis was performed in review manager 5.3 using the inverse of variance test with a random effect analysis. Additionally, publication bias was assessed by funnel plots and Egger's test. The funnel plots were created of meta-analysis with at least 10 included studies. Egger's test of $p < 0.05$ indicated the presence of publication bias. Funnel plots and Egger's test were conducted in Stata version 17.

Results

Study selection

The initial search yielded 2009 articles after removing duplicates (Figure 6.1). After title/abstract screening, 80 articles were eligible for full-text screening, of which 25 articles[16,17,20–42] met the inclusion criteria. Table 6.1 gives an overview of study characteristics of the studies included in the meta-analysis.

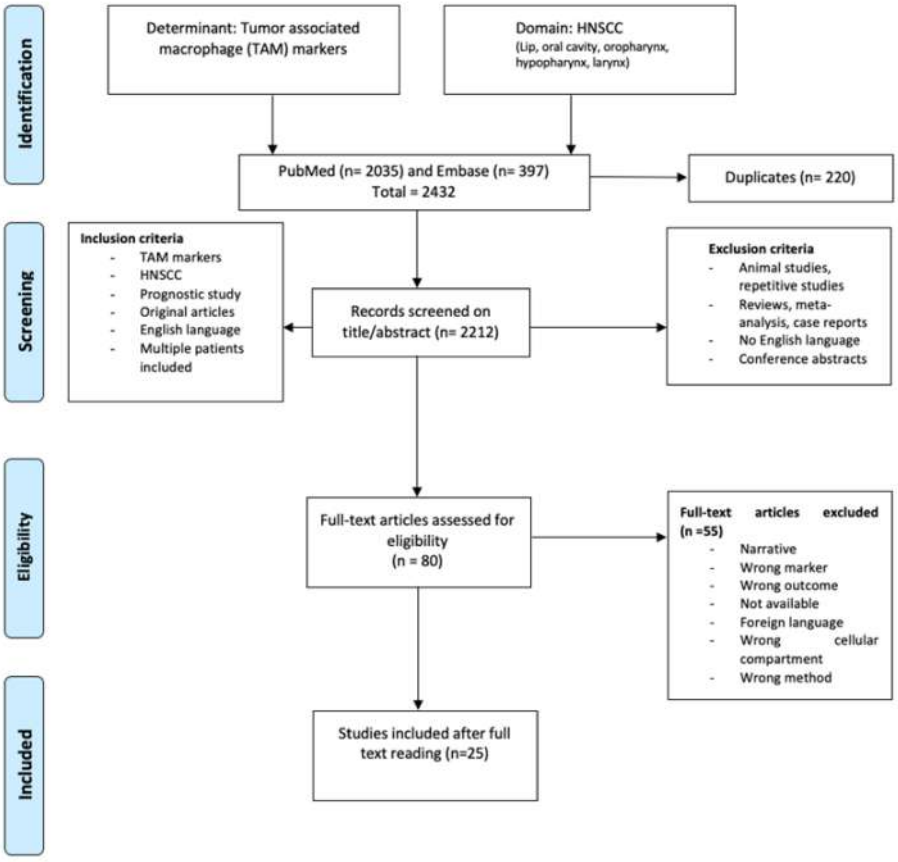


Figure 6.1. Of the 80 articles subjected to full-text reading, 25 articles were eligible for inclusion.

Table 6.1. Study characteristics of the studies included in the meta-analysis.

Study	Sample size	Subsite	HPV +/-	Stage	Treatment	Biomarkers	Material	Scoring compartment	*Technique*
Hori 2021	62	T	–	I-IVb	S, S + RR, S + CRT	CD163, Pan-CK, CD4, CD8, FOXP3, CD45RO	FFPE	IT/PT	*IHC*
Kikuchi 2020	103	OC	–	All	S, S + RT, S +,CRT	CD68, PD-1, PD-L1, CD3, CD8, CD4	FFPE	TE, TS	*IHC*
Tsakiroglou 2020	72	OP	Both	All	RT, CRT	CD68, CD8, PD1, PD-L1	FFPE	NS	*IF*
Haque 2019	44	OC	–	All	?	CD3, CD20, CD162, CD204, CD206, EGF	FFPE	NS	*IHC*
Kwon 2019	54	OC	–	All	S, S + RT, S + CRT	CD68, Stabilin-1	FFPE	NS	*IHC*
Ou 2019	95	OC, OP, L, HP	Both	III, IV	CRT, BRT	CD68, CD163, HLA 1	FFPE	TE, TS	*IHC*
Zhou 2019	71	L	–	All	S	CD68, CD163, CD3, CD8, CD4	FFPE	IT/PT	*IHC*
Ryu 2018	396	OC, OP, HP, L, NP, T	–	All	S + CT, RT	CD163, P16, CD3, CD8, C8/144B, FOXP3 ICOS/CD278 , LAG-3 , TIM-3 , CTLA-4, PD-L1, PD-1, c-MetNUT, Trk, TrkB panTrk, cyclin D1	FFPE	TE	*IHC*
Seminerio 2018	110	OC, OP, HP, L, NP	Both	All	S	CD68	FFPE	NS	*IHC*
Sun 2018	72	OC	–	I-IVb	CT, RT	CD68, CD31	FFPE/FT	NS	*IHC*
Cioni 2018	142	OP	Both	All	RT, CRT	CD4, CD8, CD68, FoxP3, CD163, panCK	FFPE/FT	TE, TS	*IF*
Fang 2017	78	OP	–	All	S	CD57, CD68, CD8, CD4, T-bet	FFPE	TS	*IHC*
Kubota 2017	46	OC	–	All	?	CD163, CD204, CD25, IL10, CD69	FFPE/FT	TE	*IHC*
Oguejiofor 2017	124	OP	Both	All	RT, S + RT, CRT, S + CRT	CD68, CD8, PDL1	FFPE	NS	*IF*
Takahashi 2017	73	T	–	All	S	CD68, CD163, a-SMA, Ki67, p53	FFPE	TS	*IHC*
Nguyen 2016	278	OC, OP, HP, L	Both	All	S, CRT, RT, P	CD68, CD104, CD8, CD4, CD1a	TMA	TE	*IHC*
Lee 2015	79	TS	Both	All	CRT	CD68, CD8, CD4	FFPE	TE, TS	*IHC*
Matsuoka 2015	60	OC	–	II,III, IV	CRT	CD163, a-SMA	FFPE	TS	*IHC*
Ni 2015	91	OC	–	All	S	CD68	FFPE	TE, TS	*IHC*
Balermpas 2014	106	OC, OP, HP, L	Both	I-IVb	CRT	CD68, CD163, CD11b, p16, CD31	FFPE	NS	*IHC*
Fuijta 2014	50	OC	–	All	S	CD163, IL8, FOXp3	FFPE	TE	*IHC*
Wang 2014	298	OC	–	All	S	CD163, IL-10, IFN-y	FFPE	NS	*IHC*
Russell 2013	35	OC, OP, HP, L, SN	Both	All	S	CD68, CD3, CD8, FOXp3, CD20, CD16,HLA-DR, HLA-A, HLA-G	FFPE	TE	*IHC*
Fujii 2012	108	OC	–	All	S	CD68, CD163, a-SMA	FFPE	TE	*IHC*
Lin 2011	*84*	*L*	–	*All*	*S*	*CD68*	*FFPE*	*TE, TS*	*IHC*

Oral Cavity (OC), Oropharynx (OP), Hypopharynx (HP), Larynx (L), Lip (Lip) Tongue (T), Sinonasal (SN), Nasopharynx (NP), Tonsil (TS), Surgery (S), Radiotherapy (RT), Chemotherapy (CT), Chemoradiotherapy (CRT), Immunotherapy (IT), Bio Radiotherapy (BRT), Formalin Fixed, Paraffin Embedded material (FFPE), Fresh tissue (FT), Tissue Microarray (TMA), Tumor epithelium (TE), Tumor stroma (TS), Intra-tumoral (IT), Peritumoral (PT), Immunohistochemistry (IHC), Immunofluorescence (IF), Not specified (NS).

Critical appraisal

The 25 studies included in the meta-analysis were critically appraised using the QUIPS-criteria by the two independent researchers.[18] Concordance between the researchers was considered fair (κ 0.04 CI 0.03-0.78). Four studies[22,27,30,40] scored moderately in the study participation domain due to non-consecutive cohorts, insufficient reporting of treatment method, or inclusion/exclusion criteria. The domain for study attrition was disregarded because only one study[20] provided information about patients' loss to follow-up. In the prognostic factor domain, many studies used the median value as a cut-off value. Since no consensus is reached about a reasonable cut-off value, the use of medians was scored as low risk. Two studies[21,34] scored moderately in this domain because they provided insufficient information about the histological techniques used to assess the prognostic factor. One study[38] scored moderately in the outcome measurement domain because no follow-up time was mentioned. In the statistical analysis and reporting domain, it was observed that several studies did not conduct multivariate analysis, and this was scored accordingly.[21,31] Data reported in the study by Lee et al. (2015)[34] could not be reproduced in our meta-analysis; this domain was scored as high risk of bias. Therefore, this study was excluded from the meta-analysis. The full quality assessment is summarized in Table 6.2.

Classical marker CD68 as a prognostic biomarker for survival

A total of nine studies reported on CD68+ TAMs and their correlation with OS in a univariate manner by the IHC technique.[16,20,24,26-29,32,33,36] The pooled meta-analysis showed no correlation between CD68+ TAMs and OS (HR 1.27 95%CI (0.90-1.79) p = 0.17, Figure 6.2a). Seven of the studies included in the univariate meta-analysis also reported multivariate HRs. Three studies only reported multivariate HRs.[37,42,43] Pooled meta-analysis of the final ten studies investigating the relationship between CD68+ TAMs and OS showed that a low number of CD68+ TAMs correlated to a better OS (HR 1.36 95%CI (1.07-1.72) p = 0.01, Figure 6.2b).

In the univariate and multivariate pooled analysis, a total of four studies assessed CD68 in both tumor epithelium and tumor stroma.[20,24,36,42] Kikuchi et al. (2020) reported a trend that low CD68+ TAMs correlated to better OS in the tumor epithelium. However, this was not the case in tumor stroma.[20] Lin et al. (2011) also supported the latter finding.[42] Ni et al. (2015) reported that low CD68+ TAMs were associated with a better OS in the tumor stroma, but tumoral CD68 expression did not have a prognostic impact.[36] Of the studies that did not specify the scoring compartment, Sun et al. (2018),

Seminerio et al. (2018), and Wang et al. (2014) also reported a positive correlation between low CD68+ TAMs and OS.[26,27,39] The remaining studies found no correlation between CD68+ TAMs and OS. In contrast with the previous studies, Ou et al. (2019) report a trend following a high number of CD68+ TAMs in tumor epithelium correlating with better OS.[24] When stratifying studies in subgroup based on tumor compartment, no difference was found between TAMs found in tumoral or surrounded stromal tissue (Figure 6.2c). However, these results need to be interpreted with caution, as high statistical heterogeneity is present.

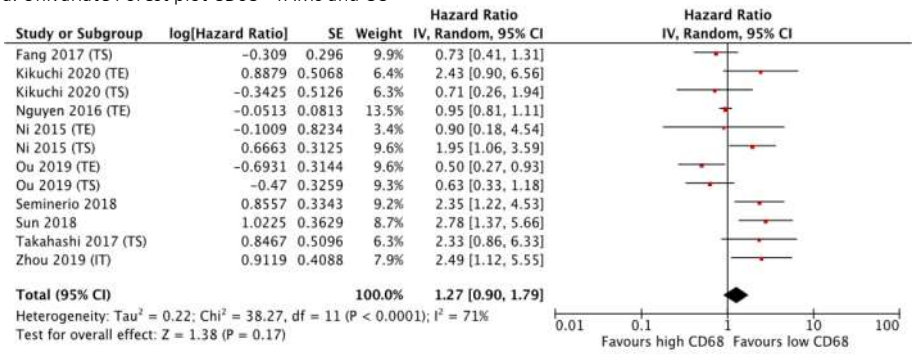
Table 6.2. Quality assessment of the studies included in the meta-analysis.

StudyStudy participation		Study attrition	Prognostic factor meassurment	Outcome meassurement	Study confounding	Statistical analysis and reporting	Total bias score
Hori 2021	○	•	○	○	○	○	Low
Kikuchi 2020	○	◐	○	○	○	○	Low
Tsakiroglou 2020	○	•	◐	○	○	◐	Moderate
Haque 2019	◐	•	○	○	○	○	Low
Kwon 2019	○	•	○	○	○	○	Low
Ou 2019	○	•	○	○	○	○	Low
Zhou 2019	○	•	○	○	○	○	Low
Ryu 2018	○	•	○	○	○	○	Low
Seminerio 2018	○	•	○	○	○	○	Low
Sun 2018	◐	•	○	○	○	○	Low
Cioni 2017	○	•	○	○	○	○	Low
Fang 2017	○	•	○	○	○	○	Low
Kubota 2017	◐	•	○	○	○	○	Low
Oguejiofor 2017	○	•	○	○	○	◐	Low
Takahashi 2017	○	•	○	○	○	○	Low
Nguyen 2016	○	•	○	○	○	○	Low
Lee 2015	○	•	◐	○	○	•	Moderate
Matsuoka 2015	○	•	○	○	○	○	Low
Ni 2015	○	•	○	○	○	○	Low
Balermpas 2014	○	•	○	○	○	○	Low
Fuijta 2014	○	•	○	◐	○	○	Low
Wang 2014	○	•	○	○	○	○	Low
Russell 2013	◐	•	○	○	○	○	Low
Fujii 2012	○	•	○	○	○	○	Low
Lin 2011	○	•	○	○	○	○	Low

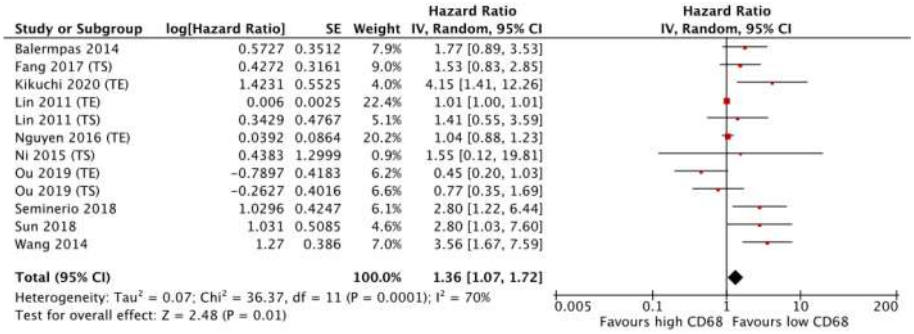
○ = low risk of bias, ◐ = moderate risk of bias, • = high risk of bias.

Three studies[21,28,31] employed IF techniques to study the relationship between CD68+ TAMs and OS and reported no significant correlation in univariate analysis (Supplementary Figure S6.1 via https://ars-els-cdn-com.proxy-ub.rug.nl/content/image/1-s2.0-S1368837522005164-mmc1.docx).

a. Univariate Forest plot CD68+ TAMs and OS



b. Multivariate Forest plot CD68+ TAMs and OS



c. Subgroup analysis of the correlation between CD68+ TAMs and OS in tumor epithelium and tumor stroma.

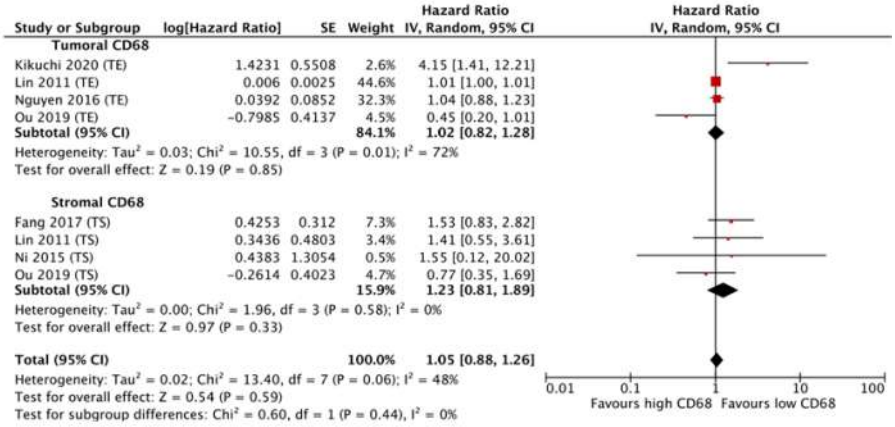


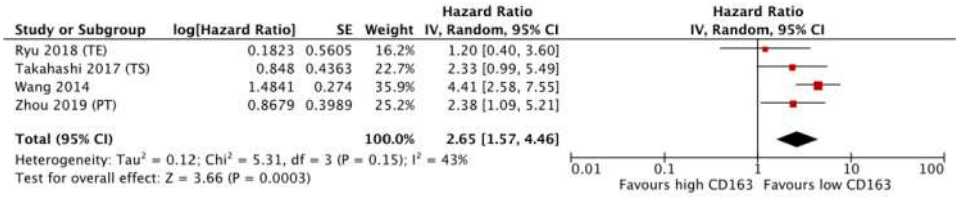
Figure 6.2. These forest plots assessed the correlation between CD68+ TAMs stained by IHC techniques and OS: 6.2a. The pooled univariate analysis revealed no correlation between CD68+ TAMs and OS. 6.2b. The pooled multivariate analysis revealed that a low number of CD68+ TAMs correlated to a better OS. 6.2c. Subgroup analysis revealed that there was no significant difference on the number of TAMs in tumoral or stromal tissue.

One study, Lin et al. (2011)[42] reported that a low number of CD68+ TAMs correlate to a better DFS, especially for stromal TAMs (HR 5.4 95%CI (1.17-25.47) p = 0.02). Three studies[23,33,40] reported on the correlation between CD68+ TAMs and DSS, and not one significant correlation was found in univariate analysis (Supplementary Figure S6.2). Another three[24,32,37] studies reported on the correlation between CD68+ TAMs and PFS. In both univariate and multivariate analysis, Ou et al. (2019) reported that high CD68+ TAMs correlated to a better PFS, whereas the two other studies did not report significant findings. The pooled meta-analysis conducted for multivariate analysis revealed no significant correlation (HR 1.04 95%CI (0.45-2.40) p = 0.93) (Supplementary Figure S6.3). Contrary to Ou et al. (2019), Takahashi et al. (2017) did report a significant univariate correlation, in which low CD68+ TAMs correlate with better PFS; however, this finding was not maintained in multivariate analysis (resp. HR 4.3 95%CI (1.26-1.47) p = 0.02 and HR 2.38 95%CI (0.63-8.90) p = 0.20).[32] Pooled analysis between three studies[23,26,33] that reported on RFS revealed no correlation between CD68+ TAMs and RFS (HR 1.62 95%CI (0.70-3.75) p = 0.25) (Supplementary Figure S6.4).

M2-like markers CD163, CD204, and CD206 as prognostic biomarkers for survival

The pooled analysis for studies reporting univariate HRs found that low CD163+ TAMs correlate to better OS (HR 2.65 95%CI (1.57-4.46) p = 0.01) (Figure 6.3a). The pooled analysis for studies that reported multivariate HRs also found that low CD163+ TAMs correlate to better OS (HR 2.42 95%CI (1.72-3.41) $p < 0.001$) (Figure 6.3b). Three studies reported on the correlation between CD163+ TAMs and DFS in a multivariate manner.[17,35,38] The pooled analysis showed that low CD163+ TAMs correlated to a better DFS (HR 2.51 95%CI (1.55-4.09) $p < 0.001$) (Figure 6.4). The pooled result of two studies[22,30] revealed no significant correlation between CD163+ TAMs and DSS (Supplementary Figure S6.5). The pooled result for both univariate and multivariate studies showed that low CD163+ TAMs correlate to a better PFS (resp. HR 1.86 95%CI (1.22-2.84) p = 0.00 and HR 1.50 95%CI (1.02-2.22) p = 0.04) (Figure 6.5). Hori et al. (2021) also reported on LRC and found that low CD163+ TAMs in tumor epithelium correlates to a better LRC (Multivariate HR 5.06 95%CI (1.12-22.88) p = 0.04).[17]

a. Univariate Forest plot CD163+ TAMs and OS.



b. Multivariate Forest plot CD163+ TAMs and OS

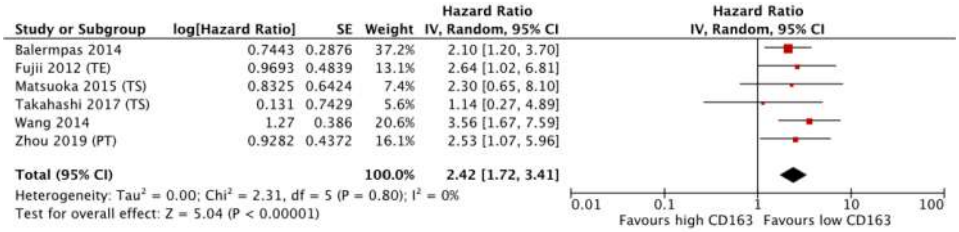


Figure 6.3. These forest plots assessed the correlation between CD163+ TAMs stained by IHC techniques and OS: 6.3a. The pooled univariate analysis revealed that a low number of CD163+ TAMs correlates to a better OS. 6.3b. The pooled multivariate analysis also revealed that a low number of CD163+ TAMs correlates to a better OS.

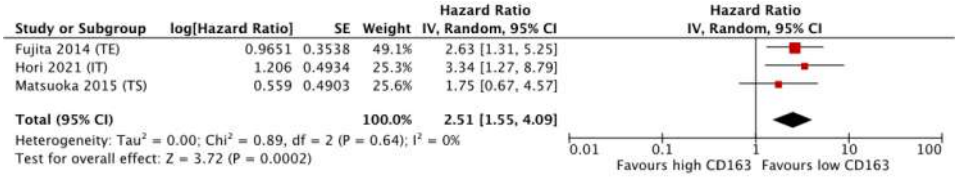
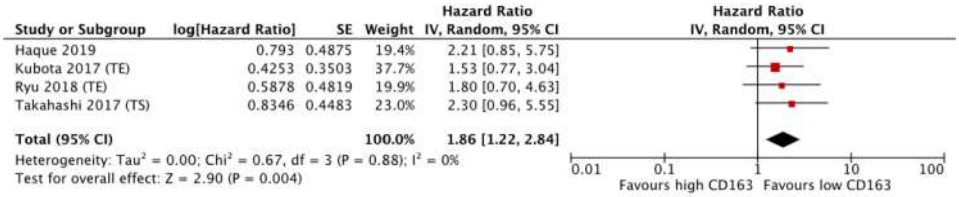


Figure 6.4. The pooled multivariate analysis revealed that a low number of CD163+ TAMs correlates to a better DFS.

a. Univariate Forest plot CD163+ TAMs and PFS.



b. Multivariate Forest plot CD163+ TAMs and PFS.

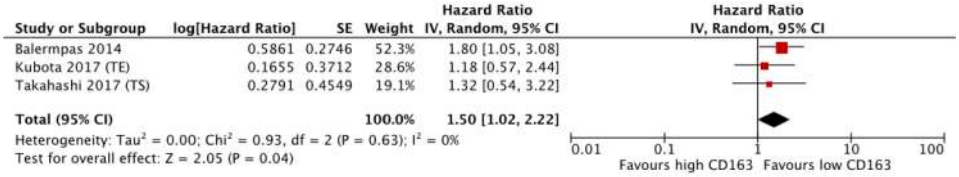


Figure 6.5. These forest plots assessed the correlation between CD163+ TAMs stained by IHC techniques and PFS: 6.5a. Even though separate studies did not report a significant correlation, the pooled univariate analysis revealed that a low number of CD163+ TAMs correlates to a better PFS, due to increased statistical power. 6.5b. The pooled multivariate analysis revealed that a low number of CD163+ TAMs correlates to a better PFS.

Haque et al. (2019) and Kubota et al. (2017) reported on the correlation between CD204+ TAMs and DSS.[22,30] The pooled result revealed no significance between CD204+ TAMs and DSS (Supplementary Figure S6.6). These two studies also reported on the correlation between CD204+ TAMs and PFS. The pooled result revealed that low CD204+ TAMs correlate to a better PFS (HR 1.96 95%CI (1.15-3.35) p = 0.02) (Supplementary Figure S6.7).

Haque et al. (2019)[22] reported on CD206+ TAMs and their correlation with DSS and PFS. For both endpoints, low CD206+TAMs correlated with better survival (HR 3.29 95%CI (1.1-14.1) p = 0.03 and HR 3.28 95%CI (1.1-14.1) p = 0.03).

M1-like markers iNOS, HLA-DR, and CD11b as prognostic biomarkers for survival

No studies reported on the correlation between iNOS, HLA-DR, or CD11b expressed by TAMs and OS, DFS, DSS, PFS, RFS or LRC.

Quality of evidence

The strongest certainty in the quality of evidence was present in PFS as outcome, based on the GRADE approach. OS, DSS and RFS had a lower GRADE score on inconsistency, due to high statistical heterogeneity in the pooled meta-analysis. DFS had a lower GRADE score on imprecision, due to the relatively smaller sample size (Supplementary Table S6.2). Publication bias is an important component of the GRADE approach. To address this, funnel plots were created of the correlation of CD68 with OS, because these meta-analyses were the only ones with at least 10 studies included. The funnel plots showed a slight asymmetric distribution, which could indicate potential publication bias for OS as outcome (Supplementary Figure S6.8A and S6.8B). However, Egger's test displayed a $p < 0.19$ for the univariate meta-analysis and a $p < 0.52$ for the multivariate meta-analysis. Our pooled result of the correlation between CD68 and OS was thus not affected by publication bias.

Discussion

This systematic review and meta-analysis demonstrate that a low number of CD68+ TAMs found in the TME of HNSCC correlate with better OS. Furthermore, a low number of CD163+ TAMs correlate to better OS, DFS, and PFS. A low number of CD204+ TAMs correlate to better PFS.

HNSCC is thought to be an immunogenic cancer type, and TAMs are the most abundant type of immune cell present in the TME.[43] In the early stages of neoplastic development, TAMs within the TME predominantly express M1-like features, probably attempting to eliminate tumor cells. As the tumor progresses, tumor cells can excrete soluble biomolecules which activate and polarize TAMs to work for the tumor's benefit, skewing them in the M2-like direction.[14,44] Tumor evasion mechanisms are characteristic traits of cancer, and TAMs have various strategies to achieve this, like the upregulation of checkpoint inhibitors like Programmed cell Death Ligand 1 (PD-L1) to deactivate cytotoxic lymphocytes and the secretion of immunosuppressive cytokines that affect other immune cells. TAMs can also promote tumor metastasis by secreting soluble factors that damage the endothelial basement membrane of blood vessels, like matrix metalloproteases (MMPs). TAMs are drawn to hypoxic tumor regions, where they often upregulate hypoxia inducible factor (HIF)-1a and HIF-2a. This hypoxic environment aids TAMs in angiogenesis by inducing the secretion of angiogenic molecules, like vascular endothelial growth factor (VEGF).[14] Due to the duality in TAM function and subsequent controversial evidence on TAMs in HNSCC, its prognostic role has yet to be determined.

This systematic review and meta-analysis fulfill an unmet need by including a broad spectrum of TAM markers (CD68, CD163, CD204, CD206, iNOS, HLA-DR and CD11b) in the quest for clarification of the prognostic role of TAMs within the TME in HNSCC.

Remarkable results of this study are that the univariate meta-analysis did not show a correlation between CD68+ TAMs and OS. In contrast, the multivariate meta-analysis showed that a low number of CD68+ TAMs correlate to better OS than a high number of CD68+ TAMs. This could be explained by greater statistical power in the multivariate analysis, due to a greater sample size. In many studies, markers are only included in the multivariate analysis if they show a certain level of significance in the univariate analysis, which could result in greater statistical power, due to narrower confidence intervals. One pattern can be recognized in the univariate- and multivariate meta-analysis; most individual studies mention that low CD68+ TAMs correlated to better survival. Other individual studies mention a trend in that low CD68+ TAMs correlates to worse survival, or they mention no correlation at all.

These inconsistencies between individual studies could have different explanations. One partial explanation could be the specificity of CD68. CD68 is a glycosylated type 1 transmembrane glycoprotein located in lysosomes, intracellularly. It is not specific for cell lineage, but for lysosomal activity. Although it is highly expressed on the monocyte/macrophage lineage, it is also incidentally expressed on fibroblasts, endothelial cells, dendritic cells, B cells, T cells, basophils, neutrophils, and osteoclasts.[10] Therefore, results on pan-macrophage marker CD68 should be interpreted with discretion.

Another explanation could be the different TAM detection and quantification techniques among studies.[11] Most studies used the 'hotspot' method, but the amount of 'hotspots' differs between studies. Some studies quantified TAMs manually, meaning that at least two pathologists manually counted the macrophages. However, other studies used automatic quantification methods. There is no consensus on a cut-off value of TAMs, resulting in some studies using median values, others using means, which could aid in differences. These limitations indicate the need for standardization of TAM detection and quantification, for example, by reaching consensus on the number of 'hotspots' and which cut-off value to use.

A third contributor to the discrepancies could be explained by differences in the tumor compartment in which the TAMs were assessed.[11] For a long time, it was believed that a high abundance of TAMs led to poor survival in various types of solid tumors. However,

in the last decade, accumulating evidence arose on the complexity of TAMs residing within the TME. In lung cancer, a high number of M2-like TAMs in the tumor stroma were associated with a poor OS. Interestingly, abundant M1-like TAMs in the tumor epithelium was associated with a favorable OS.[45,46] On the contrary, in breast cancer, a high number of TAMs correlate to worse survival, especially for TAMs found in the tumor stroma.[47] For this matter, a subgroup analysis was conducted in this study to investigate a potential difference between the prognostic value of CD68+ TAMs in tumor stroma and tumor epithelium. No difference between TAMs in the tumor or stroma was found. These results, however, need to be interpreted with caution because of high statistical heterogeneity. It is also notable that a clear definition of intra-tumoral and peritumoral compartment within HNSCC seems to be lacking, as studies used different definitions, which complicates the interpretation of the results regarding tumor compartment.

Furthermore, in this study, a low number of CD163+ TAMs correlated with better OS in both univariate and multivariate analysis. In univariate analysis, Troiano et al. (2019) found that a low number of CD163+ TAMs correlated to better OS.[48] Our study strengthens the results of the former study by the additional finding that a low number of CD163+ TAMs correlated to better OS in multivariate analysis. CD163+ TAMs also correlated to better DFS and PFS. CD163 is exclusively expressed on cells of the monocyte lineage (monocytes, macrophages, dendritic cells) and could therefore function as a good prognostic biomarker and target for therapy.[49] Based on these results, it is concluded that CD163 is potentially a stronger prognosticator for survival than CD68. Also, CD204 could potentially function as a prognostic biomarker based on the results of this meta-analysis. However, only two studies assessed the prognostic role of this marker, and they reported HRs with wide confidence intervals, which warrants careful interpretation.

Lastly, in this study, the prognostic role of M1 marker iNOS expressed on TAMs in HNSCC was aimed to be identified. However, no studies on this topic were found. Even though iNOS is a widely known M1-marker, iNOS immunohistochemistry bears challenges, like unreliable results when using paraffine-embedded blocks, due to mRNA degradation.[50] iNOS can also be expressed on tumor cells and endothelial cells, which could blur the actual effect of M1-like TAMs on survival. No studies were found on some other markers for M1-macrophages, including CD11b and HLA-DR. Agarbati et al. (2021) investigated the prognostic role of CD11c M1-like TAMs in tongue squamous cell carcinoma and reported a better DFS in a subgroup of patients with histologic grade 3 differentiation.[51] However, CD11c is a nonspecific marker, mostly expressed on dendritic cells, limiting its

specificity. This study was not included in our study because HRs were not reported. Specifically, for M1-like TAMs, a robust marker for IHC seems to be lacking.

The strength of this study lies in the large number of studies included, resulting in the possibility of pooling both univariate and multivariate results. Our study also calculated the interobserver variability between the raters of the QUIPS criteria and employed the GRADE approach to rate the quality of the body of evidence. In meta-analysis conducted on the correlation of CD68+ TAMs and OS, Egger's test revealed no publication bias was present, however high statistical heterogeneity was observed, which is most likely the result of clinical heterogeneity. Therefore, the first general limitation of this study is heterogeneity in tumor subsite, among and within studies included in this review. The studies showed heterogeneity in treatment modalities with different mechanisms of action, so the prognostic role of TAMs could also differ. This analysis included both HPV+ and HPV- HNSCC, even though we know that HPV+ HNSCC is a different entity with better survival outcomes. Furthermore, Seminerio et al. (2018) report a higher number of CD68+ TAMs in HPV+/p16+ tumors, indicating that HPV is an important confounder.[26] In multivariate analysis, correction of patient related factors is included, allowing a more accurate interpretation of results. A limitation of pooling the multivariate results together in this meta-analysis is that the studies did not all correct for the same variables. Lastly, several studies would have been eligible for inclusion if they provided hazard ratios.

In conclusion, this study showed that in multivariate meta-analysis, CD163 is a potentially better prognosticator for OS than CD68. CD163+ TAMs also correlated with DFS and PFS, outcomes that are more relevant in clinical practice. The quality of evidence regarding PFS as outcome was high, indicating strong confidence in the effect estimate. For M1-like TAMs, limited studies have been conducted, which could be attributed to the lack of a robust IHC marker.

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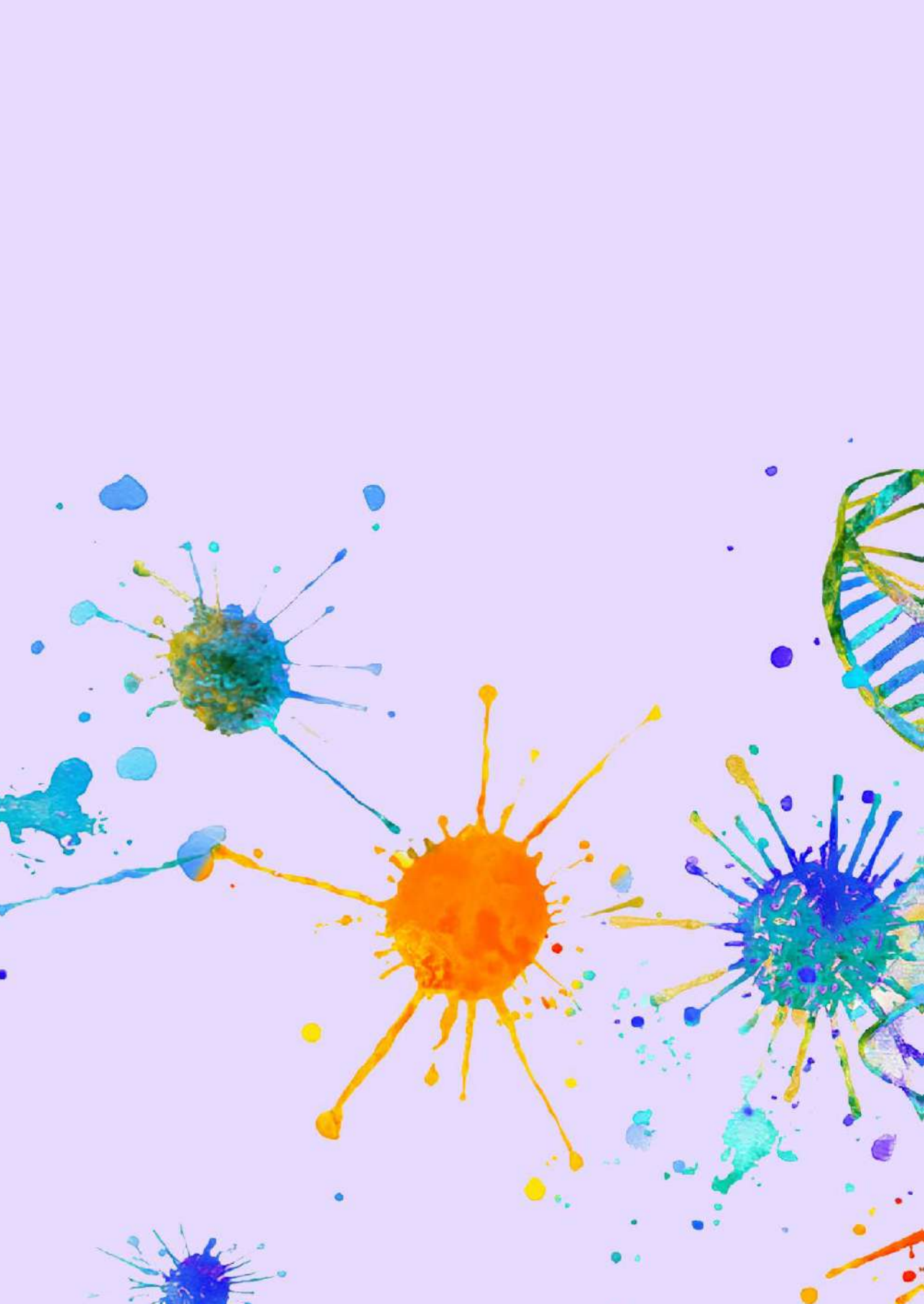

CHAPTER 7

Addition of tumor microenvironment immune cell composition to improve the performance of a predictive model for oral squamous cell carcinoma

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Abstract

Background

Conventional clinicopathological characteristics insufficiently predict prognosis in oral squamous cell carcinoma (OSCC). We aimed to assess the added predictive value of tumor microenvironment immune cell composition (TMICC) in addition to conventional clinicopathological characteristics.

Methods

Primary tumor samples of 290 OSCC patients were immunohistochemically stained for CD4, CD8, CD20, CD68, CD163, CD57, FoxP3 and Programmed cell Death Ligand 1. Additionally, clinicopathological characteristics were obtained from patients' medical files. Predictive models were trained and validated by conducting Least Absolute Shrinkage and Selection Operator (LASSO) regression analyses with cross-validation. To quantify the added predictive power of TMICC within models, receiver operating characteristic (ROC) analyses were used.

Results

Recurrence occurred in 74 patients (25.5%). Conventional clinicopathological characteristics (tumor localization, pathological T-stage, pathological N-stage, extracapsular spread, resection margin, differentiation grade, perineural invasion, lymphovascular invasion) and treatment modality, were used to build a LASSO logistic regression-based predictive model. Addition of TMICC to the model resulted in a comparable AUC of respectively 0.79 (± 0.01) and 0.76 (± 0.1) in the training and test sets. The model indicated that high numbers of CD4+ T cells protected against recurrence. Lymph node metastasis, extracapsular spread, perineural invasion, positive surgical margins and reception of adjuvant treatment were associated with increased risk for recurrence.

Conclusions

The TMICC, specifically the number of CD4+ T cells, is an independent predictor, however, addition to conventional clinicopathological characteristics does not improve the performance of a predictive model for recurrence in OSCC.

Introduction

Head and neck cancer is the seventh most common cancer worldwide and approximately 40% of head and neck squamous cell carcinoma (HNSCC) cases comprise oral cavity squamous cell carcinoma (OSCC).[1] OSCC is a major health concern, with 178,000 deaths reported worldwide in the year 2020. Locoregional recurrence and distant metastasis are frequently found in patients with advanced stage OSCC, often occurring in the first two years after treatment.[2] Even for early stage OSCC treated with curative intent, still 10-25% of the patients develop recurrent disease.[3] Strikingly, the overall survival (OS) rate of patients with locoregional recurrence and/or distant metastasis drops significantly, from 90% to approximately 30%.[4] The cornerstone of treatment for OSCC is surgery. (Chemo)radiotherapy serves as either an adjuvant treatment following surgery or as the primary treatment in cases of advanced stages or when surgery is not feasible.[5] Predicting the prognosis is often challenging, making it difficult to avoid either overtreatment or undertreatment. This emphasizes the urge of identifying patients who are at risk of developing recurrent disease, so treatment planning can be tailored for the individual patient to achieve better prognosis and to avoid overtreatment.

Traditionally, conventional prognostic risk stratification and thereby HNSCC treatment planning is based on the TNM classification, supplemented with parameters like surgical margins, perineural invasion and lymphovascular invasion. Pathological entities, as depth of invasion, bone invasion and extra-nodal extension have recently been added to the TNM system (8th edition). These additions improved the prognostic potential of the TNM classification system.[6] However, it is far from optimal for patient stratification and prognostic prediction, as patients within the same tumor stage show variable survival rates.[7,8]

The interplay between immune – and tumor cells within the tumor microenvironment (TME) is a perplexing enigma, as it either promotes or inhibits tumor progression.[9] Unravelling the complex role of immune cells within the TME could provide evidence of their prognostic ability and could potentially support the TNM classification in risk stratification. In the development of predictive models, clinical context is underrepresented and the additional role of immune biomarkers upon existing prognostic and predictive factors is often left unexplored.

Therefore, the aim of this study was to develop a predictive model for OSCC that adds the tumor microenvironment immune cell composition (TMICC) to the conventional clinicopathological characteristics.

Materials and methods

Patient and tumor characteristics

This study was conducted using prospectively gathered clinical data of head and neck cancer patients, collected in the OncoLifeS data-biobank. This data-biobank has been approved by the medical ethics committee of the University Medical Center Groningen (UMCG) (no.2010/109). It is registered in the Dutch Trial Register (NL7839) and in the UMCG research register (201900297). All patients signed informed consent before inclusion. The OncoLifeS database was supplemented with retrospectively gathered data from patients' medical files. Patients older than 18 years with OSCC, diagnosed between 1993 - 2008 in the UMCG were included. Exclusion criteria were tumor type other than squamous cell carcinoma, subsite other than oral cavity and palliative treatment.

For each patient the following clinical data was collected: gender, age, tumor subsite, pathological TNM-7 stage, surgical margins, differentiation grade, perineural growth, extra-nodal extension, lymphovascular invasion, primary treatment, adjuvant treatment, locoregional recurrence (LRR), distant metastasis (DM), survival status and follow up time. The standard treatment regimen involved curative surgery with or without elective neck lymph node dissection and adjuvant radiotherapy.

Tissue microarray construction and immunohistochemistry

Formalin fixed, paraffine embedded (FFPE) surgically resected tissue of the primary tumor was collected in a tissue micro-array (TMA) and immunohistochemically stained (IHC), as described before.[10] In short, resected tissue was stained with hematoxylin-eosin and representative tumor regions were annotated by an experienced head and neck pathologist (BvdV). For each patient, three 6 mm diameter cores were taken from the pre-annotated tumor region in the donating paraffin block and combined in a pre-assigned location in a recipient block. The TMAs were stained on a Ventana BenchMark Ultra immune Stainer (Roche Ventana) for CD4 (SP35, Ventana), CD8 (C8/144B, Dako), CD20 (SP33, Ventana), CD57 (NK-1, Ventana), CD68 (KP-1, Ventana), CD163 (MRQ-26, Ventana) and Pan Keratin (anti-PAN Keratin, CKAE1, CKAE3 and PCK26, Ventana) following the manufacturer's protocol. All antibodies were pre-diluted by the supplier.

PD-L1 (22C3, Dako) was stained on a Dako Autostainer Link 48 following the manufacturer's protocol by using EnVision FLEX visualization system.

IHC analysis

Digital Image Analysis (DIA) platform Visiopharm Integrator System (VIS) (Visiopharm, Hørsholm, Denmark) was used to analyze the IHC stains. Algorithms were developed to detect and count the number of CD4 (T-helper cells), CD8 (cytoxic T-cells), CD20 (B-cells), CD57 (natural killer cells), CD68 (macrophages), CD163 (M2-like macrophages), FoxP3 (regulatory T-cells) positive cells and to score PD-L1 IHC. The mean number of positive cells was calculated by averaging the results of the available cores of each case. Cases were excluded from further analysis if ≥ 2 cores were missing or did not contain tumor cells. For analysis of PD-L1 the Combined Positive Score (CPS) was used. A virtual double-staining (VDS) was developed specifically for this study to align the cores that were stained for PD-L1 and Pan Keratin. PD-L1 positivity was assessed using an algorithm that identifies nuclei based on their morphological characteristics and size. The algorithm then categorizes these nuclei as either positive or negative, based on the DAB staining of linear structures, which correspond to the membrane fragments on the tumor cells, along with nuclear staining observed in the lymphocytic infiltrate. The CPS was determined by dividing the count of positive tumor cells and positive lymphocytes by the total count of tumor cells. In order to address intratumoral heterogeneity, the mean CPS was derived from three cores for each case. See Figure 7.1 for IHC stained TMA cores of the different immune cells and their respective algorithm overlay.

Outcome

The primary outcome measure was recurrence, consisting of locoregional recurrence (LRR) defined as development of a new tumor less than 2 cm in distance from the primary tumor or regional progression of the primary tumor, all within a period of 3-years after completion of treatment, and/or distant metastasis (DM) defined as the spread of OSCC to distant sites, irrespective of time.

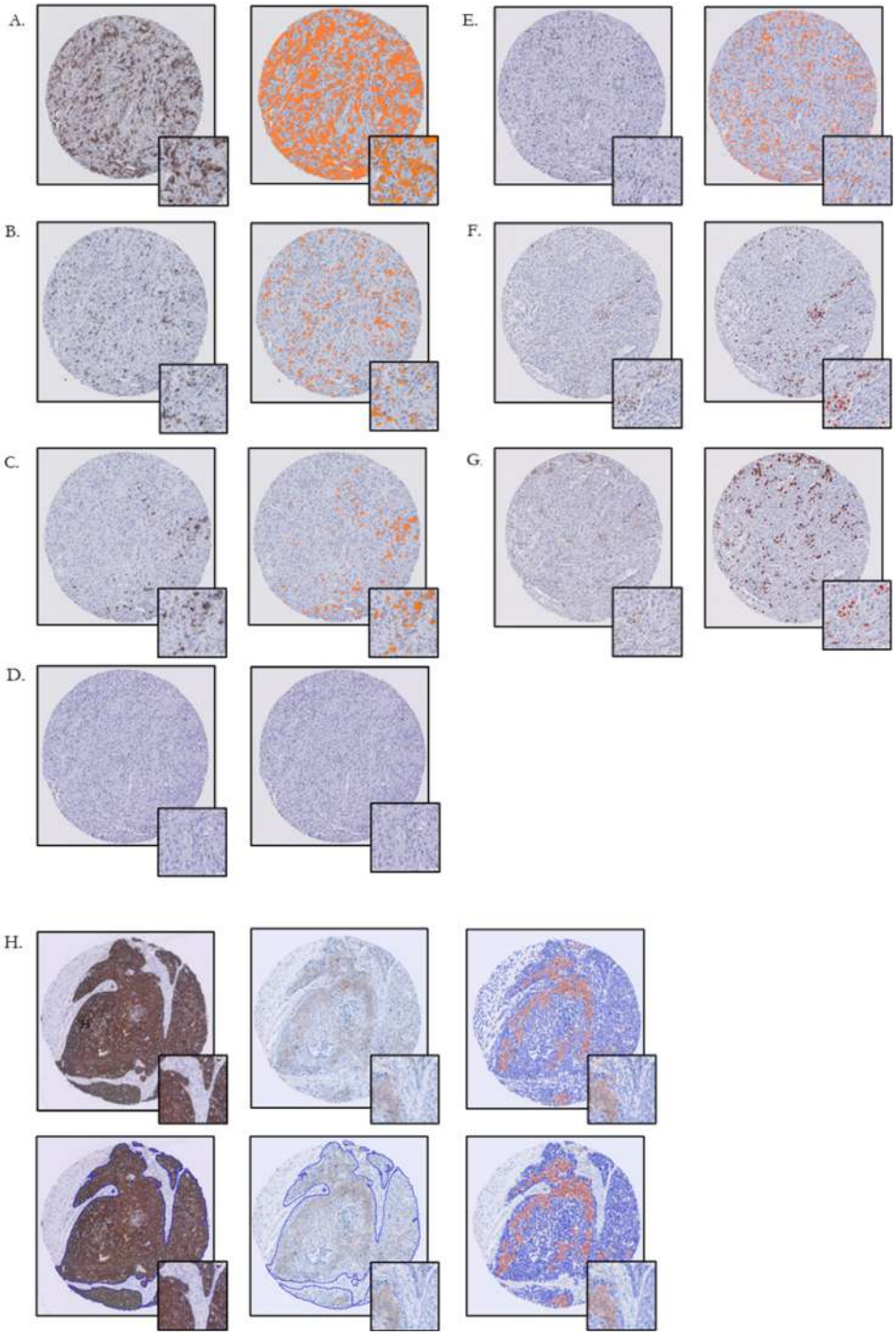
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Figure 7.1. TMA cores stained for different immune cells, on the left IHC and on the right corresponding algorithm overlay for: a. CD4, b. CD8, c.CD20, d. CD57, e. FoxP3, f. CD68, g. CD163, h: Virtual double staining components; Pan Keratin staining (upper left), PD-L1 staining (upper middle), Algorithm overlay of Pan Keratin positive cells in blue and PD-L1 positive cells in red (upper right). Tumor tissue compartment in the TMA denoted by the blue line for the Pan Keratin staining (lower left), PD-L1 staining (lower middle) and algorithm overlay of Pan Keratin positive cells in blue and PD-L1 positive cells in red. TMA: tissue microarray; IHC: immunohistochemistry.

Development of prediction models

The data exhibited some missing values (< 10% in 3 clinical variables, < 5% in all cell count variables, no missingness in remainder) which were imputed using median imputation for numerical, and most frequent imputation for categorical variables. We fitted two logistic regression models, one including all clinical variables, and one including clinical and cell count variables, respectively the clinicopathological predictive model and the combined TMICC predictive model. The cell count variables were implemented as continuous variables within the TMICC predictive model. Logistic regression using Least Absolute Shrinkage and Selection Operator (LASSO) was used for model fitting and variable selection. LASSO is a machine learning based regularization technique able to minimize variables which contribute the least to the predictive model. It does so by shrinking several model regression coefficients (β) toward zero. The optimal shrinkage parameter α was identified by plotting validation curves. These curves show the binary cross entropy loss on test data, as a function of shrinkage parameter α. Binary cross entropy loss quantifies the distance between predicted probabilities and the true classification, so the lower the binary cross entropy loss is, the better the model is at making accurate predictions. The following process was conducted to identify the best α associated with optimal binary cross entropy loss: Data were first standardized to unit variance, and for a range of possible values for the shrinkage parameter α, the binary cross entropy was calculated by dividing the data into training (7/8) and test (1/8) sets, fitting the regularized model to training data, and evaluating the binary cross entropy on both training and test set; this procedure was repeated 400 times with random splits stratified by outcome to reduce the variance of the estimate. Finally, the value of α minimizing the average test loss was chosen. This procedure guards against overfitting, and by using LASSO it additionally performs variable selection by shrinking the coefficients of several variables to 0, therefore selecting variables that contributed most to the model; see this article by Bühlmann et al.[11] for details. Unbiased Receiver-Operator Characteristic (ROC) curves were calculated for this optimal α using the same training/test resampling procedure, in order to compare the clinicopathological predictive model and the combined TMICC predictive model. See Figure 7.2 for the prediction model workflow.

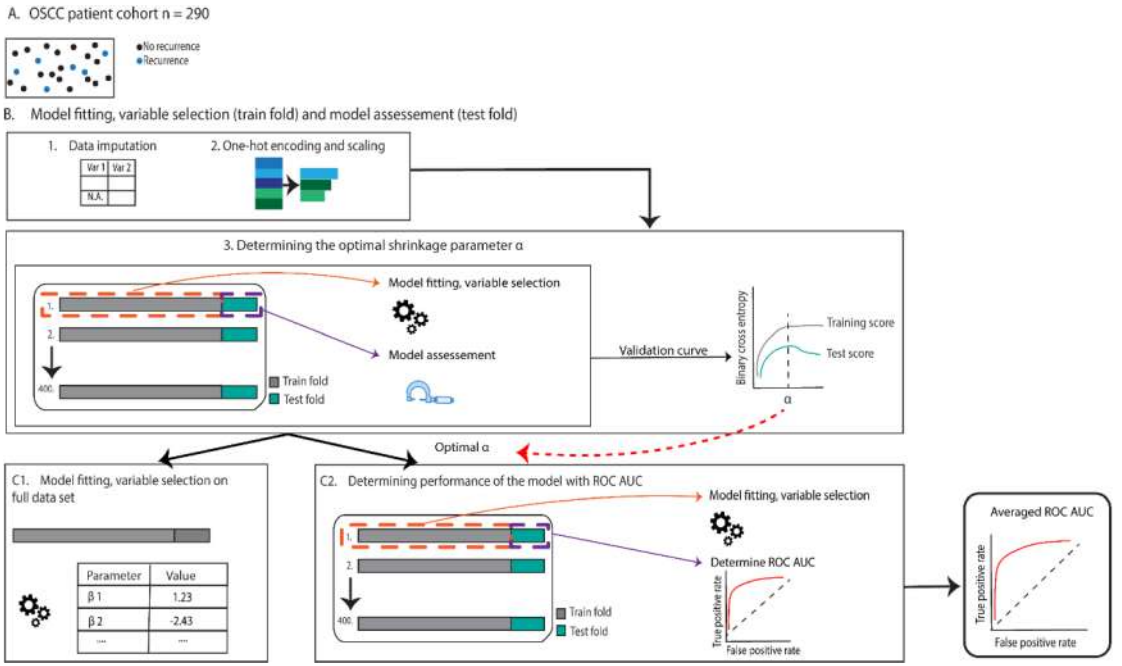

Figure 7.2. Workflow prediction model development and evaluation. A. The cohort consisted of 290 patients with OSCC. B. (1) Missing data was imputed, (2) categorical variables were converted into columns with a binary value (one-hot encoding) and standardization of numerical variables was achieved by scaling. (3) The optimal shrinkage parameter α was identified by calculating the binary cross entropy for a range of possible α values. The data was divided into training (7/8) and test sets (1/8). The training set was subject to model fitting, meaning the regression coefficients (β) of the predictors were determined and evaluated on both training and tests sets. By repeating this process 400 times, a validation curve was plotted with on the x-axis the range of possible α values, and on the y-axis the binary cross entropy score (In the figure the graph of the cross entropy score is shown, which is the negative of the cross entropy loss.) The shrinkage parameter α minimizing the binary cross entropy loss was chosen. C1. The performance of the predictive model with the optimal α was evaluated on the whole dataset and β values of important predictors were calculated. So, we identified which predictors contributed most to the predictive model, whilst the use of the optimal shrinkage parameter α made sure that several β values that did not contribute much were shrunk to 0. C2. To visualize model performance with an Receiver-Operator Characteristic (ROC) Area Under the Curve (AUC) the optimal α was subjected to the same training/test resampling procedure. This was done to be able to compare the clinicopathological predictive model with the combined TMICC predictive model. Created with Adobe Illustrator (2020).

Evaluation of prediction models

Different models were evaluated by ROC Area Under the Curve (AUC) visualization. A high ROC AUC indicates better discriminatory ability of the model. Best performing models were selected and used to assess the added predictive value of TMICC with respect to conventional clinicopathological characteristics for the binary outcome recurrence.

Results

Patient characteristics

This patient cohort consisted of 290 patients diagnosed with OSCC. The majority were localized in the floor of the mouth (111, 38.3%), followed by the tongue (93, 32.1%) and the buccal mucosa (49, 16.9%). More than half of the patients were male (172, 59.3%), and the median age was 62 years. All patients were treated with surgery and the majority received post-operative radiotherapy (*n* = 199, 68.6%). The median follow-up time was 36 months (Standard Deviation 36.7). LRR occurred in 64 patients (22.1%) and distant metastasis occurred in 17 patients (5.9%). Our primary outcome recurrence (LRR and/or DM) occurred in 74 patients (25.5%). The clinicopathological characteristics are summarized in Table 7.1.

Table 7.1. Clinicopathological variables of the included patients (n = 290).

Clinicopathological characteristics	Total *n* = 290
Age (Years)	
Median (Min, Max)	62 (25, 94)
Mean (SD)	62 (12.3)
	No. of Patients (%)
Sex	
Male	172 (59.3%)
Female	118 (40.7%)
Oral cavity subsite	
Tongue	93 (32.1%)
Gingiva	49 (16.9%)
Floor of the mouth	111 (38.3%)
Buccal mucosa	8 (2.8%)
Retromolar	23 (7.9%)
Other	6 (2.1%)

Table 7.1. (continued)

Clinicopathological characteristics	Total *n* = 290
Pathological T stage	
T1	74 (25.5%)
T2	94 (32.4%)
T3	28 (9.7%)
T4	94 (32.4%)
Pathological N stage	
N0	139 (47.9%)
N1	57 (19.7%)
N2a	3 (1%)
N2b	68 (23.4%)
N2c	20 (6.9%)
N3	1 (0.3%)
Nx	2 (0.7%)
Extra nodal spread (N+)	*n* = 149
Yes	65 (22.4%)
No	84 (29%)
Differentiation grade	
Well differentiated	66 (22.8%)
Moderately differentiated	167 (57.6%)
Poorly differentiated	46 (15.9%)
Missing	11 (3.8%)
Perineural invasion	
Yes	73 (25.2%)
No	198 (68.3%)
Missing	19 (6.6%)
Lymphovascular invasion	
Yes	28 (9.7%)
No	228 (78.6%)
Missing	34 (11.7%)
Surgical Margin	
Positive	73 (25.2%)
Close	74 (25.5%)
Negative	143 (49.3%)
Primary treatment	
Local surgery	2 (0.7%)
Local surgery + neck dissection	288 (99.3%)
Adjuvant treatment	
Post-operative radiotherapy	199 (68.6%)
None	91 (31.4%)

Clinicopathological predictive model for recurrence

First, we created a predictive model for recurrence containing the following conventional clinicopathological characteristics and treatment modality as input: tumor

subsite, TNM-7 stage, extra nodal extension, surgical margins, differentiation grade, perineural growth, lymphovascular invasion and post-operative radiotherapy. The optimal cross entropy loss for train and test sets were respectively -0.47 (±0.01) and -0.49 (±0.01). The best α associated with the optimal binary cross entropy loss was identified at 0.12. The ROC AUC of the train and test sets indicated a good discriminatory power with values of respectively 0.79 (±0.01) and 0.77 (±0.01), see **Figure 7.3**. This predictive model included a set of six key conventional clinicopathological characteristics explaining the likelihood of recurrence in this study population. The following characteristics were associated with an increased likelihood of recurrence: (1) lymph node metastasis, (2) perineural invasion, (3) lymphovascular invasion and (4) extra capsular spread, (5) positive surgical margins and (6) post-operative radiotherapy. See **Table 7.2** for predictive model regression coefficients (β).

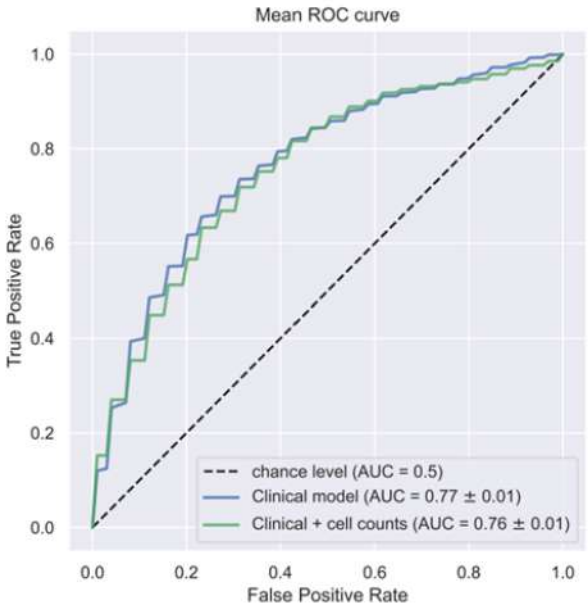

Figure 7.3. ROC AUC curve of the clinicopathological predictive model (blue line) and the combined TMICC predictive model (green line).

Table 7.2. Predictive model regression coefficients (β). Coefficients were transformed back to undo scaling and correspond to directly observed values (natural log transformed in case of CD4+ T cell counts). Positive values of β predict an increased likelihood of recurrence. Negative values of β predict a decreased likelihood of recurrence.

Characteristic	Clinicopathological predictive model	Combined TMICC predictive model
	Regression coefficients (β)	Regression coefficients (β)
Mean log CD4+ T cells	-	-0.0065
Lymph node metastasis (N0)	0.67	0.65
Perineural invasion	0.17	0.12
Lymphovascular invasion	0.069	-
Extracapsular spread	1.13	1.10
Positive surgical margins	0.25	0.19
Reception of post-operative radiotherapy	0.33	0.27

Combined TMICC predictive model for recurrence

Then, we added the TMICC, including CD4+ helper T cells, CD8+ cytotoxic T cells, FoxP3+ regulatory T cells, CD20+ B cells, CD68+ macrophages, CD163+ M2-macrophages, CD57+ NK cells and PD-L1+ cells to the predictive model. The optimal cross entropy loss for train and test sets were respectively -0.47 (± 0.01) and -0.50 (±0.01). The best α associated with the optimal binary cross entropy loss was identified at 0.10. The ROC AUC of the train and test sets were comparable to the clinicopathological predictive model with values of respectively 0.79 (± 0.01) and 0.76 (± 0.01), see Figure 7.3. The combined model again included a set of six key characteristics explaining the likelihood of recurrence. (1) Higher logarithmically transformed mean CD4+ T helper cell count was associated with a decreased likelihood of recurrence. The following characteristics were associated with an increased likelihood of recurrence: (2) lymph node metastasis, (3) perineural invasion and (4) extracapsular spread, (5) Positive surgical margins and (6) post-operative radiotherapy. See Table 2 for predictive model regression coefficients (β).

Discussion

This is the first study adding the TMICC to conventional clinicopathological characteristics in a predictive model for recurrence to assess the magnitude of their added predictive value. The TMICC was found to be an independent predictor, with high numbers of CD4+ T cells having a protective effect against the likelihood of the development of recurrence in OSCC. However, the added value of the TMICC, including

CD4+ T cells, on top of the clinicopathological predictive model remained marginal, as it did not improve the model's performance.

The clinicopathological predictive model demonstrated robust discriminatory ability and consistent performance on both the training - and test data. Current Dutch – and NCCN guidelines for OSCC recognize lymph node metastasis with extra nodal extension and positive surgical margins as major risk factors for recurrence and advise adjuvant treatment with post-operative radiotherapy.[12,13] Our study results are in line with these guidelines as lymph node metastasis, extra nodal extension, and surgical margin were important predictors for recurrence. This study additionally identified perineural invasion and lymphovascular invasion as important predictors for recurrence. The TNM-staging system, however, not yet reflects the importance of these minor risk factors.[14] Interestingly, the clinical significance of minor risk factors remains debatable and adjuvant treatment selection is often based on multi-disciplinary expert opinions in the absence of major risk factors.[15]

The TME harbors a variety of immune cells, including T cells, B cells, Natural Killer (NK) cells, Tumor Associated Macrophages (TAMs), myeloid derived suppressor cells (MDSC) and dendric cells (DCs).[16] In this study we included immunohistochemical markers for a broad variety of immune cells, namely the T-helper cells, Cytotoxic T-cells, Regulatory T-cells, B-cells, NK cells, TAMs and checkpoint inhibitor PD-L1. Intricate cross-talk between immune cells and tumor cells within the TME has been known to have clinical relevance, especially in light of the recent advances in immunotherapy.[16,17] Immune biomarkers, however, have not had a major influence selecting patients for additional therapies in routine clinical practice.[18] Current predictive models for patient survival focus on the tumor and patient characteristics, failing to recognize and incorporate the dynamic relationship with TME components in tumor development and control.[19,20] Also, studies often correlate less than five immune biomarkers with survival parameters in multi-variate analysis, insufficiently displaying the biological interactions of cells within the TME.[21] These reasons underline why we chose an approach different from our predecessors, by quantifying the additional value of the TMICC with respect to the conventional clinicopathological characteristics. Consistent with prior research, we found that the TMICC is an independent predictor for recurrence. However, in the current study the additional value besides 'classical' tumor characteristics was marginal.
The development of an immuno-score in colorectal cancer (CRC), quantifying densities of CD3+ and CD8+ TILs in tumor center and invasive margin by computational pathology, was a front runner in an attempt to improve cancer staging and risk stratification. In a large validation cohort, the immuno-score was standardized and found to be a reliable

predictor of the risk of recurrence in CRC, possibly even superior to the TNM staging system.[22] Zhang et al. (2018) utilized this principle in an HNSCC cohort and found that the immuno-score, comprising CD3+ and CD8+ TILs, was closely related to outcome, and could supplement TNM staging.[23] Zhou et al. (2019) created a prognostic score for survival in OSCC containing four T cell subtypes (CD3, CD8, CD45RO, FoxP3) and found that their classifier functioned as an independent prognostic predictor.[24] Our combined TMICC predictive model did show good ability in predicting recurrence, in line with the studies above with CD4+ T cells as independent predictor. Our main study question however was distinctively different from the studies mentioned above, and we found that our combined TMICC predictive model did not show superiority when compared to the clinicopathological predictive model in predicting recurrence risk. Aside from difference in study question, a broad variety of clinicopathological characteristics were included in this study, consisting of both major and minor risk factors, whereas the studies above only corrected for the TNM stage, location and pathological grade.

A strength of this study is the fact that it was conducted in a homogenous cohort of OSCC patients, that were primarily treated with surgery, with or without post-operative radiotherapy. Also, we investigated an important clinical question to quantify the added predictive value of the TMICC upon conventional clinicopathological characteristics. This study was retrospective and bias for patient selection inevitably remained, as seen by the variable post-operative radiotherapy and how it predicted recurrence in our model. An explanation could be that post-operative radiotherapy is reserved for patients that already show adverse features. Another important limitation is the lack of an external validation cohort which influences generalizability. Also, statistical difficulties include the relatively small patient cohort limiting statistical power and the presence of multi-collinearity, which was tackled by using LASSO regularization in the regression analysis. TNM staging is a dynamic construct, and our time frame 1993-2008 included several TNM staging versions. An important clinicopathological characteristic, depth of invasion, which is recognized as an important predictor for recurrence in the TNM version 8, was unavailable in our patient cohort which could have influenced the clinicopathological predictive model.

In conclusion, we showed that the TMICC, especially CD4+ T cells are an independent predictor, however, addition to conventional clinicopathological characteristics does not improve the performance of a predictive model for recurrence in OSCC.

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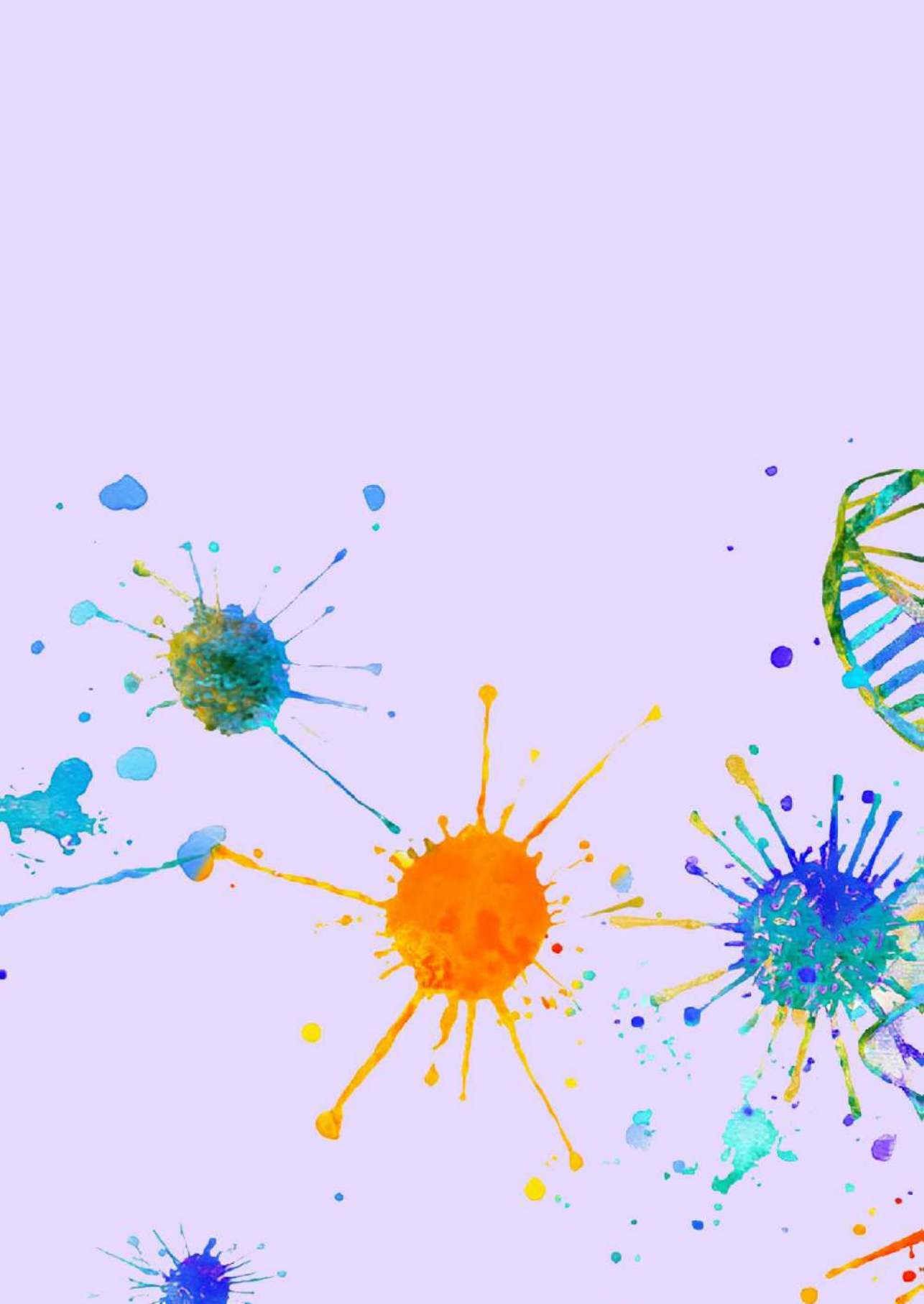

CHAPTER 8

Discussion and future perspectives

Martine F. van der Kamp

Synopsis

The prognosis of patients with Head and Neck Squamous Carcinoma (HNSCC) is traditionally determined by factors such as tumor stage, treatment modality and patient characteristics including chronological age. However, predicting individual patient outcomes remains challenging, particularly for older patients. This thesis aims to explore age-related clinical aspects, oncogenic pathways, and the tumor microenvironment (TME) in HNSCC, emphasizing their prognostic and predictive implications. The main findings are summarized in Table 8.1.

First, age-related clinical aspects in HNSCC were examined. In ***Chapter 2***, we analyzed variations in treatment patterns and survival rates of HNSCC patients between two time periods. A shift toward non-surgical treatment modalities was observed for oropharyngeal, hypopharyngeal, and advanced laryngeal cancers between 1990–1995 and 2010–2015. This shift was accompanied by improved survival for most sites, except for advanced-stage laryngeal SCC, where survival slightly declined.

In ***Chapter 3***, we investigated the relationship between age and the risk of distant metastases (DM). Male gender, hypopharyngeal location, advanced T-stage, poor differentiation, regional lymph node metastases, and extracapsular extension were identified as key prognostic factors. However, no significant association was found between age and DM.

In ***Chapter 4***, we explored age-specific oncogenic pathways in HNSCC. Through a comprehensive literature review, we identified novel age-related pathophysiological mechanisms underlying HNSCC.

We proposed a fourth subcategory of elderly, HPV-negative HNSCC patients without a history of tobacco or alcohol use, characterized by distinct carcinogenic mechanisms such as genomic instability, immunosenescence, cell cycle disruption, and telomere shortening.

Chapters 5 to 7 investigated the TME and its relationship with age and prognosis.

In ***Chapter 5,*** we described differences in immune infiltration based on biological and chronological age. Biologically older patients exhibited reduced infiltration of CD163+ macrophages, CD4+, and CD8+ lymphocytes, while chronologically older patients showed lower PD-L1 combined positive scores. Advanced tumor stage and perineural invasion were associated with worse disease-free survival (DFS); however no significant correlations were found between immunological markers and DFS.

In ***Chapter 6,*** we assessed the prognostic role of tumor-associated macrophages (TAMs) in HNSCC through a systematic review and meta-analysis. CD163+ TAMs emerged as a stronger prognostic marker than CD68+ TAMs, correlating with improved overall survival

(OS), DFS, and progression-free survival (PFS), while CD204+ TAMs showed relevance only for PFS. These findings highlighted CD163+ TAMs as a promising biomarker for future clinical applications.

In ***Chapter 7,*** we explored the predictive value of the TME beyond conventional clinicopathological characteristics. A predictive model incorporating TME components, including CD4+ T cells, revealed that high CD4+ T cell levels protected against recurrence, while factors such as lymph node metastases, extracapsular spread, and perineural invasion increased recurrence risk. However, the addition of TME components to the predictive model yielded comparable predictive value to conventional models.

Table 8.1. Main findings of this thesis.

Chapter	Aim study	Main Findings
2	Investigated changes in treatment patterns and survival outcomes in HNSCC patients across two time periods (1990–1995 and 2010–2015).	A shift toward non-surgical treatments was observed for oropharyngeal, hypopharyngeal, and advanced laryngeal cancers, with improved survival for most sites except advanced-stage laryngeal SCC.
3	Identified age and other predictive factors for the development of DM in HNSCC patients.	DM occurred in 9.3% of patients. Independent risk factors included male gender, hypopharyngeal tumors, advanced T-stage, poor differentiation, lymph node metastasis, and extra-nodal extension. No relation with age was found.
4	Provided an overview of age-specific pathophysiological mechanisms underlying HNSCC.	HNSCC patients can be categorized into four subgroups: 1. young patients with genomic aberrations or inheritable diseases, 2. an increasing population of HPV-related cases with favorable prognosis, 3. and HPV-negative tumors linked to lifestyle factors and diverse oncogenic pathways. A fourth subgroup of elderly HPV-negative HNSCC patients without tobacco or alcohol use is proposed, characterized by genomic instability, immunosenescence, cell cycle disruption, and telomere shortening.
5	Investigated the relationship between tumor characteristics, chronological and biological age, and their impact on survival in HNSCC patients.	Biological age was associated with reduced infiltration of CD163+ macrophages, CD4+, and CD8+ lymphocytes, while chronological age was linked to lower PD-L1 scores. No significant correlation was found between immunological markers and DFS.
6	Assessed the prognostic value of TAMs in HNSCC through a systematic review and meta-analysis.	Low CD163+ TAMs correlated with better OS, DFS, and PFS. CD163+ is a stronger prognostic marker compared to CD68+.
7	Evaluated the predictive value of the TME in addition to conventional clinicopathological characteristics in OSCC.	High levels of CD4+ T cells were associated with a reduced risk of recurrence. Adding the TME to the conventional clinicopathological model did not significantly improve predictive performance for recurrence.

Discussion and future perspectives

In this thesis, we aim to offer novel insights into the molecular mechanisms and immunological biomarkers associated with the aging population of head and neck squamous cell carcinoma (HNSCC) patients, highlighting their impact on clinical outcomes. Our goal was to provide insights that contribute to more effective and personalized treatment approaches for HNSCC patients, with a particular emphasis on biological age rather than chronological age.

Age-related trends in treatment and survival

Treatment protocols evolve over time as new drugs, surgical or radiotherapy techniques/equipment becomes available. However, it remains unclear how these advancements apply to older patients. Therefore, in ***Chapter 2,*** we analyzed changes in treatment regimens and survival rates over two decades in a large retrospective cohort of HNSCC patients aged ≥60 years, using data from the Netherlands Cancer Registry. A shift toward non-surgical treatment modalities was observed for oropharyngeal, hypopharyngeal, and advanced laryngeal cancers between 1990–1995 and 2010–2015. Concurrently, the proportion of SCC cases in the oropharynx, hypopharynx and oral cavity increased, accompanied by a notable improvement in survival for these sites.

However, laryngeal cancer showed a decline in survival outcomes, particularly in patients over 70 years with stage III and IV disease. This lack of improvement aligns with findings from other studies,[1–3] suggesting that organ-preserving protocols based on the VA study (1991)[4] failed to improve outcomes for advanced-stage laryngeal SCC. Another possible explanation for poorer survival rates in this cohort involves unrecognized selection biases, including comorbidities, treatment intent, patient and physician preferences, and tumor-specific factors such as volume and operability.

Older HNSCC patients often receive less aggressive treatment compared to their younger counterparts with similar disease status, a discrepancy that continuously influenced treatment protocols.[5] This could partly be explained by the historical underrepresented of older patients in clinical trials, leading to limited data on treatment efficacy and safety.[6] A review by Maria Cossu Rocca et al.[7], covering Phase II and III trials (2008–2022), found that only 20–30% of participants were over 65 years, emphasizing the ongoing underrepresentation of older patients. Given the limited representation of

this population in clinical trials, determining the optimal treatment approach remains challenging.

In the past, head and neck cancer guidelines have been revised to exclude older patients from adjuvant chemotherapy (CT), based on findings by Pignon et al.[8] and Blanchard et al.[9], which reported no survival benefit for patients over 70 years receiving chemoradiotherapy (CRT). However, only 4% of patients in the study of Pignon et al. were older than 70 years, and no age-based analyses were conducted. Additionally, biological age was not taken into consideration in this analysis. However, evidence on the effectiveness of CT in older patients remains conflicting. The RTOG 91-11 trial[10] suggested that CRT may benefit elderly patients with good performance status, showing higher larynx preservation rates compared to induction CT or radiotherapy (RT) alone. However, a subset analysis from Machtay et al. indicated older age as an independent risk factor for severe late toxicity after CRT.[11] VanderWalde et al.[12] found no survival advantage for CRT over RT alone in a cohort of older HNSCC patients. In contrast, more recent retrospective data indicate that CT may improve survival in selected older patients with lower comorbidity scores.[13] These mixed findings emphasize the need for prospective studies to refine patient selection criteria and assess functional status more comprehensively. This could result in guidelines for the management of specifically older patients with HNSCC.

A 2017 review by Porceddu et al.[14] concluded that while treatment tolerance may differ in elderly patients, there is no strong evidence that tumor response or cancer-related survival outcomes are inferior compared to younger patients. However, when selecting patients for treatment, it is essential to recognize that "chronological" age may not align with "biological" age.[7]

The latter, often referred to as frailty, is defined as “a state of increased vulnerability to poor resolution of homeostasis after a stressor event, which increases the risk of adverse outcomes”.[15] Advances in geriatric assessments have improved the ability to define frailty, enabling better risk stratification for both surgical and non-surgical treatments.[16] This multidimensional approach evaluates physical, functional, psychological, and socioenvironmental domains, providing a comprehensive understanding of a patient’s capacity to tolerate intensive therapies.[17] Patients with high frailty scores are at greater risk for toxicity, complications, and treatment interruptions, all of which can negatively impact outcomes.[18–20] Therefore, incorporating comprehensive geriatric assessments into clinical practice can help guide treatment

decisions in elderly patients, ensuring therapies are better aligned with overall health and treatment tolerance.

Reflecting on the findings of ***Chapter 2***, the reliance on chronological age as the sole inclusion criterion raises concerns regarding the appropriateness of patient selection. While this study included a large cohort, it lacked data on key factors influencing survival, such as comorbidities and, notably, geriatric assessment. Despite these limitations, our study provides a valuable overview of treatment trends and changes in head and neck cancer management over two decades in the Netherlands.

Distant metastasis and their relation to ageing

Earlier studies on the association between distant metastasis (DM) and age have yielded contradictory results, and those identifying age-related patterns often lack explanations for their finding.[21–26] In the retrospective institutional analysis of ***Chapter 3***, we investigated predictive factors for the development of DM in a large group of over 1,400 HNSCC patients, with a specific focus on age. Interestingly, no significant relationship between age and DM was identified.

The findings in ***Chapter 3*** reaffirm previously established independent predictive factors for DM, including male gender, hypopharyngeal tumor location, advanced T-stage, regional lymph node metastases, poor differentiation grade, and extranodal extension. These results suggest that tumor aggressiveness and metastatic potential are more closely linked to tumor biology rather than patient age. Additionally, treatment strategies in older patients often prioritize local disease control and quality of life over metastasis prevention, potentially influencing DM patterns. Shorter follow-up periods due to higher non-cancer-related mortality in older patients may also contribute to an underestimation of DM incidence in this group.

The presence of DM is frequently associated with an unfavorable prognosis. However, further stratification into oligometastasic disease (limited metastatic spread, typically ≤ 3 foci in ≤ 2 sites) and polymetastasic disease (widespread metastases) reveals meaningful prognostic differences.[27] Patients with oligometastasic disease generally have better outcomes. Likewise, synchronous metastases (diagnosed simultaneously with the primary tumor) are associated with a more favorable prognosis compared to metachronous metastases (occurring >6 months after diagnosis).[28,29]

The evolving concept of oligometastatic disease is reshaping treatment paradigms, offering improved prognosis and even curative potential with aggressive therapy.[27] Recent studies indicate that selected patients may benefit from metastasis-directed treatments. For example, definitive tumor-directed therapy has been associated with prolonged survival in patients with oligometastases.[30] Additionally, a systematic review of HNSCC patients undergoing pulmonary metastasectomy reported 5-year survival rates ranging from 21% to 59%, with median survival between 10 and 77 months.[31] Despite the generally poor prognosis associated with DM, these findings highlight the potential for improved outcomes in carefully selected patients, particularly those with oligometastatic disease.

Distinct oncogenic pathways in elderly HNSCC patients

Head and neck cancer exhibit significant heterogeneity in clinical behavior, which current clinical markers—such as the tumor-node-metastasis (TNM) stage, tumor location, and pathological grade—cannot accurately predict. Over the past decades, numerous studies have sought to classify HNSCC tumors based on tumor biology or genetic profile.[32–35] However, the only classification that has led to substantial changes in global staging and treatment protocols is the distinction between HPV-positive and HPV-negative tumors, which has fundamentally altered clinical management.

For elderly patients, identifying age-related differences in oncogenic pathways could provide valuable insights for treatment decision-making and facilitate the development of age-specific therapeutic strategies.

In ***Chapter 4,*** we investigated age-related oncogenic pathways in HNSCC, describing the pathophysiology of inherited, HPV-related, and HPV-negative HNSCC. A distinct subgroup of elderly, HPV-negative patients without a history of tobacco or alcohol use was identified (Figure 8.1). This subgroup is characterized by a multi-step carcinogenic process involving genomic instability, immunosenescence, cell cycle disruption, and telomere shortening.

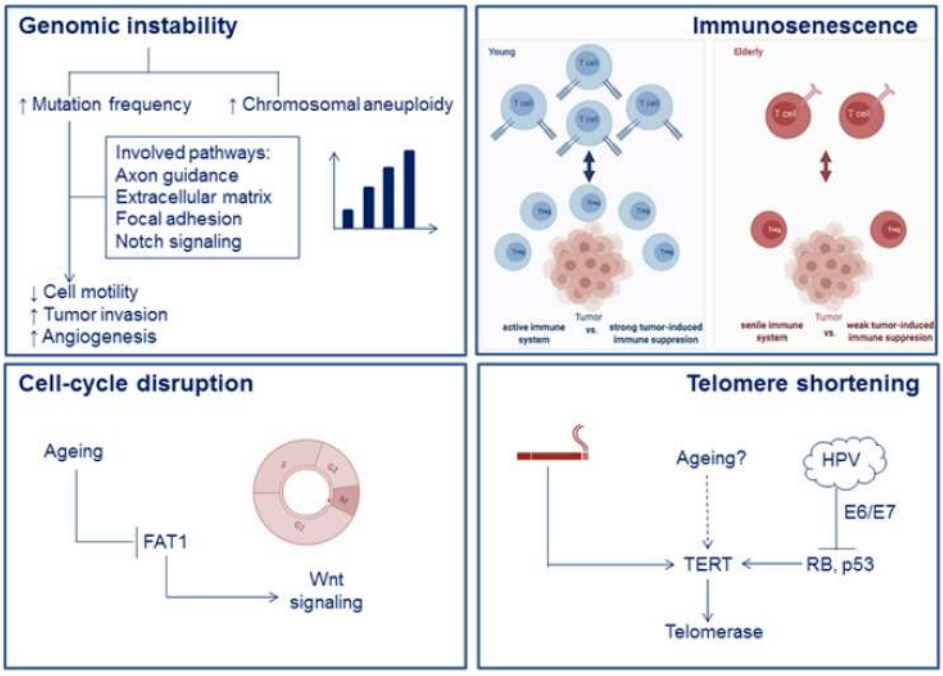

Figure 8.1. Pathophysiological mechanisms of HNSCC in elderly patients (Chapter 4 of this thesis). Genomic instability appears more frequent in elderly patients, leading to impaired cell mobility, increased tumor invasion, and enhanced angiogenesis. Immunosenescence in older patients results in a weakened immune response, making tumors less recognizable to the immune system. Aging is believed to cause inactivation of the FAT1 tumor suppressor gene, leading to activation of the Wnt signaling pathway and cell cycle disruption. While telomere shortening is a key hallmark of aging, its direct impact on HNSCC development remains unproven.

These findings suggest that the biological mechanisms driving carcinogenesis in elderly patients differ significantly from other subgroups, underscoring the need for tailored therapeutic approaches. Despite the importance of these findings, research on age-specific molecular pathways in HNSCC remains limited. Consequently, while several potential mechanisms were proposed, many insights were extrapolated from studies on other tumor types, restricting the ability to develop precise, evidence-based treatment options for elderly HNSCC patients.

Building on this, recent work by Muijlwijk et al.[33] identified a distinct subgroup of elderly HPV-negative HNSCC patients based on copy number alterations (CNAs). CNA-quiet oral cavity SCC (OCSCC), characterized by minimal or no CNAs, exhibited a unique mutational profile enriched for CASP8 and HRAS mutations, predominantly wild-type TP53, and specific histological features such as cohesive growth patterns and well-differentiated

tumors. Clinically, these tumors were more common in older patients, women, never-smokers, and non-alcohol users and were associated with improved overall survival.

This subgroup has significant clinical implications, as the authors suggested that reduced RT schedules may be feasible for CNA-quiet tumors. Additionally, these tumors display a more favorable TME, characterized by higher immune cell infiltration, which contributes to improved oncologic outcomes. However, whether this group responds differently to immunotherapy remains unclear, highlighting the need for further investigation.

TME in biologically aged HNSCC patients

Recently, immunotherapy has emerged as a promising treatment option for certain HNSCC patients, offering durable tumor responses, improved overall survival, a more manageable toxicity profile, and effectiveness in cases where conventional treatments seem to fail. The NCCN and Dutch guidelines recommend immune checkpoint inhibitors (ICIs) Pembrolizumab or Nivolumab in combination with cetuximab as a treatment option for recurrent or metastatic HNSCC, regardless of patient age.[36,37]

However, immunosenescence, which leads to reduced immune activity, may reduce the efficacy of immunotherapy in elderly patients. The relationship between TME and biological age has not been investigated so far.

Therefore, in *Chapter 5*, we explored the relationship between the TME and both biological and chronological age of HNSCC patients. We identified a significant association, with higher levels of CD163+ macrophages, CD4+ lymphocytes, and CD8+ lymphocytes linked to lower biological age, while higher PD-L1 expression was associated with higher biological age. Chronologically older patients had significantly lower PD-L1 combined positivity scores. The results are summarized in Figure 8.2.

These findings, particularly the reduced CD8+ T lymphocyte levels in biologically older patients, suggest that immune checkpoint inhibitors may be less effective in this population.

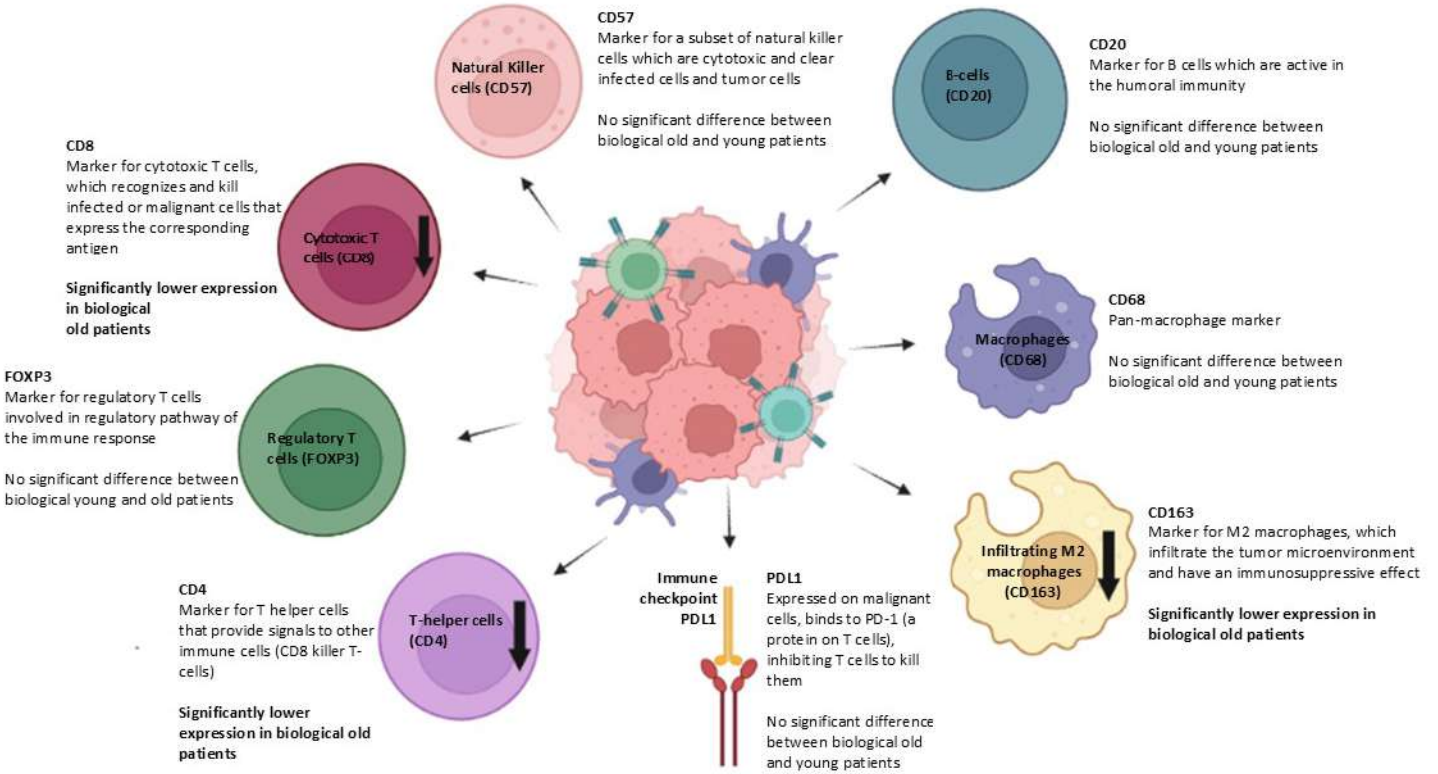

Figure 8.2. Influence of biological age on immunological markers in the TME.
(Chapter 5 of this thesis).

A recently published systematic review and meta-analysis by Salvestrini et al.[38], evaluated the efficacy and safety of immunotherapy in elderly patients (age ≥65) with locally advanced, recurrent, or metastatic HNSCC. Among 4,056 patients included in the review, 771 aged ≥65 were analyzed in the meta-analysis. Results indicated that elderly patients derived similar overall (OS) survival benefits from immunotherapy compared to younger patients. These results do not correspond to what our findings suggest. However, the limited number of older patients underscores their continued underrepresentation in clinical trials, emphasizing the need for prospective studies in this population. Additionally, biological age was not considered, leaving room for further investigation into its potential role in guiding immunotherapy strategies for biologically older HNSCC patients.

Zhang et al.[39] conducted a systematic review and meta-analysis focusing on the use of immune checkpoint inhibitors (ICIs) in a subgroup of very elderly HNSCC patients (≥75 years). The analysis included randomized trials comparing ICIs with standard-of-care (SOC) therapy for recurrent or metastatic HNSCC, specifically evaluating OS in patients aged < 75 versus ≥ 75 years. Five Phase 3 trials, involving 3,437 patients, were analyzed. The meta-analysis found no statistically significant survival benefit for patients aged ≥75 years treated with ICIs compared to SOC therapy (HR = 1.30, 95% CI: 0.93–1.81, $p = 0.127$). However, the sample size of patients aged ≥ 75 years (n = 207) fell short of the 350 patients needed to achieve 80% statistical power at the predefined significance level, resulting in insufficient power to detect a statistically significant difference. Despite these limitations, the authors concluded that current evidence is insufficient to support the use of ICIs in this specific patient population. Similarly, this study considered only chronological age, without accounting for biological age, which may have influenced the outcomes.

Several systematic reviews and meta-analyses on patients with solid cancers have shown comparable efficacy of ICIs between younger and older age groups, demonstrating survival benefits from immunotherapy compared to controls.[40–46] Based on these findings, it is suggested that chronological age-related impairments in immune function do not compromise the efficacy of ICIs in elderly patients compared to younger counterparts.

Immunosenescence represents an inevitable and complex biological process associated with aging. It involves a gradual decline in immune function, impairing tumor recognition and elimination, which results in a less effective antitumor response. While preclinical data remain controversial, evidence suggests that aging affects multiple steps of

immune surveillance, potentially contributing to differences in cancer progression and treatment response.[47] This phenomenon may partially explain discordant findings from pivotal studies.[47] However, the perceived lack of efficacy in elderly patients may also stem from their underrepresentation in clinical trials, limiting statistical power to draw robust conclusions.

Interestingly, high PD-L1 expression might serve as a predictive biomarker for ICI response, regardless of age, suggesting that older patients may still benefit from immunotherapy.[47] Additionally, data from key studies indicate that immunotherapy is generally well-tolerated in elderly patients, with safety profiles comparable to those of younger individuals and fewer adverse effects than cytotoxic therapies. Notably, none of these studies accounted for biological age, a factor that may significantly influence ICI efficacy in older patients.[47]

In our study, the relationship between the TME and biological age revealed important differences, such as reduced CD8+ T lymphocyte levels in biologically older patients, suggesting that ICIs may be less effective in this population. These findings highlight the need to incorporate biological age into future research to refine immunotherapy strategies for geriatric patients. Larger studies evaluating elderly populations through the lens of biological age and focusing on biomarkers for immunosenescence could provide critical insights into optimizing immunotherapy in this geriatric population.

The TME: predictive and prognostic implications

Traditional cancer-related prognostic factors, including histological tumor type, tumor site, tumor size, lymph node involvement, DM, and HPV status, play a central role in the clinical management of HNSCC patients. Currently, the standard treatment approach relies on risk stratification based on the eighth edition of the TNM classification.[48] However, clinical outcomes often vary significantly among patients within the same TNM stage, highlighting the need to refine the system for improved prediction of HNSCC behavior and optimization of treatment strategies.[49,50]

In this context, *Chapter 6* provided a systematic literature review and meta-analysis on the prognostic role of tumor-associated macrophages (TAMs) in HNSCC. Multivariate analysis revealed that a low count of CD68+ TAMs correlated with improved overall survival (OS), while a low count of CD163+ TAMs was associated with enhanced OS,

disease-free survival (DFS), and progression-free survival (PFS), suggesting a pro-tumor role for CD163+ TAMs in HNSCC.

Similarly, Troiano et al.[51], in their systematic review and meta-analysis, observed that a lower count of CD163+ TAMs was associated with improved OS, consistent with the findings of Chapter 6. However, both studies, included tumors from all head and neck sites, encompassing both HPV-positive and HPV-negative HNSCC, which introduced heterogeneity. Future research should address this limitation by focusing on specific HNSCC subsites and employing well-powered cohorts to account for the molecular diversity of these tumors. Nevertheless, CD163 is exclusively expressed on cells of the monocyte lineage, including monocytes, macrophages, and dendritic cells, underscoring its potential as both a prognostic biomarker and therapeutic target.[52]

Biomarkers have the potential to go beyond traditional prognostic factors, such as TNM staging and HPV status, by identifying individual differences in tumor behavior. High-throughput omics studies have advanced our understanding of the biological processes underlying HNSCC and laid the foundation for developing biomarkers that may predict individual patient prognosis.[50] Integrating biomarkers into clinical practice can not only improve the precision of prognostic models but also optimize patient-specific treatment selection. However, several challenges hinder their routine use, including insufficient evidence linking biomarkers to definitive prognostic improvements, a lack of clear clinical utility—such as the weak association between EGFR expression and response to EGFR inhibitors in earlier studies—and significant intra-tumoral heterogeneity, where expression can vary within different tumor regions. Moreover, the high costs of certain tests, such as whole-genome sequencing, remain a considerable barrier to widespread implementation.

Despite the growing number of studies utilizing training and matched validation cohorts, as well as advanced bioinformatics modeling supported by mechanistic preclinical evidence, no biomarker has yet surpassed classical prognostic markers such as TNM stage, HPV status, or patient characteristics in routine clinical practice.[50]
To bridge this gap, Chapter 7 of this thesis explored the additional predictive value of the TME alongside conventional clinicopathological characteristics in oral cavity SCC (OCSCC).This analysis included a broad range of immune cells, such as T-helper cells, cytotoxic T-cells, regulatory T-cells, B-cells, NK cells, TAMs, and the checkpoint inhibitor PD-L1. High levels of CD4+ T cells in the TME emerged as an independent protective factor against recurrence in OSCC. However, the overall added value of the TME to conventional predictive models was marginal.

Previous studies have highlighted the potential of immune-based scoring systems to improve prognostic models for HNSCC and OSCC by incorporating immune cell markers, such as tumor-infiltrating lymphocytes (TILs) and various T-cell subtypes, into predictive frameworks.[53,54] Unlike these studies, which focused solely on immune markers and adjusted primarily for basic tumor characteristics (e.g. TNM stage, location, and pathological grade), in Chapter 7, we integrated immune-based parameters into a comprehensive clinicopathological predictive model. While the marginal added value highlights the complexity of integrating novel parameters, it also underscores the potential of the TME as a complementary factor in outcome prediction.

Future research should focus on validating these findings in larger, well-powered cohorts and investigating additional (immune-related) markers to further enhance predictive precision. Ultimately, these efforts could contribute to the development of personalized treatment strategies and improved clinical outcomes for patients with HNSCC.

Future perspectives

Scientific directions

This thesis underscores the importance of considering biological age over chronological age in HNSCC research. Future studies should examine the impact of biological age on prognostic outcomes and evaluate whether treatment strategies require modification for biologically older patients to enhance efficacy and minimize toxicity. Addressing the underrepresentation of elderly patients in clinical trials—both in terms of chronological and biological age—is essential. Efforts must ensure the inclusion of participants aged 75 and older while integrating geriatric assessment to capture the diversity of this population.

Research into biomarkers of immunosenescence, such as PD-L1 expression and the broader TME, should be prioritized to improve patient stratification and optimize the use of immunotherapy. Moreover, research into reduced-intensity treatments for (biologically) older patients with favorable tumor profiles, such as CNA-quiet SCC, offers a promising avenue to achieve better therapeutic outcomes while reducing treatment burden.

Distinct oncogenic pathways identified in elderly, HPV-negative HNSCC patients warrant further investigation. Mechanisms like genomic instability, telomere shortening, and immunosenescence may play pivotal roles in the carcinogenesis of this subgroup. One

promising approach for identifying molecular pathways and therapeutic targets is multi-omics profiling. This integrative strategy combines data from multiple molecular layers, such as genomics, proteomics, transcriptomics, epigenomics, and metabolomics, to generate a comprehensive understanding of tumor biology. By linking molecular alterations across various stages—such as pre- and post-translational levels—multi-omics analyses offer a more holistic view of tumor pathophysiology compared to single-omics studies, potentially uncovering new biomarkers and therapeutic opportunities.

Clinical applications

Incorporating biological age into clinical workflows offers a practical step toward improving outcomes for elderly HNSCC patients. Tools such as geriatric screening and comprehensive geriatric assessment provide valuable insights into overall health status and treatment tolerance.[55] This approach minimizes the risk of both under- and overtreatment by aligning therapies with individual patient capacities, ultimately reducing complications and improving overall outcomes.

Tailoring immunotherapy for biologically older patients merits further exploration, as biological age-related changes in the immune system may influence treatment efficacy. Adjustments to drug dosing, scheduling, or combination regimens might be required to optimize outcomes. Additionally, integrating validated biomarkers into clinical decision-making would refine patient selection, ensuring therapies are targeted to those most likely to benefit.

The development of novel therapeutic agents, such as Xevinapant, which targets inhibitors of apoptosis proteins (IAPs), highlights the potential for integrating innovative treatments into existing modalities. Early clinical trials have shown promise[56–58], particularly in combination with chemoradiotherapy. Future research should assess its efficacy across diverse patient populations including biologically older patients.

Finally, the development of age-specific treatment guidelines that incorporate evidence on biological age, oncogenic pathways, and biomarkers has the potential to improve care for elderly patients and, by extension, all HNSCC patients. Striking the right balance between treatment intensity and preserving quality of life remains a critical goal in the management of this diverse patient population.

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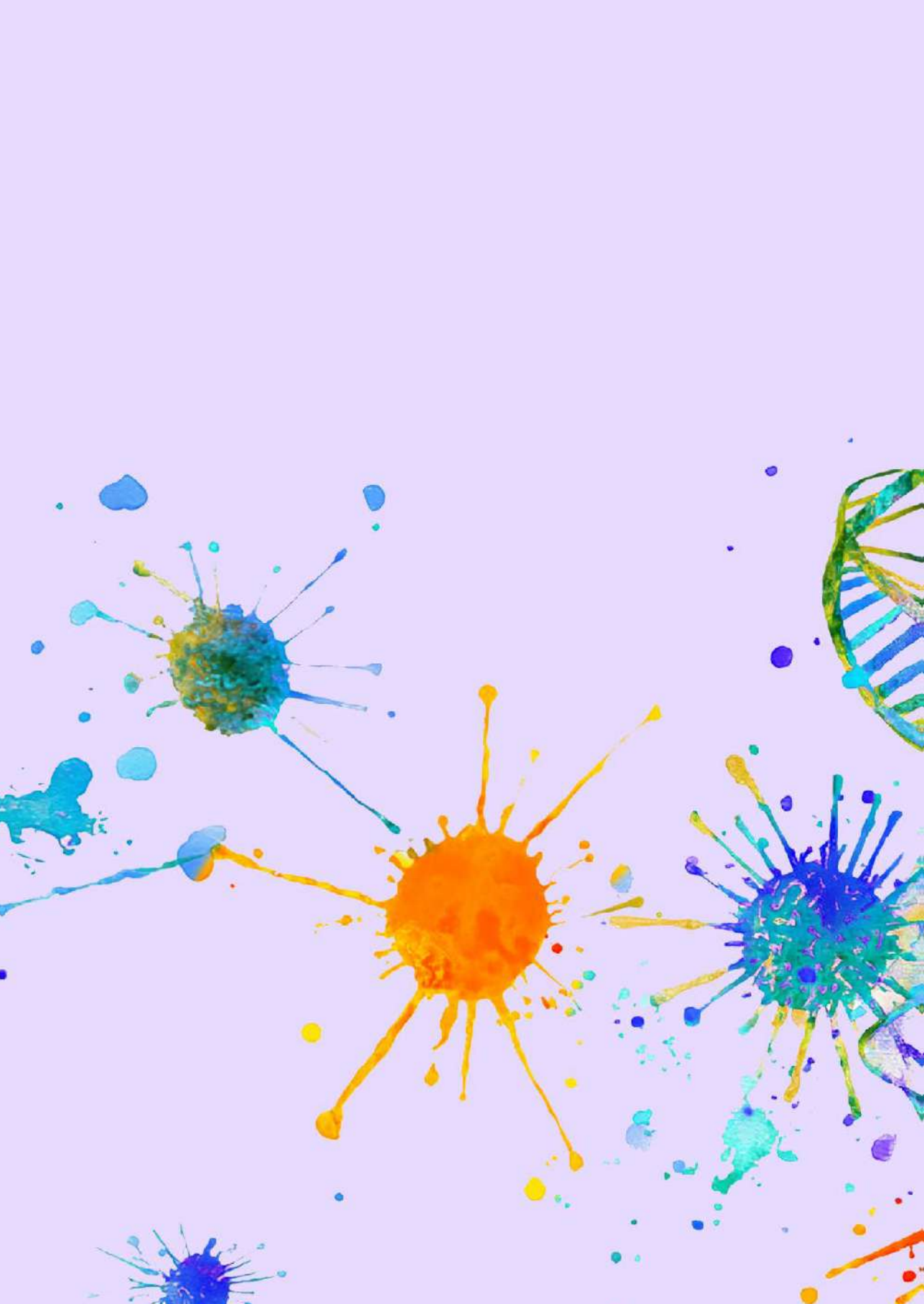


CHAPTER 9

Summary (NL)

Martine F. van der Kamp

Summary (NL)

Hoofd-halskanker is wereldwijd een van de zes meest voorkomende vormen van kanker, met een incidentie van ongeveer 890.000 per jaar en een mortaliteit van 450.000 patiënten per jaar. Meestal betreft het een plaveiselcelcarcinoom (PCC), een tumor die ontstaat uit het slijmvlies van de bovenste lucht- en voedselwegen. Vooral tumoren in de mond, het strottenhoofd en de keelholte komen vaak voor, daarom richt dit proefschrift zich op specifiek deze vormen van hoofd-halskanker.

Bekende risicofactoren voor hoofd-halskanker zijn alcoholgebruik en roken. Ook bepaalde virusinfecties spelen een rol, zoals het humaan papillomavirus (HPV) bij orofarynx (mondkeelholte) kanker en Epstein-Barr virus bij nasofarynx (neuskeelholte) kanker. Andere (zeldzamere) risicofactoren voor hoofd-halskanker zijn bepaalde erfelijke aandoeningen, maar ook slechte mondhygiëne, het kauwen van betelnoot (een in Azië voorkomend gebruik), voeding, (werk-gerelateerde) blootstelling aan carcinogene stoffen, bestraling en een verzwakt immuunsysteem dragen bij aan dit risico.

Meer dan 60% van de patiënten met hoofd-halskanker wordt gediagnosticeerd in een gevorderd stadium, waarbij lokale doorgroei en/of uitzaaiingen naar lymfeklieren of andere organen doorgaans een intensieve behandeling met meerdere behandelmodaliteiten vereisen. Meestal bestaat de behandeling uit een combinatie van chirurgie, radiotherapie en systemische therapie zoals chemo- of immunotherapie. Bij curatieve behandeling staan lokale tumorcontrole en behoud van essentiële functies van het hoofd-halsgebied - zoals slikken, ademen en spreken - centraal. In de palliatieve setting ligt de nadruk op symptoombestrijding, behoud van kwaliteit van leven en vertraging van ziekteprogressie. Een multidisciplinaire en gepersonaliseerde aanpak is hierin van belang, waarbij gezamenlijke besluitvorming een centrale rol speelt.

Een belangrijke voorspeller voor de prognose van hoofd-halskanker is de tumorlocatie en het tumorstadium (TNM-stadium), dat onder andere wordt bepaald op basis van de tumorgrootte en de aanwezigheid van lymfeklier- en afstandsmetastasen. Afstandsmetastasen vormen een belangrijke prognostische factor, met een gerapporteerde incidentie tussen 3% en 52%. De longen zijn het meest frequent aangedaan, gevolgd door botten en de lever. Daarnaast zijn verschillende histopathologische, genetische en immunologische kenmerken van invloed op de prognose. Verbeterde overlevingsuitkomsten worden voornamelijk gezien bij jongere patiënten met HPV-gerelateerde tumoren en tumoren op specifieke locaties in het hoofd-halsgebied, terwijl dergelijke verbeteringen uitblijven bij ouderen (> 75 jaar) en bij

larynx (strottenhoofd) carcinomen. Deze verbeteringen worden mede toegeschreven aan de opkomst van doelgerichte therapieën en immuuntherapie, maar ook aan verbeterde chirurgische technieken zoals robot-geassisteerde chirurgie. Geavanceerde bestralingstechnieken, waaronder protonentherapie, dragen bovendien bij aan een reductie van behandelingsgerelateerde toxiciteit.

Door de toenemende levensverwachting stijgt het aantal oudere patiënten met hoofd-halskanker. Eerdere studies tonen aan dat oudere patiënten vaak een afwijkend behandeltraject volgen ten opzichte van jongere patiënten, waarbij minder frequent een standaard- of intensieve behandeling wordt toegepast. Desondanks blijkt dat intensieve therapieën bij zorgvuldig geselecteerde ouderen doorgaans goed worden verdragen, met vergelijkbare behandeluitkomsten als bij jongere patiënten.
Bij het bepalen van de behandelingsstrategie wordt veelal uitgegaan van de chronologische leeftijd van de patiënt, terwijl deze slechts beperkt inzicht biedt in diens fysieke en mentale conditie. De biologische leeftijd (geassocieerd met kwetsbaarheid en vast te stellen door middel van een geriatrische screening) vormt een betere afspiegeling van de algehele gezondheidstoestand en blijkt een sterkere voorspeller van behandelingstolerantie. Dit is met name relevant bij patiënten met hoofd-halskanker, waarbij vaker sprake is van een verhoogde biologische leeftijd.

De afgelopen jaren is er toenemende aandacht voor de tumor micro-environment (TME), zowel vanwege het potentieel als prognostische biomarker maar ook als aangrijpingspunt voor immunotherapie. De TME bestaat uit verscheidene immuuncellen, waarvan de samenstelling varieert per tumortype en -locatie, maar ook tussen patiënten en binnen individuele tumoren. De balans tussen de verschillende immuuncellen heeft invloed op de interactie tussen immuuninfiltraat en tumorcellen, waarbij de TME zowel een tumoronderdrukkende als een tumorstimulerende rol kan vervullen. In dit proefschrift wordt dieper ingegaan op twee belangrijke componenten binnen de TME: tumorinfiltrerende lymfocyten (TILs) en tumor-geassocieerde macrofagen (TAMs).

TILs omvatten onder andere T-cellen, B-cellen en natural killercellen. Bij hoofd-hals PCC's is een hoge mate van TIL-infiltratie geassocieerd met een gunstiger prognose. Tumorcellen die PD-L1 tot expressie brengen kunnen echter de werking van T-cellen remmen en zo de immuunrespons onderdrukken. Door deze interactie te blokkeren met immuuntherapie, zoals PD-1-remmers, kan de antitumoractiviteit van T-cellen worden hersteld. Daarentegen wordt een hoge aanwezigheid van TAMs juist in verband gebracht met een slechtere prognose, mede vanwege hun rol in lokale immuunsuppressie, wat bijdraagt aan tumorprogressie.

De samenstelling en functionele activiteit van immuuncellen binnen de TME lijken dus van groot belang voor de ontwikkeling, progressie en therapierespons van hoofd-hals PCC's. Inzicht in deze variaties kan bijdragen aan verdere personalisering van behandeling.

Momenteel worden PD-1/PD-L1-immuuncheckpointremmers (zoals nivolumab en pembrolizumab) toegepast bij de behandeling van recidiverende of gemetastaseerde vormen van hoofd-halskanker. EGFR-remmers (zoals cetuximab) worden gegeven in combinatie met radiotherapie en/of chemotherapie. Een scala aan doelgerichte therapieën met verschillende aangrijpingspunten, zoals PI3K/AKT/mTOR-, VEGF- en HRAS-remmers, bevindt zich nog in de onderzoeksfase en wordt uitsluitend toegepast in studieverband.

In dit proefschrift worden klinische aspecten, tumoreigenschappen, immunologische kenmerken en pathofysiologische mechanismen van hoofd-halskanker onderzocht, met nadruk op verschillen gerelateerd aan de biologische leeftijd van de patiënt.

In **Hoofdstuk 2** van dit proefschrift onderzochten we veranderingen in behandelpatronen en overlevingsuitkomsten bij patiënten met hoofd-halskanker. Hiervoor werden gegevens uit een retrospectieve database van de Nederlandse Kankerregistratie geanalyseerd van patiënten gediagnosticeerd met hoofd-halskanker tussen de periodes 1990–1995 en 2010–2015. Er werd een verschuiving waargenomen van chirurgische naar niet-chirurgische behandelmodaliteiten voor oro-, hypofaryngeale- en gevorderde larynxcarcinomen. Deze verschuiving ging gepaard met een verbeterde overleving voor de meeste tumorlokalisaties, met uitzondering van hoog-stadium larynxcarcinomen, waarbij een lichte daling in overleving werd gezien. Dit is mogelijk te wijten aan de wereldwijde verschuiving naar orgaansparende behandelstrategieën, zoals (chemo)radiotherapie, in plaats van primaire chirurgie in deze groep. Deze benadering lijkt met name bij oudere patiënten met gevorderde stadia (stadium III en IV) minder effectief, zeker wanneer zij vanwege leeftijd of comorbiditeit uitgesloten worden van aanvullende chemotherapie. Dit suggereert dat behandelingskeuzes niet louter gebaseerd zouden moeten worden op kalenderleeftijd, maar op de algehele conditie van de patiënt.

In **Hoofdstuk 3** van dit proefschridt onderzochten we de relatie tussen de chronologische leeftijd en het risico op het ontwikkelen van afstandsmetastasen in een retrospectieve studie van bijna 1500 patiënten met hoofd-hals kanker uit het Universitair Medisch Centrum Groningen (UMCG). Mannelijk geslacht, hypofaryngeale tumorlokalisatie, een

gevorderd T-stadium, slechte differentiatiegraad, regionale lymfekliermetastasen en extranodale uitbreiding werden bevestigd als significante prognostische factoren. Er werd geen associatie gevonden tussen chronologische leeftijd en het optreden van afstandsmetastasen. Dit suggereert dat bij alle patiënten met een hoog risico op het ontwikkelen van afstandsmetastasen uitgebreide screening overwogen zou moeten worden, ongeacht de leeftijd van de patiënt.

In Hoofdstuk 4 van dit proefschrift richtten we ons op chronologische leeftijdsspecifieke oncogenetische mechanismen in hoofd-hals PCCs. In een uitgebreide literatuurstudie werden op basis van leeftijd drie verschillende pathofysiologische mechanismen beschreven. Een kleine groep jonge patiënten met erfelijke aandoeningen zoals Fanconi anemie, HPV-gerelateerde hoofdhals PCCs (voornamelijk orofarynxcarinomen) en een heterogene groep van HPV-negatieve tumoren. Deze laatste groep tumoren wordt geassocieerd met leefstijlfactoren zoals roken en alcoholgebruik, en komen met name voor bij mannen van middelbare leeftijd. Op basis van de literatuur introduceerden we een vierde subcategorie patiënten: oudere, HPV-negatieve hoofd-halskanker patiënten zonder voorgeschiedenis van tabak- of alcoholgebruik, gekenmerkt door afwijkende carcinogene mechanismen zoals genomische instabiliteit, immunosenescence, verstoorde celcyclus en telomeerverkorting. Bij deze patiëntengroep lijkt de opstapeling van mutaties de belangrijkste drijvende kracht achter carcinogenese te zijn.

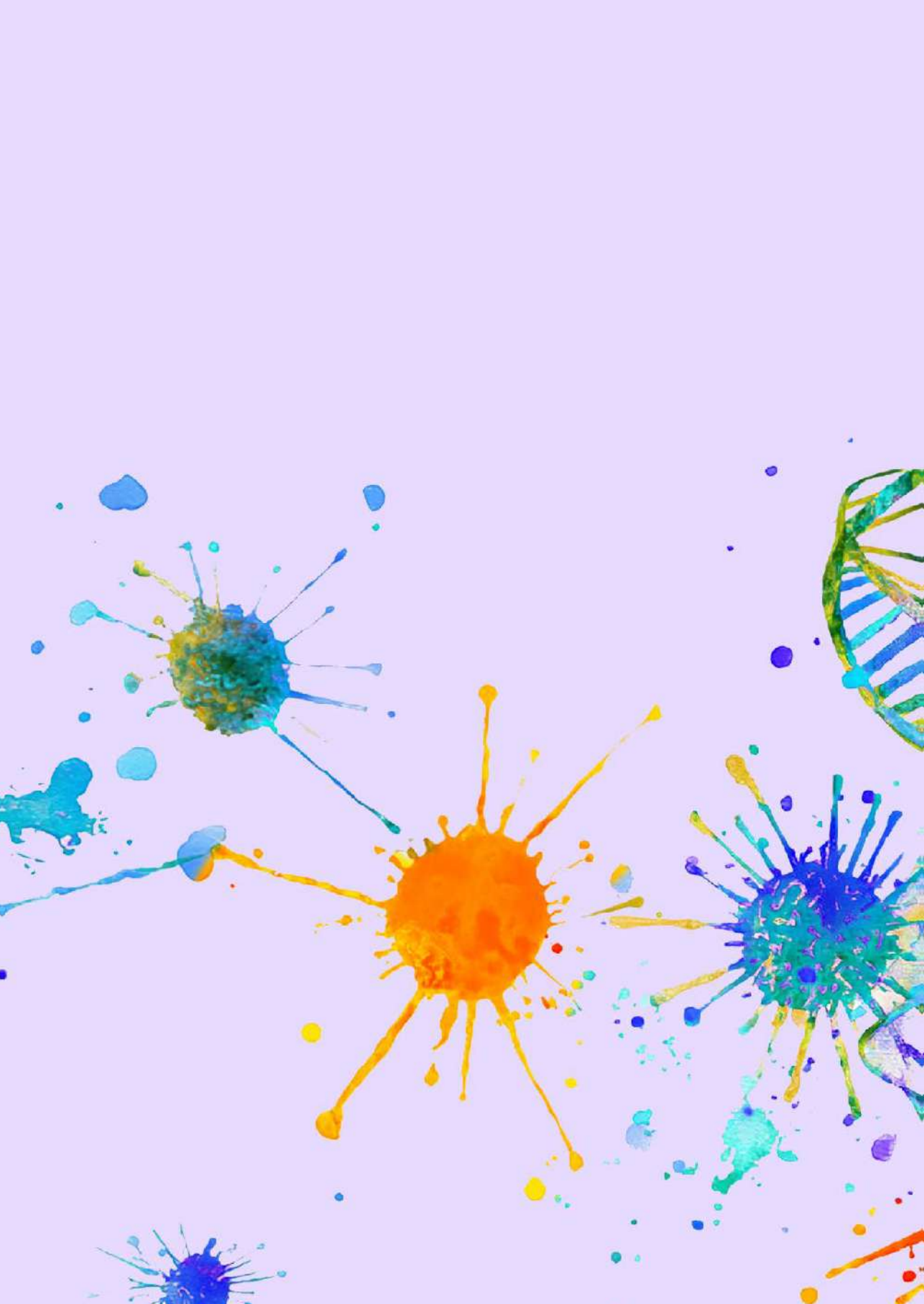
Hoofdstukken 5 t/m 7 behandelen de TME en de relatie met leeftijd en prognose bij hoofd-halskanker.

In Hoofdstuk 5 van dit proefschrift beschreven we verschillen in de samenstelling van de TME op basis van zowel biologische- als chronologische leeftijd, aan de hand van prospectief verzamelde data van 164 HNSCC-patiënten uit de OncoLifeS-database. Biologisch oudere patiënten vertoonden minder infiltratie van CD163+ macrofagen en CD4+ en CD8+ lymfocyten, terwijl chronologisch oudere patiënten lagere PD-L1-expressie vertoonden. Een gevorderd tumorstadium en perineurale invasie waren geassocieerd met slechtere ziektevrije overleving. Er werd geen significant verband gevonden tussen immunologische markers en ziektevrije overleving. Biologische leeftijd leek een sterkere invloed te hebben op de TME dan chronologische leeftijd. Deze bevindingen onderstrepen de noodzaak om vervolgonderzoek te doen naar het effect van specifieke behandelingen, zoals immunotherapie, in relatie tot de biologische leeftijd van patiënten.

In Hoofdstuk 6 van dit proefschrift werd de prognostische waarde van TAMs bij hoofd-hals PCCs onderzocht middels een systematische review en meta-analyse, waarin 25 studies met in totaal 2731 patiënten werden geïncludeerd. CD163+ TAMs bleken sterkere prognostische markers dan CD68+ TAMs voor algehele overleving, ziektevrije overleving en progressievrije overleving. Deze bevindingen suggereren dat CD163+ TAMs een veelbelovende biomarker vormen voor toekomstige klinische toepassingen.

In Hoofdstuk 7 van dit proefschrift onderzochten we in hoeverre de TME aanvullende voorspellende waarde biedt ten opzichte van conventionele klinisch-pathologische kenmerken in een studie onder 290 mondholtekankerpatiënten. Met behulp van LASSO-logistische regressie en ROC-analyse werd een voorspellend model ontwikkeld en gevalideerd. Middels dit model kon worden aangetoond dat hoge niveaus van CD4+ T-cellen geassocieerd waren met een lager risico op tumorrecidief. In deze studie werden bekende risicofactoren voor recidief zoals lymfekliermetastasen, extranodale uitbreiding en perineurale invasie, bevestigd. Hoewel de aanwezigheid van CD4+ T-cellen een onafhankelijke voorspeller bleek, leidde toevoeging van de TME aan het conventioneel klinisch-pathologisch model niet tot een significante verbetering van de voorspellende waarde.

Door deze aspecten te onderzoeken, beoogt dit proefschrift bij te dragen aan een beter begrip van hoofd-hals kanker en aan de ontwikkeling van effectievere behandelstrategieën met name gericht op de oudere patiënt.



APPENDICES

Acknowledgements (NL)

List of publications

Curriculum Vitae (NL)

Martine F. van der Kamp

Acknowledgements (NL)

Waarde patiënten, dit boek schreef ik voor jullie.

"Wat ga je doen als je later groot bent?" Als klein, blond meisje antwoordde ik: "Het medicijn tegen kanker uitvinden." Dit bleek misschien wat hoog gegrepen, maar met dit proefschrift hoop ik een bescheiden bijdrage te leveren aan de verbetering van de zorg voor hoofd-halskanker patiënten.

Bij de totstandkoming van dit proefschrift hebben velen een waardevolle bijdrage geleverd, met als belangrijkste spelers mijn promotieteam.

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Martine van der Kamp - Wuisman, 1 juli 2025

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**Shared first author*

About the author

Martine Froukje van der Kamp - Wuisman was born on August 21st in Arnhem, the Netherlands, where she grew up with her parents and brother. During secondary school, she participated in a dedicated sports program and played field hockey at the national level. After graduating in 2007, she spent a year in Australia, where she studied English and practiced her surfing skills.

In 2008, she commenced her medical studies at the University of Utrecht. During her time as a medical student, she held an active role within the student association U.V.S.V./N.V.V.S.U., where she participated in several committees. She enjoyed travelling and was able to combine this passion with her medical training by completing a clinical internship in Social Medicine at the White Yellow cross in Curaçao. In her final year, she focused on Otorhinolaryngology and Head & Neck Surgery through clinical and research internships at the VU University Medical Center and the Netherlands Cancer Institute, which marked the beginning of her academic career.

After obtaining her Master's degree in 2015, she worked as a surgical resident (non-training) at the Flevoziekenhuis and as a medical researcher in Head & Neck Surgery at the Erasmus University Medical Center. In 2019, she started her PhD trajectory at the University Medical Center Groningen (UMCG) under the supervision of prof. dr. Gyuri Halmos, prof. dr. Bernard van der Laan, prof. dr. Ed Schuuring, and dr. Bert van der Vegt, of which this thesis is the result. Martine has presented her research at several national and international conferences.

She completed the thesis during her residency training in Otorhinolaryngology and Head & Neck Surgery, which she began in 2021 at the UMCG under supervision of dr. Inge Wegner and dr. Astrid Korsten-Meijer. Martine currently serves on the national board of the Dutch Otorhinolaryngology residents' association. During her residency, she completed part of her training at the Ommelander Hospital (OZG) under supervision of dr. Noortje Smale and at the Isala Hospital in Zwolle under supervision of drs. Glen Kemps, dr. Hilke van Det-Bartels and dr. Dirk Dietz de Loos, where she currently lives with her husband Ies and their daughter Emma. The family will return to Groningen to complete the final part of Martine's residency training.

Just keep swimming