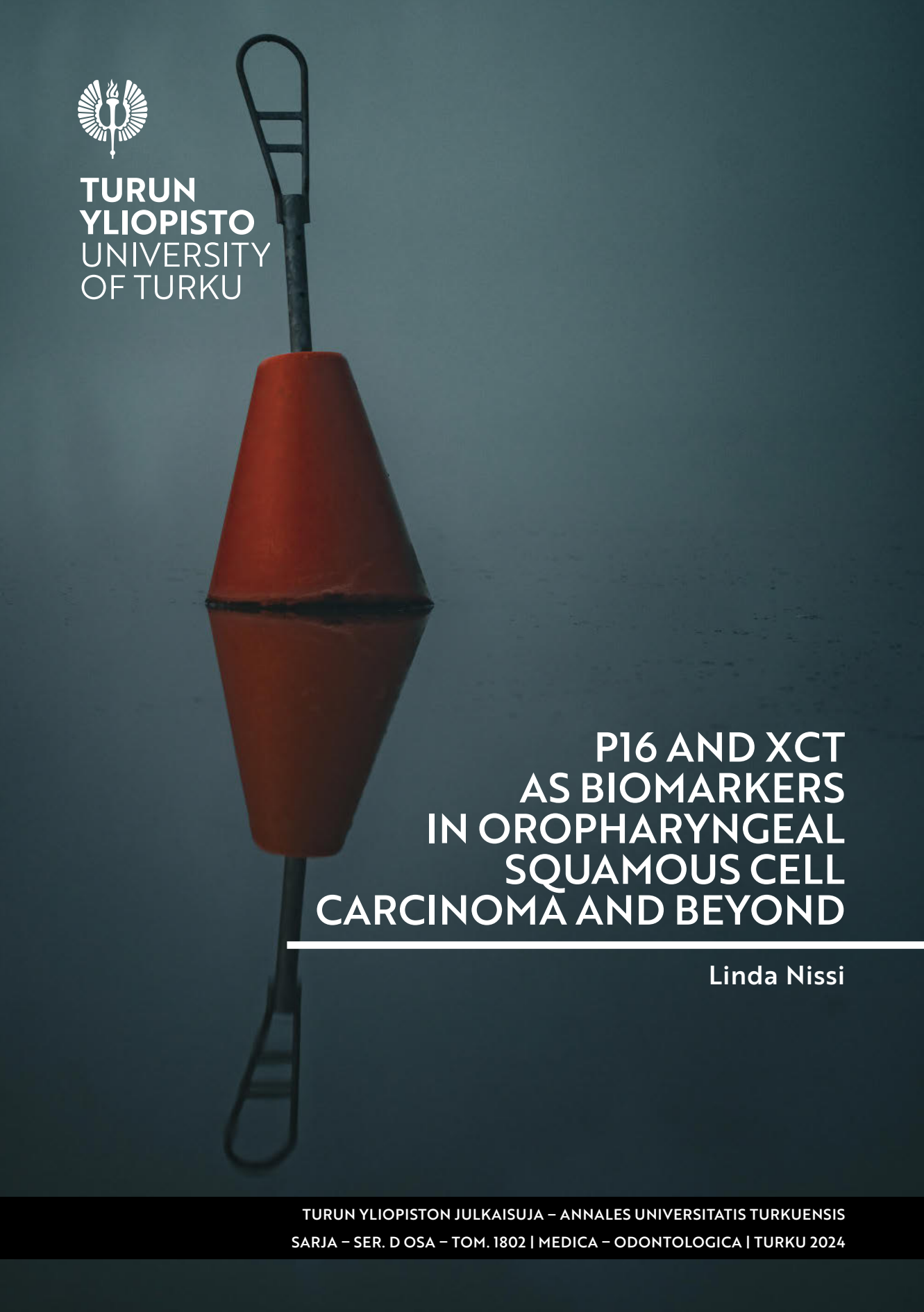

TURUN YLIOPISTO
UNIVERSITY OF TURKU

P16 AND XCT AS BIOMARKERS IN OROPHARYNGEAL SQUAMOUS CELL CARCINOMA AND BEYOND

Linda Nissi

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
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Cover Image: Aleksi Hakala

ISBN 978-951-29-9771-8 (PRINT)
ISBN 978-951-29-9772-5 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2024

To my family and friends,

UNIVERSITY OF TURKU
Faculty of Medicine
Department of Clinical Medicine
Clinical Oncology
LINDA NISSI: P16 and xCT as Biomarkers in Oropharyngeal Squamous Cell Carcinoma and Beyond
Doctoral Dissertation, 173 pp.
Doctoral Programme in Clinical Research
August 2024

ABSTRACT

Current therapy stratification of head and neck squamous cell carcinomas (HNSCCs) is based on clinical features. This approach often fails to capture the enormous biologic heterogeneity of the disease entity. Therefore, biomarkers which would act as indicators of treatment sensitivity could be extremely helpful in tailoring therapy on a more individualized basis. Human papillomavirus (HPV) has emerged as a biomarker in oropharyngeal squamous cell carcinoma (OPSCC). De-escalation of treatment based on p16 as a surrogate marker for HPV infection is a matter of debate.

The aim of this thesis was to evaluate p16 and xCT, an amino acid transporter that mediates programmed cell death, as biomarkers in HNSCC. Clinical data of all patients treated for HNSCC at Turku University Hospital during 2005 – 2015 were investigated. Expression of p16 and xCT were evaluated using immunohistochemistry in a population-based tissue microarray. Moreover, patterns of recurrence were studied using dose distribution analyses of hybrid positron emission tomography - computed tomography (PET-CT) images from radiotherapy (RT) treatment planning co-registered with PET-CT/MRI (magnetic resonance imaging) images obtained at the time of relapse.

HNSCCs were observed to respond heterogeneously to RT, and a small subset of p16-positive diseases relapsed within the high-risk treatment volume despite the common view of their high radiosensitivity and better prognosis. Moreover, results from p16-negative tumours suggested that even meticulous treatment planning with multimodality imaging may fail to detect all clinically significant disease. In OPSCC, p16 was an independent prognostic factor when adjusted for age, treatment modality, T class, nodal positivity, and consumption of alcohol and tobacco. The expression and prognostic value of xCT varied markedly among different primary tumour sites. In OPSCC, xCT was a powerful prognostic factor.

The thesis suggests that successful treatment de-escalation of patients with p16-positive OPSCC likely requires further biomarkers predictive of RT response. Also, the findings encouraged for further studies on therapeutic targeting of xCT to overcome radioresistance.

KEYWORDS: p16; human papillomavirus; xCT; SLC7A11; biomarker; recurrence; head and neck squamous cell carcinoma; oropharyngeal cancer; radioresistance; tissue microarray; population-based; imaging

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Kliininen laitos
Kliininen syöpätautioppi
LINDA NISSI: P16 ja xCT biomarkkereina suunielun levyepiteelisyövissä
Väitöskirja, 173 s.
Turun kliininen tohtoriohjelma
Elokuu 2024

TIIVISTELMÄ

Hoitoa koskeva päätöksenteko pään ja kaulan alueen levyepiteelisyövissä perustuu edelleen kliinisiin seikkoihin. Tautiryhmä on kuitenkin hyvin monimuotoinen ja syövän käyttäytymistä ennustavat biomarkkerit voisivat mahdollistaa yksilöllisen ja oikein mitoitetun hoidon. Ihmisen papilloomaviruksen (human papillomavirus; HPV) tunnistamiseen käytettyä p16-proteiinia on viimeisen vuosikymmenen ajan hyödynnetty biomarkkerina suunielun syövissä. Hoidon keventäminen p16-proteiinin perusteella on kuitenkin herättänyt runsaasti keskustelua.

Tämän tutkimuksen tavoite on arvioida p16- ja xCT-proteiineja biomarkkereina pään ja kaulan alueen levyepiteelisyövissä. Tutkimuksessa käytettiin Turun yliopistollisessa keskussairaalassa 2005–2015 diagnosoitujen pään ja kaulan alueen syöpää sairastavien potilaiden kliinisiä tietoja. P16- ja xCT-proteiinien ilmentymistä arvioitiin immunohistokemiallisin menetelmin väestöpohjaisella kudosmikrosirulla. Syöpien uusiutumistapaa selvitettiin myös annosjakaumalaskelmilla, jotka saatiin yhdistämällä sädehoidon annossuunnittelukuvat niiden positroniemissiotomografia-tietokonetomografia (PET-TT) tai PET-magneettikuvien (PET-MRI) kanssa, joissa uusiutuminen todettiin.

Vaste sädehoidolle todettiin vaihtelevaksi, ja pienen p16-positiivisen alajoukon todettiin uusiutuvan korkean riskin annosjakaumassa siitä huolimatta, että p16-positiivisia tauteja pidetään yleisesti ottaen sädeherkkinä ja parempiennusteisina. Tulokset viittaavat myös siihen, että seikkaperäisestä, useaa eri kuvantamis-menetelmää hyödyntävästä hoitosuunnittelusta huolimatta osa kliinisesti merkittävistä alueista jää riittävän annosjakauman ulkopuolelle. P16 todettiin olevan suunielun syövässä itsenäinen ennusteellinen tekijä silloinkin, kun potilaan iän, tupakoinnin, alkoholinkäytön, hoitomuodon, kasvaimen koon, ja imusolmuke-levinnäisyyden vaikutus ennusteeseen huomioitiin. P16-positiivisten suunielu-kasvainten onnistunut hoidon keventäminen edellyttänee kuitenkin täydentäviä sädehoitovastetta ennustavia biomarkkereita. xCT-proteiinin ilmentymisen ja ennusteellisen arvon todettiin riippuvan merkittävästi kasvaimen sijainnista. Ennusteellinen vaikutus oli voimakkainta suunielusyövissä. XCT-proteiinia hillitsevät hoidot saattaisivat edistää kasvainten säderherkkyyttä.

AVAINSANAT: p16; ihmisen papilloomavirus; xCT; SLC7A11; biomarkkeri; uusiutuminen; pään ja kaulan alueen levyepiteelikarsinooma; suunielusyöpä; säderesistenssi; kudosmikrosiru; kuvantaminen

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Abbreviations

AJCC	American Joint Committee on Cancer
BTV	Biological Target Volume
CI	Confidence interval
CNA	Copy number alteration
CPS	Combined positive score
CRT	Chemoradiotherapy
CSC	Cancer stem cell
CT	Computed tomography
CTV	Clinical target volume
DFS	Disease-free survival
DPBC	Dose painting by contours
DPBN	Dose painting by numbers
DSS	Disease-specific survival
EBV	Epstein – Barr virus
EGFR	Epidermal growth factor receptor
ENE	Extracapsular nodal extension
FDA	Food and Drug Administration
^{18}F-FDG	Fluorine-18-fluorodeoxy-D-glucose
FFPE	Formalin-fixed paraffin-embedded
GSH	Glutathione
GTV	Gross tumour volume
HIF1α	Hypoxia-inducible factor 1-alpha
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Hazard ratio
IHC	Immunohistochemistry
IMRT	Intensity-modulated radiotherapy
ISH	In situ hybridization
MRI	Magnetic resonance imaging
NT	Non-targeting

OAS	Overall survival
OPSCC	Oropharyngeal squamous cell carcinoma
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PD-L1	Programmed death-ligand 1
PET	Positron emission tomography
PTV	Planning target volume
PVTMA	Population-validated tissue microarray
PY	Pack year
Rb	Retinoblastoma
RT	Radiotherapy
RT-qPCR	Real-time quantitative polymerase chain reaction
SCC	Squamous cell carcinoma
siRNA	RNA silencing
TCGA	The Cancer Genome Atlas
TMA	Tissue microarray
TNM	Tumour, node, metastasis
TORS	Transoral robotic surgery
TUH	Turku University Hospital
UICC	Union for International Cancer Control

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Linda Nissi, Sami Suilamo, Eero Kytö, Samuli Vaittinen, Heikki Irjala, Heikki Minn. Recurrence of head and neck squamous cell carcinoma in relation to high-risk treatment volume. *Clinical and Translational Radiation Oncology*, 2021; 27: 139–146.

II Mari Mylly*, Linda Nissi*, Teemu Huusko, Johannes Routila, Samuli Vaittinen, Heikki Irjala, Ilmo Leivo, Sami Ventelä. Epidemiological Study of p16 Incidence in Head and Neck Squamous Cell Carcinoma 2005 – 2015 in a Representative Northern European Population. *Cancers*, 2022; 14: 5717. *These authors contributed equally to this work.

III Manuscript: Linda Nissi, Sanni Tuominen, Johannes Routila, Teemu Huusko, Petra Ketonen, Maria Sundvall, Ilmo Leivo, Heikki Irjala, Heikki Minn, Tove J. Grönroos, and Sami Ventelä. xCT as a Predictor for Survival in a Population-Based Cohort of Head and Neck Squamous Cell Carcinoma.

1 Introduction

Head and neck cancers (HNCs) encompass a diverse group of malignant neoplasms occurring in different anatomical subsites of the upper aerodigestive tract that together comprise the sixth most common cancer globally. (Sung et al., 2021) HNCs are categorized according to their anatomical localization as carcinomas of the lips, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, salivary glands, and sinonasal area, as illustrated in **Figure 1**. The majority of all HNC cases, more than 90% arise from the squamous epithelium and are thus classified as squamous cell carcinomas (SCCs). The histopathological origins of cancers of the salivary glands and the sinonasal cavities are more diverse, and they are thus often considered as own disease entities. The discussion in this thesis will be limited to HNSCC (head and neck squamous cell carcinoma) arising from squamous epithelia.

Although all HNSCCs originate from mucosal epithelial cells, the anatomically diverse subsites of the head and neck together with several potential etiological agents give rise to a highly heterogenous disease entity. The current treatment modalities include surgery, radiotherapy, chemotherapy, targeted agents, and immune checkpoint inhibition. Despite appropriate multimodal therapy, prognosis of patients with HNSCC has remained modest, as 50–65% of patients with stage III–IV disease relapse locoregionally, as reviewed by Mody et al., 2021. Moreover, treatment response is often unpredictable even in tumours of the same site and stage. Thus, appropriate clinical management remains a major challenge. In addition, treatment-related toxicities affect long-term function and quality of life in many patients who survived.

In this context, predictive biomarkers aiding in patient selection for targeted cancer therapies and prognostic biomarkers indicating the biologic behaviour of the cancer would be highly beneficial in improving cancer survival and avoiding unnecessary treatment-related toxicity. To date, HPV (human papillomavirus) status as an indicator for more favourable prognosis, is the only clinically established biomarker in HNSCC. The use of p16 as a surrogate marker for HPV infection is a matter of debate. Still, it is the most widely applied method for HPV detection. At present, treatment de-escalation of HPV-related diseases is not recommended outside well-designed clinical trials, owing to differing results on its safety.

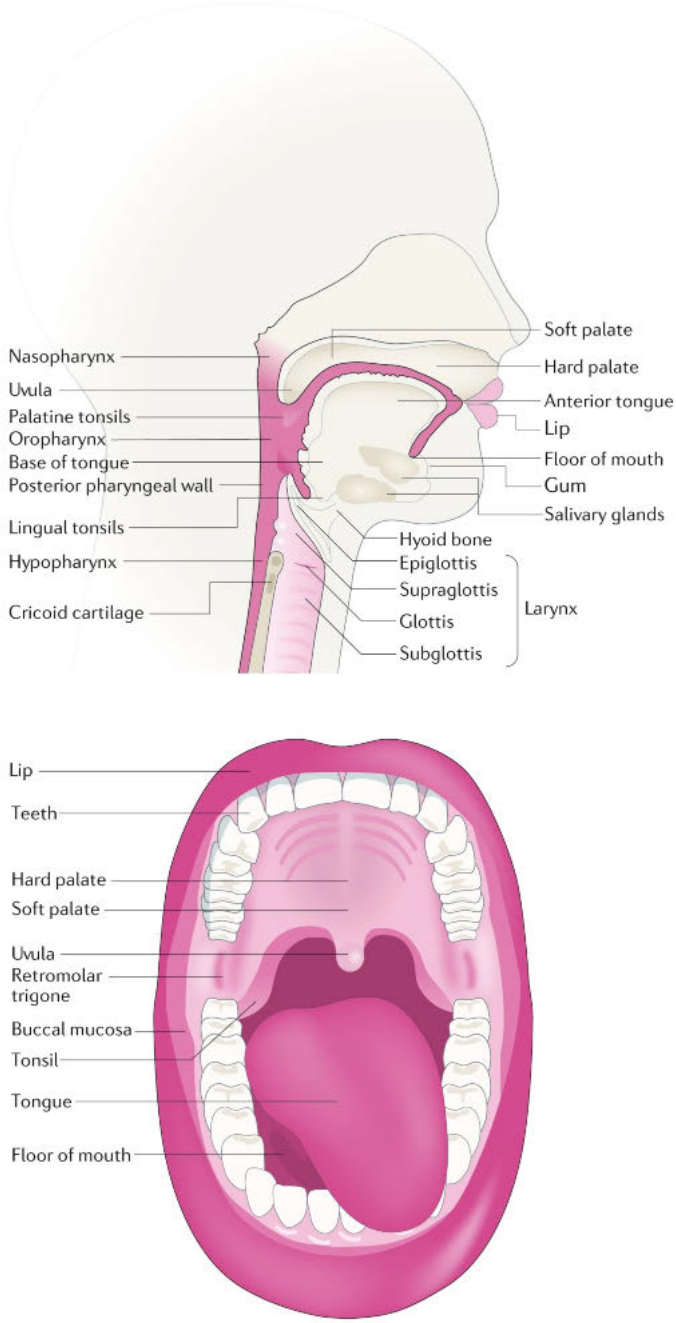

Figure 1. Head and neck squamous cell carcinoma (HNSCC) arises from the mucosal epithelium of the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, and the sinonasal areas. Modified from: *Johnson, D. E., Burtness, B., Leemans, C. R., Lui, V. W. Y., Bauman, J. E., & Grandis, J. R. (2020). Head and neck squamous cell carcinoma. Nature Reviews Disease Primers 2020 6:1, 6(1), 1–22*, with permission of Springer Nature.

2 Review of the Literature

2.1 Epidemiology

Head and neck cancer is the sixth most common cancer globally, with over 878,000 new cases and over 444,000 deaths annually (Sung et al., 2021). The overall incidence of HNC continues to rise and it is anticipated to increase by 30% by the year 2030. (Bray et al., 2018) Cancers of the lip and oral cavity are highly frequent in South Central Asia (e.g., India, Pakistan, and Sri Lanka) and Papua New Guinea, due to popularity of betel nut chewing as reviewed by Gupta et al., 2018. Incidence rates of HNC are also high in Europe, Australia, and New Zealand. (Sung et al., 2021). The increase in HNC rates in developed regions, such as the USA and Western Europe, has been attributed to a rise in oropharyngeal cancer, associated to high-risk strains of human papillomavirus, as reviewed by Chatuverdi et al., 2011 and H. Mehanna et al., 2013. There is a global trend towards increasing incidence in HPV-related HNC in countries such as the USA, South Korea, and Canada, as reviewed by Menezes, et al., 2021.

Table 1 presents the incidence percentages of tumours originating from different primary sites of the head and neck in Nordic countries in 2017–2021 together with estimated annual change in incidence. Of note is the rising incidence of oropharyngeal cancer.

Table 1. Age-standardized incidence rates per 100 000 persons per year for all new HNCs in Nordic countries* in 2017–2021. Data retrieved from the NORDCAN database (*Nordcan 2.0*, accessed 27.11.2023.)

Site	Incidence rate in females	Incidence rate in males	Annual change in females (%)	Annual change in males (%)
Lip	0.8	1.3	-0.3	-3.1
Oral cavity	3.6	5.2	+0.2	+0.2
Oropharynx	2.4	6.6	+2.8	+3.0
Hypopharynx	0.3	1.2	-1.3	-0.4
Larynx	0.7	3.6	-1.0	-2.0
Nasopharynx	0.2	0.4	-1.0	-1.3
Nasal cavity, middle ear, and sinuses	0.7	1.0	-0.7	-1.3

* Denmark, Faroe Islands, Finland, Greenland, Iceland, Norway, and Sweden.

2.2 Etiology and risk factors

Historically, the major risk factors for HNSCC have been smoking and heavy alcohol consumption which may also act as synergistic risk factors. (Blot et al., 1988; Maier et al., 1992) Tobacco smoke contains a diverse array of chemicals among which more than 60 cause cancer by inducing DNA damage, leading to an increased chance of acquiring driver mutations in cancer-related genes, as reviewed by Hecht, 2003. Smoking-related mutational signatures appear to be site-specific, with laryngeal SCC presenting the strongest mutational signature from smoking among all HNSCC subsites. Moreover, indirect effects of tobacco smoking including epigenetic changes, immune system-related events such as inflammation and metabolic disturbances may confer an elevated risk for HNSCC. (Alexandrov et al., 2016)

Alcohol use independently increases the risk of HNSCC. This association may be stronger among cancers of hypopharynx and oropharynx compared with other sites. (Menvielle et al., 2004) Although the mechanisms for alcohol-related carcinogenesis are not fully understood, several carcinogenic effects of alcohol and acetaldehyde, its major metabolite, have been suggested. These include disruption of DNA synthesis and repair, altered methylation and nutritional deficiencies. (Garro et al., 1986; Liu et al., 2001; Manari et al., 2003; Seitz & Stickel, 2006) Additionally, larger impact of alcohol is observed in interaction with tobacco use. The combined effects of alcohol and tobacco increase the risk of cancer in a synergistic manner. (Anantharaman et al., 2011; Hashibe et al., 2009).

The overall incidence of HNSCCs has been slowly decreasing in Europe, United States and Australia over the past decades while a simultaneous decrease has occurred in tobacco exposure. However, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been increasing in many Western countries. (Chaturvedi et al., 2011, 2013) This trend is mainly attributable to the etiological involvement of high-risk strains of sexually transmitted human papillomavirus that play a pathogenic role especially in OPSCC. (Chaturvedi et al., 2008; Rietbergen et al., 2013). The most common type involved in HNSCC carcinogenesis is HPV-16, that is estimated to account for 83% of HPV-positive OPSCC. (Castellsagué et al., 2016) HPV is also detectable at varying frequencies in other HNSCCs but its pathogenic role in them remains less clear. (Chung et al., 2014)

Other risk factors for HNSCC include exposure to environmental pollutants, diets lacking in vegetables, genetic susceptibility, and smokeless tobacco. (*IARC Monographs on the Identification of Carcinogenic Hazards to Humans*.; Chuang et al., 2012; Hashim et al., 2016) Also, unfavourable changes in the oral microbiome, that are often related to poor oral health, have been attributed to oncogenesis in 7–15% of oral cancers, not explainable by known risk factors. (Banerjee et al., 2017)

Lastly, Epstein – Barr virus (EBV) is a known etiological factor for HNSCC arising from the nasopharynx. Nasopharyngeal carcinomas have unique

lymphoepithelial-like histological features, pattern of growth and prognosis, and they are thus considered a distinct disease entity within HNSCC. The highest incidence of nasopharyngeal carcinoma is found in South China, Southeast Asia, North Africa, and some arctic regions, as reviewed by Tsang et al., 2020.

2.3 Pathogenesis

2.3.1 Pathogenesis of HNSCC

Generally, cancer arises through the accumulation of genetic and epigenetic changes, leading to the acquisition of tumorigenic properties, as reviewed by Hanahan & Weinberg, 2000. These include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Inflammation fosters many of these hallmarks. Later, reprogramming of energy metabolism and evading immune destruction were added to the list of the hallmarks of cancer. (Hanahan & Weinberg, 2011) In addition to these properties of cancer cells themselves, the development of a malignant tumour also requires certain interactions with the surrounding normal tissue, leading to a tumour microenvironment favorable for cancer cell invasion, as reviewed by Hanahan, 2022 and G. Wang et al., 2021.

Defining the genetic and epigenetic changes that confer tumorigenic properties in HNSCC cells has proven to be highly complex. However, the oncogenic transformation likely starts with the accumulation of mutations in a single adult stem cell or a stem cell progenitor, giving rise to cancer stem cells (CSCs). (Lim et al., 2013; Tomasetti et al., 2017; Tomasetti & Vogelstein, 2015; van Houten et al., 2002) Genetic instability with frequent loss or gain of chromosomal regions is an undisputed hallmark of HNSCC. A detailed analysis of 279 HNSCC tumours, 243 HPV-negative and 35 HPV-positive, included in the The Cancer Genome Atlas (TCGA), revealed an average of 141 copy number alterations (CNA) and 62 chromosomal structural abnormalities. (Lawrence et al., 2015) Most frequent alterations are losses of chromosomes 3p and 9p. (Califano et al., 1996) A multitude of other changed chromosome regions have been reported too, with chromosomes 12 and 16 being the only ones that do not seem to be involved in HNSCC, as reviewed by Leemans et al., 2011.

Next-generation sequencing has allowed the identification of an exploding number of candidate cancer driver genes in HNSCC. **Table 2** presents genes frequently mutated in HNSCC as listed by Lawrence et al. based on the TCGA data and established cancer driver genes as defined by Leemans et al. (Lawrence et al., 2015; Leemans et al., 2011) The most mutated gene in HNSCC is the tumour suppressor and cell cycle regulator *TP53.* Somatic mutations in *TP53*, that may be either activating or inactivating, are found in 84% of HPV-negative HNSCCs.

(Lawrence et al., 2015) Unlike in other solid malignancies that are commonly driven by mutations in oncogenes, many of the frequently mutated genes in HNSCC, including *TP53, CDKN2A, FAT1, NOTCH1, KMT2D, NSD1, TGFBR2, PTEN and SMAD4,* are considered tumour suppressors. Besides mutations, genes may be activated by amplification or inactivated by homozygous or heterozygous losses. Examples of these are the amplification of epidermal growth factor receptor (*EGFR*), leading to overexpression of the EGFR protein in 80–90% of HNSCCs, and loss of the tumour suppressor cyclin-dependent kinase inhibitor 2A (*CDKN2A*). (Grandis et al., 1998; Lawrence et al., 2015)

In addition, epigenetic changes, such as hyper- and hypomethylation of DNA, also take place in HNSCC oncogenesis. (Foy et al., 2015; Viswanathan et al., 2003) Frequently mutated genes related to epigenetic regulation include *NSD1* and *KMT2D.* Both of these encode histone-lysine *N*-methyltransferases that affect the accessibility of DNA during transcription. (Lawrence et al., 2015) The effect of both genetic and epigenetic changes can be studied in signalling pathway analysis, in which individual alterations are put to a clinically more meaningful context. In HNSCC, the most frequently altered signalling pathway is the mitogenic

Table 2. Left side, genes significantly enriched in mutations as identified by Lawrence et al. Mutation percentage of these genes in Tumour Cancer Genome Atlas (TCGA) is presented on the left side of the list. *The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 517, 576–582 (2015).* Right side, list of established genes affecting the tumorigenesis of HNSCC as defined by Leemans et al. *Leemans, C., Braakhuis, B. & Brakenhoff, R. The molecular biology of head and neck cancer. Nat Rev Cancer 11, 9–22 (2011),* accompanied by the effect on the cellular process and type of gene.

Mutation % in TCGA	**Gene** (Lawrence et al., 2015)	**Gene** (Leemans et al., 2011)	**Cellular process**	**Type of gene**
72%	*TP53*	*TP53*	cell cycle	tumour suppressor
23%	*FAT1*		WNT signalling	tumour suppressor
22%	*CDKN2A*	*CDKN2A*	cell cycle	tumour suppressor
21%	*PIK3CA*	*PIK3CA*	survival	oncogene
19%	*NOTCH1*		WNT signalling	tumour suppressor
18%	*KMT2D*		epigenetic regulation	tumour suppressor
10%	*NSD1*		epigenetic regulation	tumour suppressor
9%	*CASP8*		cell cycle	tumour suppressor
6%	*AJUBA*		WNT signalling	tumour suppressor
4%	*TGFBR2*		growth signalling	tumour suppressor
3%	*HLA-A*		immune surveillance	tumour suppressor
		PTEN	survival	tumour suppressor
		SMAD4	growth signalling	tumour suppressor
		EGFR	growth signalling	oncogene
		MET	growth signalling	oncogene
		CCND1	cell cycle	oncogene

PI3K – AKT – mTOR, with 30.5% of HNSCC tumours harbouring mutations of this pathway. (Lui et al., 2013) Other remarkable signalling pathways often altered in HNSCC include IL-6 – JAK – STAT3 axis and WNT – β-catenin pathway. The IL-6 – JAK – STAT3 promotes cellular proliferation and survival along with suppressing the antitumour immune response. Whereas the WNT – β-catenin pathway that affects multiple cellular processes and endows tumour cells with the ability to maintain and expand immature stem-like phenotypes, proliferate, extend survival and acquire aggressive characteristics by adopting mesenchymal traits, as reviewed by Alamoud & Kukuruzinska, 2018; and Johnson et al., 2018.

Regarding signalling proteins, in addition to EGFR, overexpression of other receptor tyrosine kinases, such as HER2 and MET also occurs. (Chung et al., 2017; Knowles et al., 2009) Furthermore, HNSCC are characterized by hypoxia, which induces the expression of hypoxia-inducible factor 1-alpha (HIF1α), that in turn drives the expression of multiple genes encoding proteins that promote angiogenesis and remodelling of the extracellular matrix. (J. L. Lin et al., 2008; Ravi et al., 2000) Moreover, HIF1 upregulates metabolic reprogramming of tumour cells resulting in increased glucose uptake and enhanced glycolysis instead of oxidative phosphorylation, known as the Warburg effect. (Stegeman et al., 2016)

2.3.2 HPV-negative HNSCC

Although most patients present with tumours *de novo,* the development of precancerous lesions needs to be considered in HPV-negative HNSCC. Leucoplakia and erythroplakia are visible white and red areas in the mucosal linings that may develop into invasive cancer. (Bouquot et al., 1988; Brouns et al., 2014) In addition to the macroscopically recognizable lesions, microscopic premalignant mucosal abnormalities also exist. Several carcinogens, such as tobacco smoke and alcohol, affect large areas of the head and neck mucosa simultaneously. Field cancerization, proposed by Slaugther et al in 1953, describes the presence of persistent preneoplastic fields in the clinically normal mucosae surrounding the primary tumour. These preneoplastic fields may develop further into invasive tumours in a synchronous or metachronous manner. (Slaughter et al., 1953). Moreover, they can be markedly larger than the primary tumour and are generally difficult to detect without genomic analyses due to their appearance that may be morphologically normal. (Lydiatt et al., 1998; Poh et al., 2006) The high rate of local recurrence after successful curative treatment in HNSCC has been postulated to be due to field cancerization. In addition, the presence of preneoplastic fields in the mucosae may be the source of second primary tumors of the head and neck region, as reviewed by Leemans et al., 2011.

Typical key alterations in HPV-negative HNSCC are mutations of *TP53*, encoding p53, loss of *CDKN2A*, encoding p16, and a frequently observed

amplification of the proto-oncogene *CCND1,* encoding cyclin D1. Loss of p53 function leads to disruption of DNA repair, cell growth and apoptosis. Deletion in *CDKN2A* leads to loss of p16 that plays an essential role in cell cycle regulation. Amplification of *CCND1* causes increased activation of cyclin-dependent kinases eventually leading into phosphorylation of retinoblastoma (Rb) which loses its suppressive effect on transcription factor E2F. Amplification of E2F in turn leads to DNA damage and changes in transcription. (Lechner et al., 2013; Levine & Oren, 2009) Other remarkable alterations in HPV-negative HNSCC occur in genes related to the WNT – β-catenin signalling pathway. These include *FAT1, AJUBA,* and *NOTCH1*. (Lawrence et al., 2015)

Molecular profiling studies have categorized HPV-negative tumours into further subgroups. For instance, a separate subgroup characterized by very few CNAs and wild-type *TP53*, along with a better prognosis has been reported. (Gross et al., 2014) This subgroup of HPV-negative and CNA-silent tumours, occurring more frequently in females without a history of smoking or alcohol consumption, typically display activating *HRAS* and inactivating *CASP8* mutations. (Lawrence et al., 2015) Furthermore, RNA profiling has divided HPV-negative HNSCCs into three clusters described as basal, mesenchymal, classical. (Chung et al., 2004; Walter et al., 2013) The clinical impact of these clusters, also presented in **Figure 2**, remains yet uncertain, but they may become increasingly relevant in the search for individualized therapeutic strategies.

2.3.3 HPV-positive HNSCC

HPV is a relatively novel risk factor attributing particularly to SCCs of the oropharynx. Crypt cells of palatine and lingual tonsils are organised in a discontinuous single-layer epithelium that is more susceptible to carcinogenic transformation. In HPV-related SCC, this transformation is attributed to HPV's two oncogenic viral protein products E6 and E7 that affect carcinogenesis by their inactivating effects on p53 and Rb proteins, that normally regulate the cell cycle negatively. The oncoprotein E6 binds to p53, leading to degradation of p53 and prevention of apoptosis. The oncoprotein E7 binds to Rb and inactivates it, leading to cessation of its repressive function on E2F. (Pagano M et al., 1992; Scheffner et al., 1993) Consequently, p16 protein is upregulated as an unsuccessful attempt to inhibit the inactivation of Rb. (Reuschenbach et al., 2008) It has been estimated that in two thirds of HPV-positive HNSCC the viral genome is integrated into the genome of the host cell. Whereas in the rest one third of the cases HPV DNA acts as an episome without DNA integration. (Parfenov et al., 2014)

Genes frequently altered in HPV-negative HNSCC, including *TP53* and *CDKN2A* are mainly unaffected in the HPV-positive diseases. (Chung et al., 2015; Lawrence et al., 2015) Instead, the inactivation of the p53 and Rb pathways is

executed virally. Likewise, the amplification of *EGFR* is absent. The most common alterations in HPV-positive HNSCC consist of mutations in the PI3K pathway. Furthermore, the loss of genes encoding TRAF3 (tumour necrosis factor receptor-associated factor 3) and ATM (ataxia telangectasia mutated) kinase is specific for HPV-driven HNSCC. (Lawrence et al., 2015)

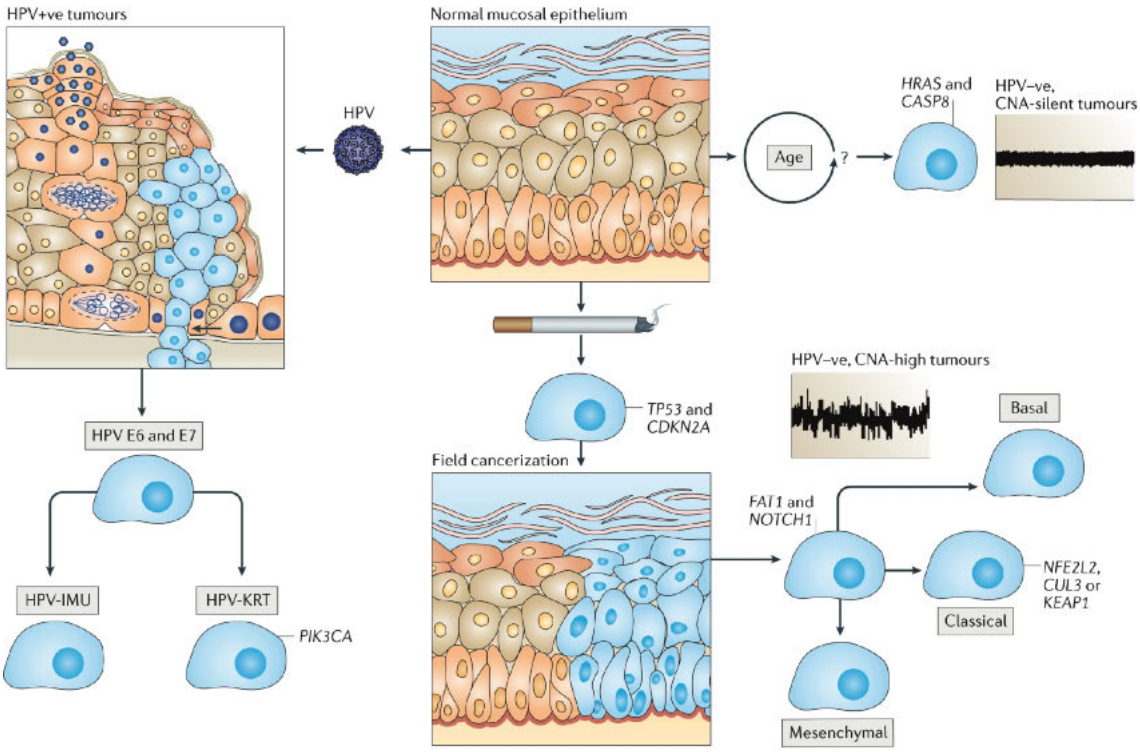

Figure 2. An overview of carcinogenesis in head and neck squamous cell carcinoma (HNSCC). The main distinctive features are the involvement of human papillomavirus (HPV) and the number of copy number alterations (CNAs). HPV-related tumours can be divided into HPV-KRT (keratinocyte differentiation and oxidative reduction process) and HPV-IMU (immune response and mesenchymal cell differentiation). In HPV-unrelated HNSCC, the p53 and Rb pathways are frequently abrogated, except in CNA-silent tumours. The etiology of CNA-silent tumours remains elusive with ageing as a hypothesized risk factor. In other types of HPV-unrelated HNSCCs, smoking is a known risk factor. A plethora of genes and pathways seem to be involved in the progression of HPV-unrelated tumours with a high number of CNAs. One of the well-established genes in this process include *NOTCH1* and *FAT1* that act in the WNT – β-catenin pathway. At least three subgroups can be identified based on expression profiling: classical, basal and mesenchymal. The group defined as classical is characterized by mutations of nuclear factor erythroid 2-related factor 2 (NFE2L2) pathway, cullin 3 (*CUL3*), and kelch-like ECH-associated protein 1 (*KEAP1*). *CASP8,* caspase 8; *CDKN2A,* cyclin-dependent kinase inhibitor 2A. Adopted from *Leemans, C., Snijders, P. & Brakenhoff, R. The molecular landscape of head and neck cancer. Nat Rev Cancer 18, 269–282 (2018)*, with the permission of Springer Nature.

Molecular profiling studies have subclassified HPV-positive HNSCC into two groups named HPV-IMU (immune response and mesenchymal cell differentiation) and HPV-KRT (keratinocyte differentiation and oxidative reduction process), as presented in **Figure 2**. The HPV-IMU group, also referred as inflamed/mesenchymal (IMS), group is characterized by an expression signature of mesenchymal and immunological response genes. Meanwhile, the HPV-KRT, also referred as classical HPV-related group, is characterized by upregulated keratinocyte differentiation and oxidative stress related genes. (Keck et al., 2015; Zhang et al., 2016) Moreover, tumours of the HPV-KRT subtype presented more often with HPV genome integration to host cell which has been associated with unfavourable survival. (Koneva et al., 2018; Pinatti et al., 2021; Qin et al., 2020) Validation of the survival difference between these subgroups could lead to a more refined prognostic stratification of patients with HPV-positive HNSCC.

2.4 Diagnostic evaluation

2.4.1 Symptoms and clinical presence

The risk of HNC increases with age, with most cases occurring among individuals over 50 years old. Overall, men are at two to four times higher risk for developing HNSCC than women. The presenting symptoms vary according to the anatomical site of the primary tumour and its aetiology. Classical symptoms of tumours originating from different primary sites are presented in **Table 3**.

The first step in patients presenting with concerning symptoms is an examination of the oral cavity and endoscopic evaluation of the nasopharynx, oropharynx, and larynx. In smokers, the elevated risk of a second tobacco-related primary tumour may justify panendoscopy of the upper aerodigestive tract, as reviewed by Coca-Pelaz et al., 2020. Tumours of the oral cavity are often diagnosed at an early stage due to patient's self-identification or routine dental examinations and symptoms that interfere with eating and speaking. Also, laryngeal tumours are often diagnosed at an early stage owing to voice changes. Whereas tumours of the oropharynx and hypopharynx typically become symptomatic first at a later stage, as reviewed by Johnson et al., 2020. Approximately 40% of HNSCC patients present with asymptomatic neck metastasis, while distant metastasis is rare at the time of diagnosis. (Routila et al., 2021; Zhang et al., 2022)

Table 3. Classical symptoms of HNSCC arising from different primary sites. Human papilloma virus (HPV), Epstein – Barr virus (EBV).

Site	Symptoms
Lip	discoloured lesion, sore, bleeding or pain of the lip
Oral cavity	non-healing sore or ulcer, chewing pain, dysarthria
Oropharynx	HPV-negative: dysphagia, ear pain. HPV-positive: painless neck mass
Hypopharynx	Dysphagia, voice changes, haemoptysis
Larynx	Hoarseness and haemoptysis; in later stages dyspnoea and airway obstruction
Nasopharynx, nasal cavity, paranasal sinuses	Nasal bleeding, unilateral nose obstruction, symptoms related to dysfunction of the Eustachian tube, cranial nerve palsies, neck mass in EBV-positive cases

2.4.2 Histopathology

Diagnosis of HNSCC is based on a biopsy of the primary tumour. If a neck mass presents without a primary tumour, it should undergo an fine needle aspiration. (Pynnonen et al., 2017) The biopsy specimen can usually be evaluated by routine histopathology, but immunohistochemistry may be necessary in case of poorly differentiated or basaloid tumours. The histopathology of HNSCC is characterized by cellular atypia and loss of squamous differentiation of varying extent. Features indicating aggressiveness include lymphatic or perineural invasion and poor differentiation. Of note, despite the more favourable prognosis, HPV-positive tumours are mostly poorly differentiated and non-keratinizing. In contrast, HPV-negative tumours are more often well or moderately differentiated, with preserved keratinization and stratification of the epithelium. (Ang et al., 2010a; Fakhry et al., 2008; Gondim et al., 2016; Grønhøj et al., 2018)

In OPSCC and an unknown primary tumour setting, HPV status is routinely evaluated, as described in the next chapter. Patients with nasopharyngeal carcinoma are tested for EBV. Programmed death-ligand 1 (PD-L1) testing is currently pursued in patients with metastatic or recurrent disease.

2.4.3 Detection of HPV

Considering the renewed staging system, discussed in more detail later, that considers the more favourable prognosis of HPV-positive OPSCC and related de-escalation trials, HPV-testing with verified methodology is essential for tumours of the oropharynx and pathological lymph nodes of unknown primary. The expression of the E6 and E7 oncoproteins appears to be fundamental for the carcinogenic process and maintenance of the malignant transformed phenotype in HPV-positive HNSCC. Thereby, in the absence of fully reliable immunohistochemical probes for

E6 and E7 proteins, detecting mRNA of E6 and E7 is the current gold standard to identify HPV-related disease. Unfortunately, this method is ideally limited to fresh samples that are not routinely available in standard clinical practices, as reviewed by Bussu et al., 2019; and Gallus et al., 2023. Regarding standard formalin-fixed paraffin-embedded (FFPE) samples, consensus about the most appropriate method for HPV-detection in HNSCC is still missing. Current options for FFPE samples are based on the detection of viral DNA with or without polymerase chain reaction (PCR), detection of mRNA or DNA with in situ hybridization (ISH), and detection of surrogate markers, as reviewed by Venuti & Paolini, 2012.

Immunohistochemistry (IHC) of the p16 protein has been widely used as a surrogate marker for HPV-positive OPSCC, owing to its many advantages, such as simplicity and cost efficiency. Moreover, p16 is used for HPV-identification in the newest, 8th edition of TNM staging guideline of UICC (Union for International Cancer Control) and AJCC (American Joint Committee on Cancer). (J. D. Brierley et al., 2017) However, p16 IHC has also disadvantages, including low specificity, as p16 can be overexpressed also by mutations and other viruses as reviewed by El-Naggar & Westra, 2012; and Romagosa et al., 2011. Furthermore, p16 lacks sensitivity in HNSCC sites other than the oropharynx. (Bishop et al., 2012)

Detection of HPV DNA by PCR is highly sensitive but poorly specific due to the risk of contamination and inability to distinguish between transcriptionally active and clinically irrelevant HPV infections. (Braakhuis et al., 2009; Smeets et al., 2007; Weinberger et al., 2006) ISH-based assays, such as detection of HPV DNA and mRNA, are rather specific but the sensitivity is considered too low for these methods to be used alone in clinical practice. (Schache et al., 2011; Schlecht et al., 2011) Moreover, ISH-based methods are more costly and require complex laboratory procedures as well as an experienced pathologist for the interpretation of the results. (Gallus et al., 2023)

As none of the detection methods suitable for FFPE samples has demonstrated sufficient sensitivity and specificity to be used as the single test for HPV evaluation, alternative approaches combining two different methods have been proposed. These sequential strategies, include the confirmation of a positive p16 IHC staining by HPV DNA detection with PCR or ISH. (Grønhøj et al., 2018; Jordan et al., 2012; Rietbergen et al., 2013; Schache et al., 2011; Smeets et al., 2007)

2.4.4 Imaging

Occasionally, imaging may be pursued to guide biopsies. After confirmation of diagnosis by histopathology, imaging is conducted to delineate the extent of the tumour and involvement of adjacent structures, and to detect regional metastases. This information is essential for accurate staging evaluation and treatment planning. The specific extent of locoregional disease is evaluated by computed tomography

(CT) or magnetic resonance imaging (MRI). While positron emission tomography (PET) combined with either CT or MRI achieves highest accuracy in identification of nodal and distant metastases. (Rohde et al., 2017; Schöder & Yeung, 2004; Schwartz et al., 2003) PET imaging also guides the identification of disease targets for radiotherapy planning, as reviewed by Grégoire et al., 2007 and Minn et al., 2010.

The choice of imaging modality for the primary tumour depends on factors related to the tumour, the patient, the resources of the treating clinic, as well as the information desired to be known by the clinician. CT is widely available and relatively inexpensive. Moreover, shorter scanning times allow to extend the scan to cover large body areas for staging purposes. Meanwhile MRI is often superior for focused evaluation of smaller regions. In MRI, functional pulse sequences, such as diffusion-weighted imaging and perfusion scans, can be conducted to obtain more specific information when needed. MRI has higher soft tissue contrast and is usually preferred in patients with suspected cranial nerve involvement or tumours encroaching on the base of the skull. CT, in turn, is better for detection of calcifications and air, as well as evaluation of cortical or periosteal bony erosion and cartilage invasion. MRI, however, is useful for assessment of bone marrow invasion in patients with oral cavity cancer. Lastly, the choice of imaging modality is also affected by relative and absolute contraindications, such as presence of cardiac pacemakers, other ferromagnetic devices, claustrophobia, and inability to stay still to MRI. Meanwhile renal failure and allergy to the iodinated contrast media are the two main contraindications to CT examinations, as reviewed by Kim et al., 2021; and Pfister et al., 2020.

PET technology uses various labelled tracers to generate functional 3-dimensional information of the disease process. This information is fused with conventional anatomical images that may be acquired either by CT or MRI. The most common used tracer in head and neck cancer is fluorine-18-fluorodeoxy-D-glucose (^{18}F FDG) that provides relevant metabolic information based on the increased glucose uptake and glycolysis of tumour cells. Major advantages of PET include the high sensitivity in detection of malignant lesions revealed by metabolic abnormalities that often occur before morphological alterations. Furthermore, combined imaging decreases susceptibility to artifacts. The main disadvantage of PET is the low specificity, as glucose uptake may be increased also by inflammation. False positive uptake may arise also due to other factors, including biopsy or treatment related changes, unilateral abnormalities, such as cranial nerve palsy, and physiological glucose uptake by lymphoid tissue, as reviewed by Purohit et al., 2014.

Accurate definition of tumour and target volumes by pretreatment imaging is vital to ensure an adequate dose of radiation to target volumes while simultaneously avoiding normal tissues. This is particularly relevant in the setting of modern, highly conformal irradiation techniques and, more recently, particle therapy. Three main volumes used for radiotherapy planning in HNSCC are: gross tumour volume

(GTV), clinical target volume (CTV), and the planning target volume (PTV). The delineation of these volumes and organs at risk is described by detailed consensus guidelines. (Brouwer et al., 2015; Grégoire et al., 2018; A. W. Lee et al., 2018) In 2000, Ling et al. proposed the concept of biological target volume (BTV) which represents a subvolume of a tumour with higher tumour burden or greater radioresistance as identified by molecular or functional imaging. (Ling et al., 2000) Thus, PET-CT and PET-MRI -based treatment planning has been widely adopted by large referral centres to assist in the dose optimisation and contouring processes. **Figure 3** depicts PET-MRI -based radiotherapy treatment planning.

Finally, repeated imaging during treatment allows for adaptive radiotherapy, in which dose distribution is re-adjusted for anatomical changes occurring during the treatment course that typically lasts from six to seven weeks, as reviewed by Kim et al., 2021.

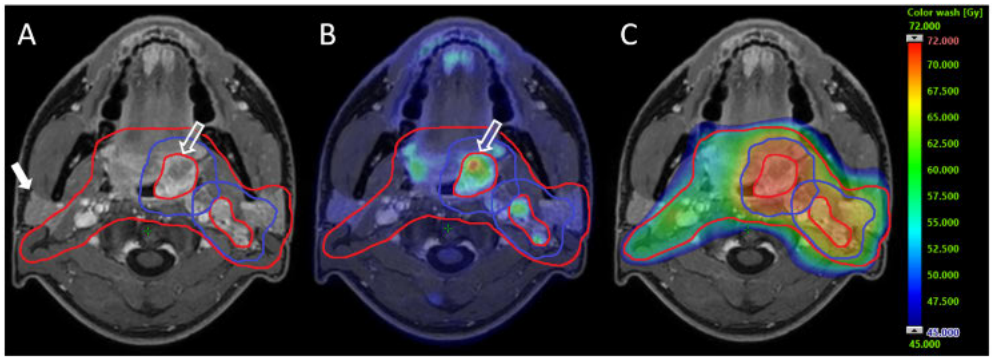

Figure 3. PET-MRI-based treatment planning for a 39-year male diagnosed with squamous cell carcinoma of the left tonsil (open arrow) and spread to cervical lymph nodes. Dose distribution has been modified using volumetric modulated arc therapy (VMAT) technique to reduce the dose received by the right parotid gland (filled arrow). Gross tumor volume (GTV) delineated by the inner red lines and clinical target volume 1 (CTV1) delineated by the blue lines in gadolinium-enhanced and T1-weighted magnetic resonance imaging (MRI) **(A)** and in hybrid imaging by positron emission tomography (PET) and MRI **(B).** Image **C** presents the exclusion of the right parotid gland, front of oral cavity, and teeth outside the planned target volume (PTV) delineated by the outer red line. PTV usually consists of the CTV1 and elective areas with an additional margin of 1 cm. Modified (by enhancing the red and blue lines) from *Ranta P., Irjala H., Minn H., and Kinnunen I. Suunielusyöpä ja elämänlaatu. Duodecim. 2023;139(17):1359–1366* with the permission of Duodecim and all authors. Original picture by Heikki Minn and Sami Suilamo.

2.4.5 Staging

Once all necessary diagnostic information is obtained, staging criteria released by the UICC and AJCC are followed for TNM (tumour, node, metastasis) classification and staging. Generally, earlier stages (I and II) encompass smaller tumours without prominent involvement of cervical lymph nodes. Later stages (III and IV) are characterized by locally advanced disease and increased number of lymph nodes

involved, or distant metastatic spread, distinctive for stage IV. Staging differs at each primary tumour site. Additionally, in 2017 separate staging systems were introduced for p16-positive and p16-negative oropharyngeal cancer patients, resulting in relative downstaging of HPV-positive disease. Moreover, extracapsular nodal extension (ENE) was included to nodal staging in non-viral HNSCC. (J. Brierley et al., 2017) **Table 4** demonstrates clinical TNM classification of oropharyngeal cancers according to the UICC/AJCC 8th edition. Next, TNM classification is used for staging as demonstrated in **Table 5** for p16-negative oropharyngeal cancers, and in **Table 6** for p16-positive diseases.

Table 4. Clinical TNM classification of oropharyngeal cancers according to the 8th edition of UICC/AJCC staging manual. (J. Brierley et al., 2017) p16 is used as a surrogate marker for human papilloma virus (HPV). Extracapsular nodal extension (ENE).

	P16 -	**P16 +**
Tumour		
TIS	carcinoma in situ	-
T0	no tumour identified	no tumour identified
T1	≤ 2 cm	≤ 2 cm
T2	> 2 cm but < 4cm	> 2 cm but < 4cm
T3	> 4 cm or extension to lingual surface of epiglottis	> 4 cm or extension to lingual surface of epiglottis
T4	-	moderately advanced disease: tumour invades larynx, extrinsic muscle of tongue, medial pterygoid muscle, hard palate, or mandible *
T4A	moderately advanced disease: tumour invades larynx, extrinsic muscle of tongue, medial pterygoid muscle, hard palate, or mandible *	-
T4B	Very advanced disease: tumour invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases carotid artery	-
NODE	**P16 -**	**P16 +**
N0	no regional lymph node metastases	no regional lymph node metastases
N1	single ipsilateral lymph node, ≤ 3 cm, without ENE	1 or more ipsilateral lymph nodes, none > 6 cm
N2	-	contralateral or bilateral lymph nodes, none > 6 cm
N2A	single ipsilateral node, > 3 cm but < 6 cm, without ENE	-
N2B	multiple ipsilateral lymph nodes, none > 6 cm, without ENE	-
N2C	bilateral or contralateral lymph nodes, none > 6 cm, without ENE	-
N3		One or more lymph nodes, > 6 cm
N3A	> 6 cm, without ENE	-
N3B	one or more lymph nodes with ENE	-
Metastasis	**P16 -**	**P16 +**
M0	no distant metastasis	no distant metastasis
M1	distant metastasis	distant metastasis

* Mucosal extension of primary tumours of the base of the tongue and vallecula to the lingual surface of epiglottis does not constitute invasion of the larynx

Table 5. Prognostic stages of p16-negative oropharyngeal cancers according to the 8th edition of UICC/AJCC staging manual. (J. Brierley et al., 2017)

Clinical TNM	N0	N1	N2	N3
T0		Stage III	Stage IVA	Stage IVB
T1	Stage I			
T2	Stage II			
T3				
T4A				
T4B				
	Any M1: Stage IVC			

Table 6. Prognostic stages of p16-positive oropharyngeal cancers according to the 8th edition of UICC/AJCC staging manual. (J. Brierley et al., 2017)

Clinical TNM	N0	N1	N2	N3
T0		Stage I	Stage II	Stage III
T1				
T2				
T3				
T4				
	Any M1: Stage IV			

2.5 Treatment

The choice of treatment is guided by the anatomical subsite, stage, and characteristics of the disease, along health and performance status of the patient. Given the intricacy of HNSCCs, treatment at a high-volume, tertiary referral centre with a multidisciplinary approach is recommended. (Eskander et al., 2014; Wuthrick et al., 2015) The principal modalities of curative therapy are surgical resection, radiotherapy, and systemic therapy. Treatment planning should aim for a highly curative approach, while optimizing preservation of organs and function. Local tumours are preferably treated with single modality therapy, while locally advanced tumours are usually treated with a combination of surgery and radiotherapy.

2.5.1 Surgery

Surgery remains the primary treatment strategy for oral cavity. (D. Adelstein et al., 2017) Also, treatment with minimally invasive transoral surgical techniques, such as transoral robotic surgery (TORS) has become an option especially for small (T1–2) oropharyngeal tumours with minimal involvement of cervical lymph nodes (N0–1), as reviewed by C. Lin et al., 2022. Regarding laryngeal cancers, definitive surgical

therapy may be suitable for small supraglottic tumours (T1–2), and for glottic tumours when partial cordectomy is considered sufficient. Surgical management for larger (T3) laryngeal tumours can be justified for lesions with poor function. Lastly, total laryngectomy remains the standard of care for patients with T4 tumours, as reviewed by Steuer et al., 2017.

Surgical management of the neck is defined by lymphatic drainage of the primary site and risk of metastatic spread. Generally, a primary tumour resection is often accompanied by ipsilateral, or if considered necessary, bilateral neck dissection. (Simon et al., 2020) Elective neck dissection in patients with early-stage oral cavity has been shown to result in higher rates of overall survival (OAS) and disease-free survival (DFS) compared to watchful waiting followed by therapeutic neck dissection. (D'Cruz et al., 2015) However, sentinel lymph node biopsy is a feasible alternative to neck dissection in early-stage HNSCCs. (Samant, 2014) Finally, reconstructive surgery is an essential component in the management of head and neck cancers.

2.5.2 Radiotherapy

In radiotherapy (RT), ionizing radiation is used to eradicate or control the growth of tumour cells. Ionizing radiation affects tumour cells by damaging DNA either directly, or indirectly by generating highly reactive oxygen species. These events may lead to single and double strand DNA breaks, which eventually lead to apoptosis. In addition, exposure to radiation stimulates several other molecular reactions in both the neoplastic and the surrounding healthy tissue.

Radiotherapeutic methods have developed from conformal to intensity-modulated radiotherapy (IMRT), allowing the delivery of higher doses to the tumour while minimizing exposure of normal tissues. IMRT utilizes several rotating beamlets of varying shapes and intensities to create an optimal dose distribution. For curative intent, radiotherapy is commonly delivered as a fractionated regimen consisting of 2.0 Gy fractions administered daily for 5 days per week to a cumulative dose of 66–70 Gy. In postoperative setting, the typical dose for the high-risk area is 60–66 Gy depending on adverse features identified after surgery, such as high T class, adequacy of the surgical resection, perineural and lymphatic invasion, and histological growth features. (Cooper et al., 2004; *Finnish Soc. Head Neck Oncol. Treat. Guidel.* 2023) Also, alternative fractionation schemes have been evaluated with an aim to optimize radiotherapy response. The standard schedule can be accelerated, or the irradiation dose may be delivered in either smaller (hyperfractionation) or larger fractions (hypofractionation). Studies on glottal cancer suggest that a hypofractionated regimen may result in better local control with the advantage of shorter treatment time. (Kachhwaha et al., 2021; T. H. Lee et al., 2022)

In contrast, a meta-analysis by Lacas et al. demonstrated a benefit on overall survival in head and neck cancer patients who received hyperfractionated radiotherapy. (Lacas et al., 2017) However, the standard regimen remains the most used.

In the primary treatment setting, radiotherapy is often preferred over surgical resection in early-stage and locally advanced laryngeal cancers, owing to better chances of preserving laryngeal function. Radiotherapy may provide similar results to surgery in some other types of HNSCCs as well. An ongoing trial (NCT02984410) compares these treatment modalities for early-stage oropharyngeal, hypopharyngeal, and supraglottic cancers. Regarding locally advanced diseases, radiotherapy plays a fundamental role in multimodality therapy and it may be utilized in primary, preoperative, adjuvant, and palliative settings, as reviewed by Mody et al., 2021.

Definitive radiotherapy with concurrent chemotherapy, i.e. chemoradiotherapy (CRT), has traditionally been the standard of care for oropharyngeal cancers. However, radiotherapy use in the oropharynx has been rapidly evolving and CRT has been partially replaced by other approaches, such as minimally invasive transoral surgery. This is due to the rise in HPV-related cases and subsequent changes in patient features and prognosis. As patients with HPV-related OPSCC are typically younger and have a long life expectancy, special emphasis needs to be set on limiting adverse effects and improving quality of life after treatment, as reviewed by Ranta et al., 2023. For T3 stage laryngeal tumours, definitive CRT has remained as the cornerstone of cancer management. (Forastiere et al., 2003)

2.5.3 Systemic therapy

Systemic therapies for HNSCCs include chemotherapy, targeted therapy, and immunotherapy, and they may be administered in concurrent, adjuvant, metastatic, and recurrent settings. Data on the use of induction chemotherapy in HNSCC are controversial. In Finland, induction chemotherapy is used occasionally for symptomatic patients with locally advanced and rapidly growing disease of especially nasopharynx. (Ghi et al., 2017; Haddad et al., 2013; Qian et al., 2015)

The standard chemotherapy regimen given concurrently with radiotherapy is single-agent cisplatin administered at 100 mg/m^2 every three weeks for three cycles. (D. J. Adelstein et al., 2003) However, toxic effects of high-dose cisplatin regimen are substantial and limit its use notably. Thereby, an alternative strategy with weekly administration of cisplatin at 40 mg/m^2 for six cycles, suggested to have comparable outcome, has been adopted by the Finnish national treatment guidelines. (Bauml et al., 2019; *Finnish Soc. Head Neck Oncol. Treat. Guidel.*, 2023; Mohamed et al., 2019) For patients who have contraindications to cisplatin, such as chronic kidney disease, hearing loss, or borderline performance status, carboplatin combined with paclitaxel may be used. (Sun et al., 2022)

In adjuvant setting, postoperative chemoradiotherapy with cisplatin has been established as the standard of care in high-risk patients with high stage, positive surgical margins, or extranodal invasion. (Bernier et al., 2004, 2005; Cooper et al., 2004) In addition, patients with perineural or lymphovascular invasion may be offered adjuvant CRT or radiotherapy selectively, based on the overall risk of relapse.

Despite the growing understanding of the genomic landscape in HNSCC, cetuximab, a monoclonal antibody targeting EGFR, remains practically the only targeted therapy for HNSCC. Cetuximab-enhanced radiotherapy has been shown to improve survival compared to radiotherapy alone. (Bonner et al., 2010) However, the use of cetuximab in concurrent setting is generally reserved for patients ineligible for cisplatin, as the results of cetuximab are inferior to cytotoxic chemotherapy. (Gillison et al., 2019; H. Mehanna et al., 2019; Xiang et al., 2018; Zandberg et al., 2018)

In recurrent and metastatic diseases that cannot be treated with salvage surgery or reirradiation, systemic therapy is the mainstay of palliative treatment. The choice between agents or a combination of them depends on the adverse effect profiles of the treatment, and characteristics related to the patient and prior therapy. The first-line standard of care in recurrent and metastatic HNSCC has, until recently, consisted of cetuximab added to chemotherapy with fluorouracil and a platinum-agent. This regimen is referred to as the EXTREME regimen. (Vermorken et al., 2008; Lynggaard et al., 2015; Sano et al., 2019)

Although the idea of boosting natural immune defences to eradicate cancer can be traced back to 150 years ago, the breakthrough of immunotherapy into clinical oncology has taken place just lately, as reviewed by Zhang & Zhang, 2020. The treatment of HNSCC has been affected particularly by the development of programmed death 1 (PD-1) immune checkpoint inhibitors. Pembrolizumab and nivolumab are IgG4 humanized antibodies to PD-1, that were approved for the treatment of recurrent or metastatic HNSCC by the Food and Drug Administration (FDA) in 2016.

Pembrolizumab has shown durable responses in patients with recurrent or metastatic disease (≥ 6 months: 82–85% of the patients who responded). However, the response rates were relatively low, 16–18%. (L. Q. M. Chow et al., 2016; Mehra et al., 2018; Seiwert et al., 2016) The phase III KEYNOTE-048 trial compared pembrolizumab monotherapy or combination of pembrolizumab and chemotherapy with a platinum-based agent and fluorouracil with the same chemotherapy combined with cetuximab. Both treatment groups that included pembrolizumab improved OAS in patients with PD-L1-expressing tumours compared with the EXTREME regimen. In the whole trial population, irrespective of PD-L1 status, the addition of pembrolizumab to chemotherapy resulted in improved OAS compared with the EXTREME regimen, while monotherapy with pembrolizumab did not. The associated toxic effects were significantly less in the group receiving pembrolizumab as monotherapy. (Burtness et al., 2019) Based on these results, pembrolizumab plus chemotherapy has been approved

as the new standard of care for first-line treatment in recurrent and metastatic HNSCC. Monotherapy with pembrolizumab may be considered in patients with tumours that express PD-L1 with combined positive scores (CPSs) of ≥1%. The CPS assesses PD-L1 staining on both tumour cells and tumour-infiltrating immune cells. Finally, the use of immune checkpoint inhibition in the treatment of curable HNSCC remains yet investigational, as reviewed by (Bhatia & Burtness, 2023)

The role of PD-L1 as a predictive biomarker is yet debatable because of unstandardized testing methodology. (Schildhaus, 2018) The CPS has shown to better predict clinical benefit in patients receiving pembrolizumab than assessing the PD-L1 staining on the tumour cells only. (Cohen et al., 2019; Seiwert et al., 2016) Nonetheless, the available data suggest that even patients with low PD-L1 expression may benefit of immunotherapy. (Burtness et al., 2019) Therefore, pembrolizumab might be justified regardless of PD-L1 status.

2.5.4 Treatment-related adverse effects

Besides achieving the highest cure rates possible, preserving organ function and minimising therapy-related morbidity is a fundamental aspect of treatment. Given the variety of daily functions within the head and neck area, the consequences of HNSCCs and their treatment substantially affect the quality of life in patients and their families. Therapy-related adverse effects depend on the site of the primary tumour and may affect swallowing, communication, and identity, for example. As an example, speech and swallowing impairments occur in approximately 50% of patients treated with radiotherapy. (Ranta et al., 2021; Rinkel et al., 2016) Although treatment-related adverse effects often heal afterwards, long-time reduction in quality of life is also common. (H. M. Mehanna & Morton, 2006) Multimodality treatment, in particular, is associated with an elevated risk of acute and late toxicities, including dysphagia with subsequent risk of aspiration pneumonia and mortality unrelated to cancer. (Forastiere et al., 2013; Trotti et al., 2007) Immunotherapeutic agents have relative favourable toxicity profiles. Nevertheless, immunotherapy-related adverse effects, resulting of overstimulation of the immune system, are remarkably different and occur later compared with those of cytotoxic chemotherapy. Dermatological side effects are the most common, but also more severe immune-related adverse effects, such as colitis, hepatitis, and endocrinopathies occur. (Long et al., 2020)

2.6 Follow-up and posttreatment imaging

Posttreatment surveillance is usually provided by ear, nose, and throat specialist with complementary input from imaging. According to the current knowledge, the follow-

up of HNSCC patients does not prolong survival but it may improve quality of life. Most societies recommend for more intensive surveillance during the first 2–3 years. (Kytö et al., 2019; Szturz et al., 2020) The national guidelines of Finnish Society for Head and Neck Oncology recommend follow-up visits to continue for 3 years in patients with a moderately good prognosis and up to 5 or more years in patients with poor prognosis. (*Finnish Soc. Head Neck Oncol. Treat. Guidel.*, 2023)

Posttreatment imaging is complicated by treatment-related effects, including oedema of soft tissues, fibrosis, and atrophy. The NCCN 2.2020 guideline for head and neck cancers recommends imaging three or four months after the end of definitive-intent treatment for locoregionally advanced disease. (Pfister et al., 2020) PET-CT has high sensitivity for detecting recurrent locoregional disease and distant metastases. In 2016, Mehanna et al. demonstrated equal survival among patients who underwent PET-CT guided surveillance compared to those who had a planned neck dissection after chemoradiotherapy for locally advanced HNSCC. (H. Mehanna, Wong, et al., 2016) However, positive PET results should be confirmed by a biopsy, as the rate of false positive results is high due to posttreatment inflammation.

There is little evidence to support routine long-term imaging surveillance in patients who have negative results in initial posttreatment imaging. (Heineman et al., 2017; Ho et al., 2013). Instead, imaging, as well as follow-up in general, should be pursued based on tumour site, prognostic factors, presence of clinical symptoms, and changes observed on clinical examination. For areas difficult to visualize by clinical examination, routine imaging may be justified.

2.7 Prognosis, survival, and recurrence

Prognosis of HNSCCs depends on a variety of factors, including primary tumour site and growth characteristics, stage at presentation, epidemiological risk factors, and patient-related features. Outcomes are better in female compared with male patients. Moreover, higher age at diagnosis decreases survival. (Gatta et al., 2015) Also, smoking worsens survival, especially if continued despite the HNSCC diagnosis. (Browman et al., 1993; Grønhøj et al., 2019; Karlsson et al., 2021) In OPSCC, prognosis is largely affected by HPV-status. (Ang et al., 2010b; Chaturvedi et al., 2011) In early-stage (I–II) HNSCC survival rates of approximately 70–90% can be achieved with single modality intervention by either surgery or radiotherapy. (Carvalho et al., 2005) Whereas locally advanced disease has poorer prognosis with survival rates of <50% in HPV-negative disease. (Braakhuis et al., 2012) **Table 7** presents the 5-year age-standardized relative survival rates for cancers originating from different subsites of the head and neck.

Table 7. Five-year age-standardized relative survival rates for head and neck cancers diagnosed in 1999–2007 in the European population. Results from the EUROCARE-5 study: *Gatta, G., Botta, L., Sánchez, M. J., Anderson, L. A., Pierannunzio, D., Licitra, L., & EUROCARE Working Group: (2015). Prognoses and improvement for head and neck cancers diagnosed in Europe in early 2000s: The EUROCARE-5 population-based study. European journal of cancer (Oxford, England:1990), 51(15), 2130–2143*.

Site	5-year age-standardised relative survival
Hypopharynx	25%
Oropharynx	39%
Tongue	43%
Oral cavity	45%
Nasopharynx	49%
Larynx	59%

Despite appropriate multimodal therapy, 50–65% of patients with stage III–IV HNSCC relapse locoregionally, as reviewed by Mody et al., 2021. The appearance of cancer recurrence after initially successful therapy is the most common type of treatment failure and implies poor prognosis. (H. Mehanna, Kong, et al., 2016)

2.8 Radioresistance

Radioresistance limits the chances of tumour control, and it is therefore a substantial clinical problem especially in patients with locally advanced HNSCC. Several mechanisms, including both intrinsic and extrinsic factors, have been acknowledged to affect radioresistance in tumour cells. Firstly, the effects of ionizing radiation are enhanced by the presence of oxygen. Hypoxic areas, typical in rapidly growing tumours with insufficient vascular circulation, are more refractory to the damage caused by ionizing radiation, as reviewed by Perri et al., 2015. The development of an aggressive cancer phenotype in hypoxic conditions is strongly mediated by HIF-1α, as reviewed by Semenza, 2000. Moreover, smoking contributes to poor oxygenation, as reviewed by Bredell et al., 2016.

Several intracellular pathways have been shown to hinder radiotherapy-induced cell death. These cascades are often interlinked and partially overlap with the general hallmarks of cancer. Among these pathways, EGFR overexpression is one of the most studied in HNSCC. (Ang et al., 2002) Intriguingly, EGFR can be activated by irradiation in the absence of its ligands through autophosphorylation of intracellular domains. (Tinhofer et al., 2012) Another signalling pathway contributing to radioresistance in HNSCC is the PI3K – Akt – mTOR cascade which can be upregulated by several mechanisms, including EGFR over expression, RAS activation, PI3K mutation, and Akt gene amplification, as reviewed by Dent et al., 2003. The upregulation of PI3K – Akt – mTOR inhibits apoptosis, activates the

DNA-repair machinery, and induces epithelial to mesenchymal transition. (Harris et al., 2019; H. Wang et al., 2021; Zhan & Han, 2004) Also, alterations in p53 have been shown to play a role in radioresistance of HNSCC. (Couture et al., 2002; Skinner et al., 2012) However, results from studies examining radioresistance in HPV-positive, wild-type p53 tumours are incoherent. (Arenz et al., 2014; Nagel et al., 2013) Generally, tumours with a high number of genetic mutations have been considered less sensitive to radiotherapy. Prognostic multi-gene expression models of tumour radiosensitivity have been identified and initially validated. (Eschrich et al., 2009; C. Lu et al., 2023)

On the other hand, irradiation can trigger cellular senescence that affects tumour cell repopulation during radiotherapy and correlates positively with radioresistance in HNSCC. (Gorgoulis et al., 2019; Schoetz et al., 2021) Also, the presence of cancer stem cells (CSCs) is demonstrated in HNSCC. (Routila et al., 2022) CSCs are distinguished by over-activated DNA damage repair systems, redistribution of the cell cycle, robust capacity for tumour cell repopulation, and independence of cellular oxygen. Owing to these properties, CSCs are thought to play an essential role in tumour maintenance, as reviewed by S. Y. Lee et al., 2017. Moreover, cancer stem cells have the ability to invade and migrate, increasing the risk of locoregional relapse and metastasis. (C. Chen et al., 2013) Regarding different molecular subtypes of HNSCC, a study that classified tumours as basal, mesenchymal, atypical, or classical found that tumours of the mesenchymal subtype showed a lower locoregional control in a postoperative (chemo)radiotherapy setting. These tumours presented with increased epithelial – mesenchymal transition and overexpression of a gene signature enriched in DNA repair genes. (Patil et al., 2022) Finally, also extrinsic factors such as the competence of the immune system and nutritional status contribute to successful radiotherapy, as reviewed by Rückert et al., 2021; and Soldati et al., 2018.

2.9 Biomarkers in HNSCC

The National Cancer Institute of USA defines a biomarker as “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease; and may be used to see how well the body responds to a treatment for a disease or condition”. (*NCI Dictionary of Cancer Terms - NCI*) Predictive biomarkers identify patients who will benefit from a particular therapy. Whereas prognostic biomarkers are associated with a clinical outcome in the absence of therapy or in the context of standard therapy that all patients likely receive. Traditional, hypothesis-driven biomarker discovery has based on the biological processes that the potential biomarker is thought to reflect. Recently, modern systems biology based on “omics” approaches has become a new tool for

discovering biomarker molecules and molecular signatures. Moreover, molecular profiling may be conducted on different levels: genome, transcriptome, proteome, and metabolome. (Wood et al., 2014) These high-throughput technologies have produced an astonishing number of candidate biomarkers. However, the specific clinical context in which the biomarker is desired to be used needs to be considered too. Moreover, simply demonstrating that a biomarker can distinguish patient groups with different outcome is not sufficient to establish its clinical utility. A feasible biomarker should provide information that affects clinical decision making and thereby results in markedly improved patient survival or quality of life. Furthermore, the improved outcome should outweighs the costs and possible risks of testing. (K. Y. Kim et al., 2014)

Except for HPV in oropharyngeal cancer, therapy stratification of HNSCCs is still based on clinical features, including TNM staging. (L. Q. M. M. Chow et al., 2020) This approach often fails to capture the enormous biologic heterogeneity of the disease entity. Thus, appropriate clinical management and evaluating the risk of relapse after primary treatment remain major challenges. On the other hand, for the many patients who are cured, late adverse effects of treatment can lead to decreased function and quality of life, or even cause non-cancer mortality. (Forastiere et al., 2013; Ranta et al., 2021; Trotti, 2000) Therefore, indicators of treatment sensitivity could be extremely helpful in tailoring therapy, including treatment modality, technique, and dose on an individualized basis. (Eze et al., 2017; Sawyers, 2008)

2.9.1 Development of cancer biomarkers

Understanding tumour biology and identification of molecular alterations has offered a backbone for the discovery of biomarkers also in HNSCC. Once a biological event has been identified as a potential biomarker, an assay to evaluate it is needed. For this purpose, a wide range of methodology exists. The gold standard for detection of mutations comprises PCR-based amplification and sequencing of the amplified gene product. However, this method is ideally limited to fresh samples. Also, detection of mRNA through PCR and protein levels by Western blotting require fresh samples, as reviewed by Cazzato et al., 2021.

Most clinical biomarker applications used to evaluate genomic status are based on IHC or ISH due to their suitability for FFPE samples. As discussed earlier in the chapter concerning HPV detection, IHC is simple, cost-efficient, and widely available. However, it is strongly dependent on the reliability of the antibody used. Therefore, a thorough validation process is needed, as reviewed by O'Hurley et al., 2014; Simpson & Browning, 2017; Smith & Womack, 2014. For this purpose, several techniques are available. The recommended validation techniques largely depend on the cost and time that can be spent, and the laboratory's access to tissues

and certain equipment. In all cases, the first step involves the identification and selection of an appropriate antibody, followed by a literature and database search to fully understand the target protein and to identify positive and negative controls for IHC. The validation should ideally be complemented by Western blotting, in which antibody specificity is demonstrated by the presence of a single band corresponding to the predicted molecular weight of the protein, as reviewed by O'Hurley et al., 2014. **Figure 4** presents a schematic representation of recommended techniques for antibody validation in mainstream biomarker development projects, oriented towards clinical application.

Once a potential biomarker is discovered and a reliable and reproducible assay to evaluate it has been established, the following step is to test its clinical benefit. First, the intended use of the biomarker and its target population need to be precisely defined. Next, the biomarker is investigated in clinical samples from cancer patients. This type of preliminary evaluation is often conducted in a retrospective cohort. During the process of biomarker development, the representativeness of the specimens and datasets needs to be carefully considered. Moreover, data analysis should account for clinical, pathological, and molecular confounders. Also, the biomarkers performance should be compared to already established biomarkers and clinicopathological factors that are in current use to guide treatment. A useful marker should provide added value to already existing methods. Finally, the biomarker should be validated in an independent external cohort and tested in a prospective trial study, as reviewed by Hsieh et al., 2019; K. Y. Kim et al., 2014; and Pepe et al., 2008.

In large-scale, tissue-based biomarker studies, tissue microarrays (TMAs) have become a popular method, as they allow large numbers of cases to be tested quickly in a single block. In addition to time and cost effectiveness, TMA sampling spares tissue specimens to be tested multiple times. Moreover, technical sources of variation between cores on the same slide can be mostly eliminated in the TMA format. Although tests used for whole tissue sections may be performed on TMAs, some techniques or methods may require TMA-specific optimisation. Disadvantages of TMA include laborious initial set-up. Also, highly heterogenous tissue may require many cores per case. Furthermore, approximately 10 – 15 % of the cores are expected to be lost while cutting TMA sections. Finally, given the initial time and cost of construction, TMAs are often reused. Thus, the representativeness of the TMA needs to be carefully considered each time a new research question is proposed. (Ilyas et al., 2013)

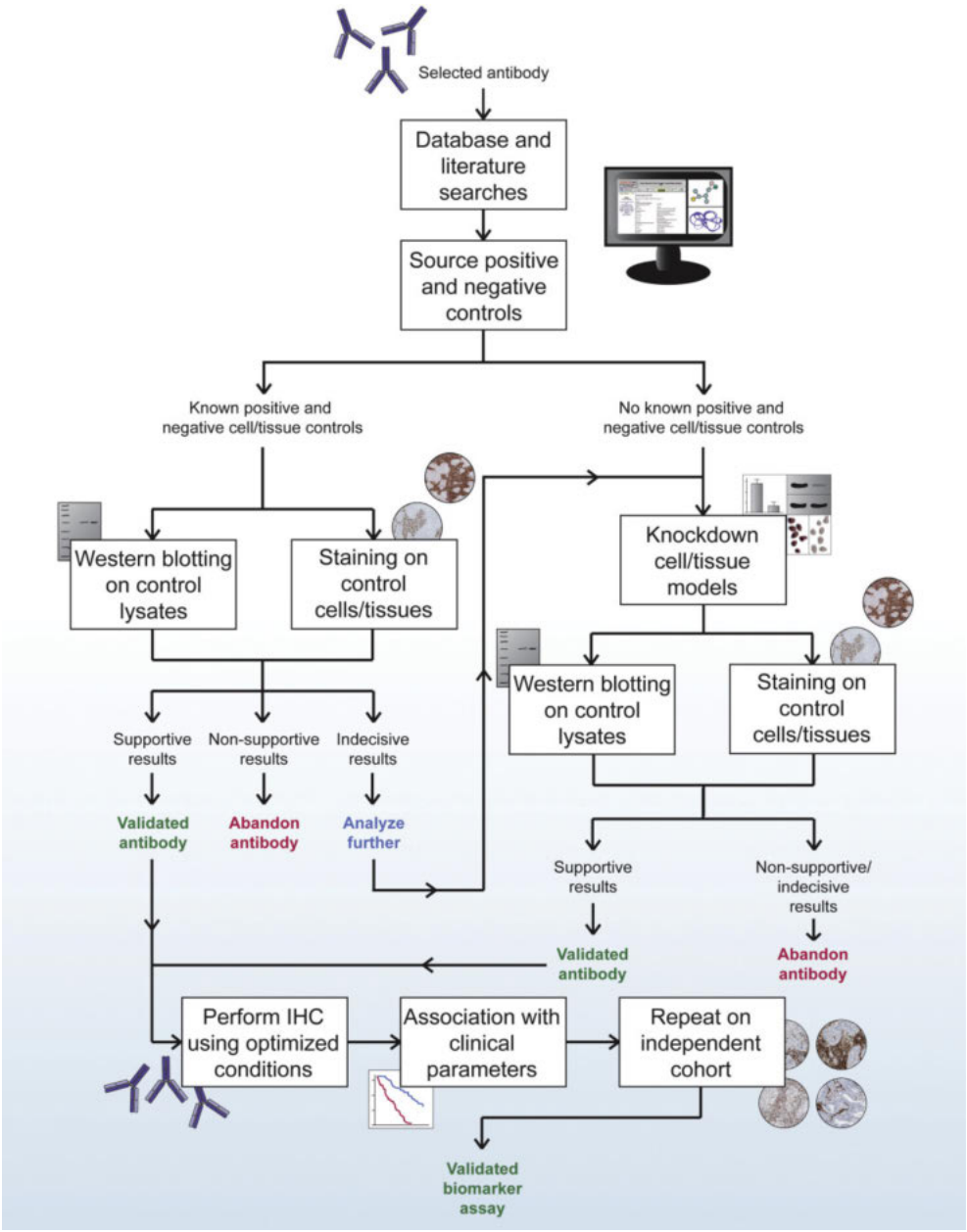

Figure 4 Recommended techniques for antibody validation in biomarker development projects oriented towards clinical application. Adopted from *O'Hurley, G., Sjöstedt, E., Rahman, A., Li, B., Kampf, C., Pontén, F., Gallagher, W. M., & Lindskog, C. (2014). Garbage in, garbage out: a critical evaluation of strategies used for validation of immunohistochemical biomarkers. Molecular oncology, 8(4), 783–798,* under the terms of the Creative Commons License CC BY-NC-ND 4.0.

There are relatively few biomarkers in oncology that have passed the rigorous validation process and then been adopted for clinical use. This is particularly true for HNSCC, despite the growing knowledge on its biology and the staggering amount of biomarker candidates suggested, as reviewed by Goossens et al., 2015; Hsieh et al., 2019; and Kang et al., 2015. Results of biomarker analyses reported by different research groups are often inconsistent. Challenges related to biomarker development include:

- variations in the assay used, such as technological platforms, reagents (especially antibodies), tissue sample qualities, and scoring procedures
- biomarker instability
- convenience sampling
- small sample size
- bias in patient inclusion to study cohorts
- differing study populations with true clinical variability
- differing statistical methods

Moreover, molecular alterations in tumour cells are not limited merely to the expression of genes and proteins. Instead, post-translational modifications, such as phosphorylation, methylation, and glycosylation may occur too, as reviewed by J. M. Lee et al., 2023. As an example, over 300 post-translational modification sites have been annotated for the tumour suppressor p53. (Hafner et al., 2019) However, detection and quantification of post-translational modifications is challenging which remains a major obstacle in the translation of these kind of biomarkers into clinical practice, as reviewed by Hermann et al., 2022. Finally, intratumoural heterogeneity may act as a confounding factor in biomarker research as well. (Mroz et al., 2015)

2.9.2 HPV as a biomarker in HNSCC

HPV-driven HNSCC is considered a biologically distinct disease among head and neck cancers, and HPV16 infection is accepted as an independent risk factor in patients with oropharyngeal cancer. To date, HPV status is the only clinically established biomarker in HNSCC. As discussed previously, the use of p16 as a surrogate marker for HPV infection remains the most widely applied method for HPV detection. Several studies have demonstrated that patients with an HPV-positive OPSCC have better prognosis compared to patients with HPV-negative diseases. In patients with HPV-associated OPSCC, the risk of death has been estimated 40–60% lower and the risk of relapse has been estimated 60–70% lower

than in patients whose OPSCC is not related to HPV. (Fakhry et al., 2008; Ragin & Taioli, 2007; Rosenthal et al., 2016)

Regarding recurrence, HPV-positive OPSCCs have shown to develop distant relapses more often than their HPV-negative counterparts. (Huang et al., 2013; Trosman et al., 2015; Wendt et al., 2021) Generally, HPV-positive tumours are considered to respond well to radiotherapy, as reviewed by Leemans et al., 2011. However, also HPV-positive OPSCCs do recur. The location of recurrences in relation to irradiation fields has not been studied in-depth. In a study by Chen et al., 40% of the recurrences in HPV-positive OPSCC occurred within the high-risk radiation field. Overall, 15–20% of patients with HPV-positive primary OPSCC are considered to have a high risk of recurrence, as reviewed by Lechner et al., 2022. Identifying these patients remains a key challenge, especially when considering de-intensified treatment. Interestingly, detection of HPV DNA in saliva or plasma samples has been suggested for monitoring post-treatment disease status in HPV-associated OPSCCs. (Chera et al., 2020; Fakhry et al., 2019)

The use of p16 IHC as a surrogate marker for HPV has been a matter of debate over the recent years. Importantly, a p16-positive but HPV DNA-negative subgroup of patients exists. A study by Wendt el al., in which HPV DNA PCR was used as the method for HPV detection, suggested that the prognosis of the $p16^{+}/HPVDNA^{-}$ group was better than the prognosis of the $p16^{-}/HPVDNA^{-}$ group but worse than the prognosis of the $p16^{+}/HPVDNA^{+}$ group. Moreover, the prognostic benefit of p16 was limited to tumours of the tonsils and the base of tongue. (Wendt et al., 2021) In concordance with Wendt et al., also Mehanna et al. found patients with discordant p16/HPV status to have intermediate prognosis. In addition, the prognosis of these patients was shown to be dependent on their smoking status. In this study HPV was tested by PCR or ISH to detect either HPV DNA or HPV RNA. (H. Mehanna et al., 2023) In contrast to these results, in a randomized controlled trial by Lilja-Fischer et al., loco-regional failure and overall survival were similar whether patients were separated by p16 IHC or HPV DNA status. In this study, a next generation sequencing panel was used to assess HPV DNA. (Lilja-Fischer et al., 2023) The impact of p16/HPV discordance seemed to be more prominent in areas with low HPV-attributable fractions, in the study by Mehanna et al. Thus, the differing results from these two studies may be partly explained by the geographic variation of the HPV-attributable fractions.

Another matter of debate is the significance of HPV and p16 in anatomic sites other than the oropharynx. Some studies have shown that patients with p16-positive non-OPSCC have better prognosis compared to their p16-negative counterparts. Nonetheless, patients with p16-positive non-OPSCC had worse prognosis than patients with p16-positive OPSCC. (Bryant et al., 2018; Chung et al., 2014) Whereas some studies suggested that the prognostic impact of p16 is limited to OPSCC.

(Castellsagué et al., 2016; Lassen et al., 2017) Furthermore, the prevalence of p16 and etiologic involvement of HPV appear to be low in non-OPSCCs. (Castellsagué et al., 2016; Lingen et al., 2013; Ndiaye et al., 2014; Taberna et al., 2016) As the prognostic implications of p16 or HPV have not yet been established, consensus guidelines do not recommend routine testing in non-OPSCC. (Lewis et al., 2018)

2.9.3 De-escalation of treatment in HPV-related OPSCC

As patients with HPV-related OPSCC are generally younger and have more favourable prognosis, multiple ongoing trials are assessing the safety of treatment de-intensification to improve quality of life during and after therapy. De-intensification strategies have included the reduction of radiotherapy dose or volume, TORS instead of chemoradiotherapy, and alternative systemic therapy regimens. The ORATOR trial, that assessed outcomes in patients who received either primary TORS or primary chemoradiotherapy, was unable to determine definitive differences in survival or quality of life. (Nichols et al., 2022) Whereas preliminary results from the ORATOR 2 trial suggested transoral surgery to be associated with fatal side effects. (Palma et al., 2022) On the other hand, a previous systematic review showed promising swallowing outcomes in patients treated with TORS. (Hutcheson et al., 2015)

Concerning radiotherapy, in a randomized clinical trial investigating dose reduction to elective nodal sites, differences in survival and recurrence rates were not statistically significant between two patient groups that received either standard dose (50 Gy) or lower dose (40 Gy) Furthermore, a significant reduction was observed in salivary gland toxicity. (Nevens et al., 2017) Also, ipsilateral irradiation instead of bilateral irradiation of the neck has been studied. Ipsilateral irradiation resulted in improved quality of life and it was suggested to be a safe approach in selected patients. (Huang et al., 2017; Jellema et al., 2007) Moreover, in a clinical trial of 39 patients, IMRT without chemotherapy resulted in a 90% complete response rate and a 94% 2-year progression-free survival rate as well as reduced toxicity compared to chemoradiotherapy. (Takemoto et al., 2021) Lastly, considering systemic therapy, the use of cetuximab instead of cisplatin as radiosensitizer had detrimental effects on 2-year OAS and recurrence. (H. Mehanna et al., 2019)

Finally, the detection method used for HPV-detection needs to be considered also when evaluating results of de-escalation studies. Although p16 has been demonstrated to be an independent prognostic factor in OPSCC, several trials have demonstrated a decrease in its prognostic benefit when treatment de-intensification strategies are implemented. (Chera & Amdur, 2018; Gillison et al., 2019; X. J. D. Lu et al., 2022; H. Mehanna et al., 2019; Wagner et al., 2020)

2.10 xCT

2.10.1 xCT and ferroptosis in cancer

Solute carrier family 7, membrane 11 (SLC7A11), also known as xCT, is one of the two subunits of system x_c^- , depicted in **Figure 5**. xCT is an amino-acid transporter that is responsible for Na^+-independent uptake of cystine into cells in exchange for glutamate. Cysteine, the reduced form of cystine, is the rate-limiting precursor for glutathione (GSH), an abundant cellular antioxidant, which plays a central role in the prevention of oxidative stress signalling that is strongly associated with cell proliferation and tumour growth, as reviewed by Lewerenz et al., 2013; and Stipanuk et al., 2006.

Moreover, xCT-mediated cystine uptake suppresses ferroptosis, an iron-mediated form of regulated cell death. Ferroptosis is characterized by generation of reactive oxygen species that can react with polyunsaturated fatty acids which leads to lethal accumulation of lipid peroxides in the cellular membrane. (Dixon et al., 2012; Jiang et al., 2015; Zhang et al., 2018) Recently, ferroptosis has been revealed to be a key tumour suppressor mechanism. (Jiang et al., 2015; Lang et al., 2019; Stockwell et al., 2017; Zhang et al., 2018) Furthermore, ferroptosis has been shown to play a key role in radiotherapy-induced cell death and to mediate the synergy between radiotherapy and immunotherapy. (Lang et al., 2019; Lei et al., 2020; Pearson et al., 2021) It has also been established that xCT promotes radioresistance and resistance to therapeutic drugs, such as cisplatin, through enhanced GSH synthesis and inhibition of ferroptosis. (Cobler et al., 2018; Nagane et al., 2018; Okuno et al., 2003) On the other hand, high xCT expression results in metabolic reprogramming, leading to dependency on glucose and glutamine, which could make cells more vulnerable to therapeutic targeting, as reviewed by Koppula et al., 2020.

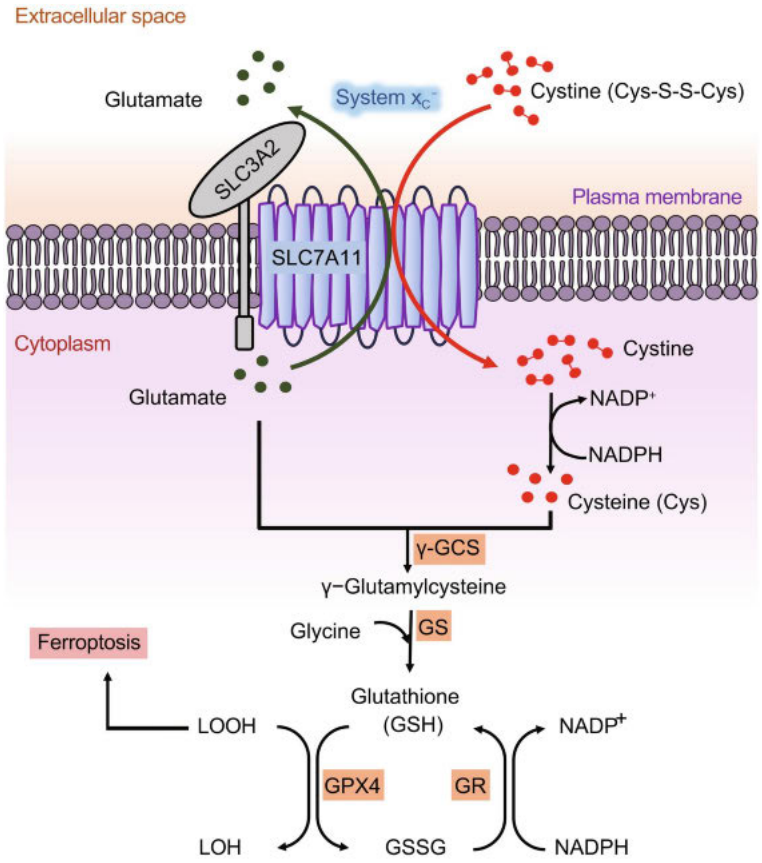

Figure 5. Structure and function of xCT/SLC7A11. System x_c^- is a heterodimer consisting of a light chain subunit SLC7A11, that mediates the uptake of cystine, and a heavy chain subunit SLC3A2 that acts as a chaperone to SLC7A11. Once extracellular cystine is imported into the cell, it is converted to cysteine through NADPH-consuming reduction. Subsequently, cysteine is used to synthesize the reduced form of glutathione (GSH) through a two-step process. First, cysteine and glutamate form γ-glutamylcysteine in a reaction catalyzed by γ-glutamylcysteine synthetase (γ-GCS). Secondly, a glutathione synthetase (GS) -mediated enzymatic addition of a glycine molecule produces GSH, a robust antioxidant. Furthermore, GSH may promote cell growth by suppressing ferroptosis, a form of regulated cell death that is induced by excessive accumulation of lipid hydroperoxides in the cell membrane. Glutathione peroxidase 4 (GPX4) uses GSH to reduce lipid hydroperoxides (LOOH) to lipid alcohols (LOH), and thereby hinders ferroptosis. GSH in turn is oxidized into GSSG (oxidized glutathione) and then converted back to GSH via glutathione reductase (GR). NADPH; nicotinamide adenine dinucleotide phosphate. Adopted from *Pranavi Koppula, Li Zhuang, Boyi Gan. Cystine transporter SLC7A11/xCT in cancer: ferroptosis, nutrient dependency, and cancer therapy.* Protein & Cell *2021;12(8):599–620,* under the Creative Commons CC BY license.

Consequently, xCT has attracted considerable interest in understanding tumour biology. Moreover, it has been shown to be involved in multiple human carcinomas, including glioma, acute myeloid leukaemia, and prostate, oesophageal, ovarian,

pancreatic, breast, and colorectal cancer. Furthermore, high expression of xCT is often associated with poor prognosis. (Badgley et al., 2020; Lo et al., 2010; M. Z. Ma et al., 2015; Okuno et al., 2003; Robert et al., 2015; Shiozaki et al., 2014; Timmerman et al., 2013; Zhao et al., 2018; Zhong et al., 2018)

Expression of xCT is regulated through a variety of mechanisms, including transcriptional, post-transcriptional, and post-translational regulation. xCT transcription can be upregulated under various conditions, such as oxidative and metabolic stress. Activating transcription factor 4 (ATF4) and nuclear factor erythroid 2-related factor 2 (NRF2) are two important transcription factors identified to mediate stress-induced xCT expression, as reviewed by Koppula et al., 2020. In contrast, p53 has been demonstrated to repress the expression of xCT and to promote ferroptosis. (Jiang et al., 2015) Still, some p53 mutants have been shown to retain their tumour suppressive function by repressing xCT. It has been suggested that p53-mediated tumour suppression at least partly depends on the p53 mutant's ability to downregulate xCT and induce ferroptosis. (Jiang et al., 2015; T. Li et al., 2012; S. J. Wang et al., 2016)

2.10.2 xCT in HNSCC

Recent results suggest that also HNSCC tumour cells might gain uncontrollable proliferation capacity through xCT upregulation to resist ferroptosis (M. Li et al., 2022), which in turn presents potential for therapeutic targeting. xCT has been evaluated as a potential prognostic biomarker in cancers of the larynx and those of the oral cavity. Lee et al. have suggested xCT to predict posttreatment survival and recurrence in surgically treated patients with oral cavity SCC. (J. R. Lee et al., 2018) Contrastingly, Toyoda et al. did not find xCT to be prognostic for overall or progression-free survival in surgically resected tongue cancer. (Toyoda et al., 2014) On the other hand, xCT has been reported to predict overall and recurrence-free survival in laryngeal SCC. (Z. Ma et al., 2017) Despite the remarkable interest in xCT, its role in HNSCC remains enigmatic due to a small number of studies that are partly contradictory.

2.10.3 Therapeutic targeting of xCT and ferroptosis

xCT has also emerged as a promising therapeutic target in cancer therapy. Several compounds, such as sulfasalazine, erastin, sorafenib, and imidazole ketone erastin (IKE) have been demonstrated to act as xCT inhibitors. (Feng & Stockwell, 2018; Hu et al., 2020) These compounds can induce ferroptosis by blocking xCT-mediated cystine uptake and are collectively called class 1 ferroptosis inducers (FINs). Another strategy is to target the previously discussed metabolic vulnerabilities

associated with high expression of xCT. This approach includes inhibition of glucose uptake by glucose transporter (GLUT) inhibitors and targeting glutamine dependency by glutaminase inhibitors, as reviewed by Koppula et al., 2020.

xCT inhibition has attracted interest as a potential adjuvant therapy for radiotherapy. (Feng et al., 2022; Sarowar et al., 2022; Yang et al., 2021) Targeting ferroptosis-associated metabolism in cancer cells has also been suggested to improve the efficacy of immunotherapy (W. Wang et al., 2019). Regarding head and neck cancer, Li et al. have reported that xCT inhibition could arrest HNSCC proliferation by promoting ferroptosis. (M. Li et al., 2022) Moreover, Roh et al. have demonstrated that both genetic silencing of the SLC7A11 gene and pharmacological inhibition of xCT by sulfasalazine significantly sensitize cisplatin-resistant HNC cells by inducing ferroptosis. (Roh et al., 2016) Interestingly, as HNSCC cells often present an increased intracellular iron concentration, inducing ferroptosis can be expected to effectively promote death of tumour cells while sparing normal tissue. This is due to upregulation of transferrin receptor 1 (TFRC1), that is responsible for iron uptake, and downregulation of ferroportin that is responsible for iron efflux. (Lenarduzzi et al., 2013; Shan et al., 2008)

3 Aims

The aims of the three studies were:

Study I:

1. To investigate geographic distribution of recurrent tumours in relation to radiotherapy high-risk treatment volume.
2. To compare patterns of recurrence in relation to p16 status and treatment modality.

Study II:

3. To study the incidence of HNSCC and p16-positive OPSCC in Southwestern Finland during the years 2005–2015.
4. To investigate locoregional variation of p16 expression in different primary tumour sites of the head and neck region.
5. To compare survival outcomes between patients with p16-positive and p16-negative HNSCC in a population-based setting.

Study III:

6. To study xCT expression in tumours originating from different anatomic subsites of the head and neck.
7. To evaluate the utility of xCT as a biomarker in HNSCC.

4 Materials and Methods

4.1 Patients and study design

4.1.1 Study I

The medical records of all 507 patients who were diagnosed with HNSCC at Hospital District of Southwest Finland during 2010–2015 were retrospectively reviewed. Overall survival and disease-free survival were analyzed, and patients with a recurrent disease were identified. P16 immunohistochemistry of the primary tumor, whenever available was used as a surrogate for HPV-positivity.

Patient selection for more detailed analysis is described in **Figure 6**. Inclusion criteria to be fulfilled were:

I. Locoregional recurrent tumor that appeared at least 3 months after end of first-line treatment

II. RT as part of a curative treatment plan for primary tumor

III. Diagnosis of local and/or regional recurrence at hybrid PET/ CT and/or MRI that was technically possible to co-register with radiotherapy dose plans

IV. Known p16 status.

All patients had treatment plans based on PET/CT imaging with 18F-FDG and IMRT was used for irradiation. The patients' follow-up schedule has been described in detail by Kytö et al., 2019. In brief, clinical, and radiological assessment of patients after multimodality treatment was the responsibility of the head and neck surgeon and during study period no systematic radiological imaging protocol was included in the schedule. The follow-up PET/CT performed three months after the end of treatment became a routine first in 2016. Therefore, imaging without a clinical suspicion of recurrence was performed only occasionally. Recurrence of study patients was detected on PET/CT or PET/MRI referred to by the head and neck surgeon and subsequently confirmed by biopsy.

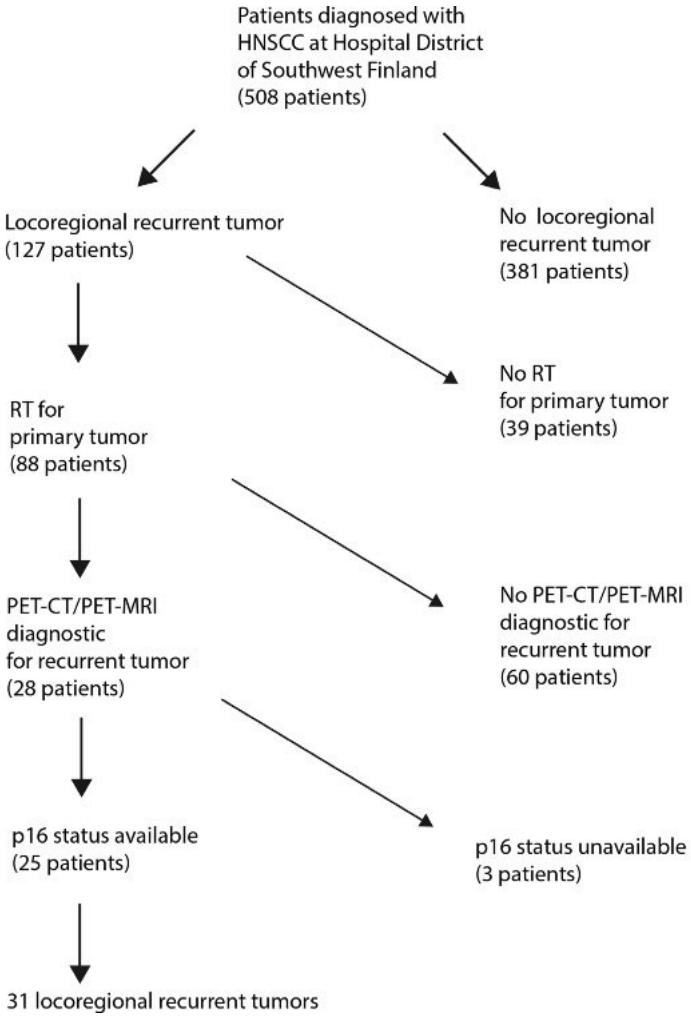

Figure 6. Patient selection in study I. HNSCC, head and neck squamous cell carcinoma; MRI, magnetic resonance imaging; PET, positron emission tomography; CT, computed tomography; RT, radiation therapy. Adopted from publication I under the Creative Commons CC-BY-NC-ND license.

4.1.2 Studies II–III

In study II, a population-based cohort was composed of all new HNSCC patients who were treated between 2005 and 2015 in the Southwest Finland tertiary referral center of Turku University Hospital (TUH). This cohort was used as the basis for a population-validated TMA used in studies II and III. The design of study II is shown in **Figure 7**. Treatment protocols and TNM status were collected from meetings of the multidisciplinary tumour board for head and neck cancer.

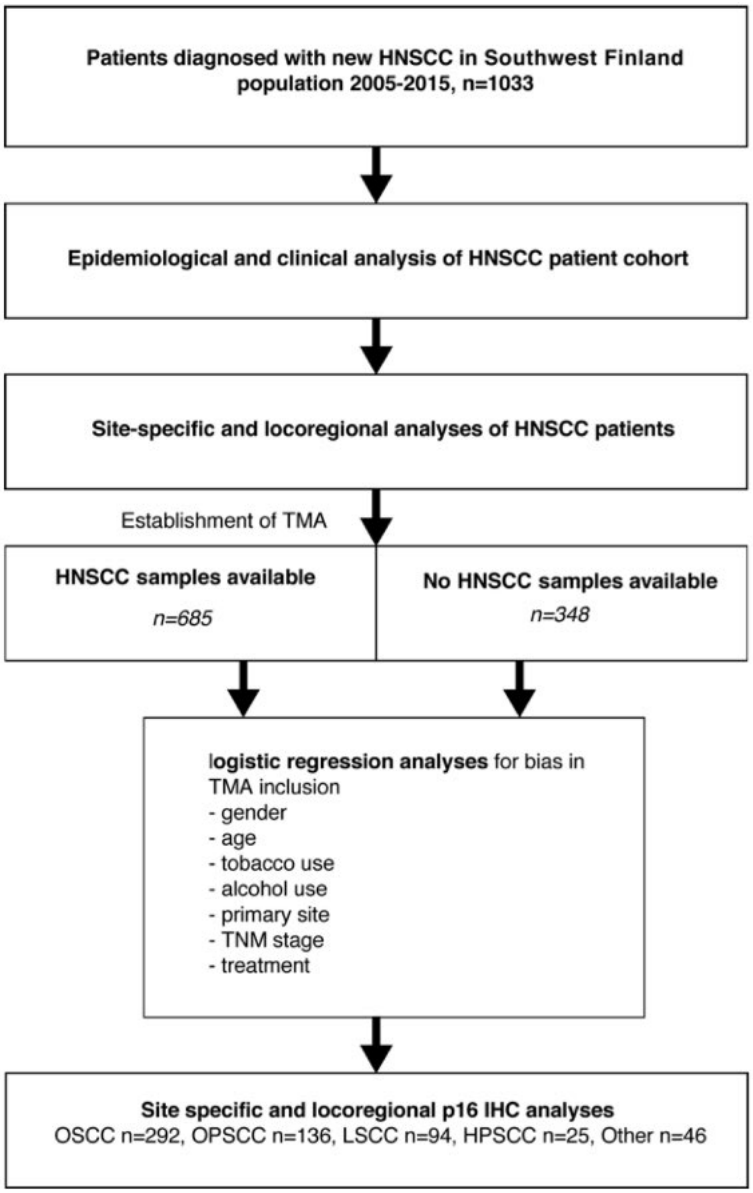

Figure 7. Patient identification and analysis in study II. All patients diagnosed with new head and neck squamous cell carcinoma (HNSCC) in Southwestern Finland between 2005 and 2015 were identified and included in the clinical cohort. This cohort was used as the basis for a population-validated tissue microarray (TMA) and immunohistochemical (IHC) analyses of p16. OSCC, oral cavity squamous cell carcinoma; OPSCC, oropharyngeal squamous cell carcinoma; LSCC, laryngeal squamous cell carcinoma. Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

Survival was defined from end-of-treatment to end-of-follow up or death. Survival status was gathered from medical records of TUH, which is connected to the Finnish National Population Information System database. Patients' end-of-follow-up dates were recorded from the time of last data in the TUH records. Information on alcohol and tobacco use was also collected from the patient records.

All the patients in the 2005–2015 clinical cohort who had a primary tumour tissue sample available (n=685) were included in the TMA. Formalin-fixed and paraffin-embedded (FFPE) tumor samples were obtained from the pathology archives of Auria Biobank. Final TMA blocks of duplicate 0.6 mm cores were made in TMA Grand Master (3D Histech). Normal liver samples were included in each block for orientation.

In study III, all patients of the TMA described in study II, who had an adequate primary tumour sample left for IHC analyses of xCT were included as illustrated in **Figure 8**.

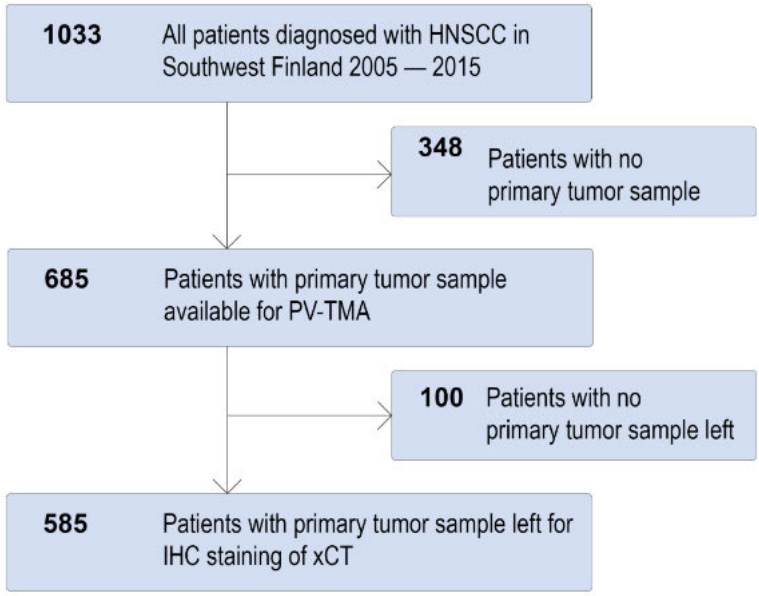

Figure 8. Patient selection for study III. Adopted from manuscript III. HNSCC, head and neck squamous cell carcinoma; PV-TMA, population-validated tissue microarray; IHC, immunohistochemistry.

4.2 Image registration for defining overlap of recurrent tumours and high-risk treatment volumes (Study I)

Eclipse 15.6 (Varian Medical Systems, Paolo Alto, CA) was used to fuse the RT treatment planning CTs with the PET/CT or PET/MRI images obtained at the time of relapse. Normal tissue regions deemed stable were utilized to achieve optimal image coregistration. Rigid registration was adequate for the paired image sets of 22 patients. Deformable image registration was necessary for the remaining 3 patients who had experienced major changes in tissue structures caused by combined modality treatment. The recurrent tumor volume was derived from the 50% SUV_{max} (maximum standardized uptake volume) threshold obtained 60 min from injection of FDG as described by Minn et al., 2010. After the recurrent tumor volume was copied on the planning CT, the dose of radiation received by the recurrent tumor volume was calculated using dose-volume histograms. Overlap volume of the recurrence and high-risk treatment volume was then determined. The high-risk treatment volume was defined as 95% isodose volume of the mean dose of the high-risk planning target volume. Delineation of high-risk treatment volume is described in more detail by Minn et al., 2010.

The recurrence volumes were classified as in-field (95% or more of recurrence volume encompassing the 95% isodose), marginal miss (20–95% of recurrence volume encompassing the 95% isodose), or true miss (less than 20% of recurrence encompassing the 95% isodose) as previously described by Dawson et al., 2000. In 5 patients more than one recurrent tumour was identified. Thereby, multiple recurrence volumes were delineated and analyzed independently.

4.3 Anti-xCT antibody validation (Study III)

4.3.1 Cell culture and RNA silencing

FaDu and Cal33 cells were a kind gift from Professor Anna Dubrovska (OncoRay–National Center for Radiation Research in Oncology, Medizinische Fakultät Dresden, Germany). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS (E.U.-approved), 1 × GlutaMAX™, 0.5% penicillin-streptomycin (10,000 U/mL), and 1 × MEM NEAA (all from Gibco™) in 5% CO_2.

FaDu and Cal33 cells (10 × 104 cells/well) were plated on 6-well plates in normal culture medium and allowed to attach overnight. Next day, cells were silenced with Accell Human xCT siRNA SMARTPool or Accell Non-targeting Control Pool (Dharmacon™ Reagents, Horizon Discovery Ltd) according to the manufacturer's protocol. Briefly, media was replaced with serum-free Accell siRNA Delivery Media

(Dharmacon™ Reagents, Horizon Discovery Ltd) with NT or xCT siRNA at a final concentration of 1 µM for 72 h. After silencing, cells were collected in RIPA Lysis and Extraction Buffer supplemented with protease and phosphatase inhibitors (all from Life Technologies Europe BV).

4.3.2 Real-time quantitative polymerase chain reaction (RT-qPCR)

Cell samples were lysed in 1:1 (v/v) RLT buffer (Qiagen) and 96% ethanol. Total RNA was isolated using the RNeasy® Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was converted into cDNA using Oligo d(T) 18 mRNA Primer (New England BioLabs), dNTP Mix, RiboLock RNAse Inhibitor, RT Buffer, and Maxima Reverse Transcriptase (all from Thermo Scientific). For RT-qPCR, 30 ng of cDNA was used with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The primers were hxCT (Linher-Melville et al., 2019) and hTBP (Menschikowski et al., 2012). Raw C_t values were normalized to the housekeeping gene (TBP) and then to the expression of NT siRNA sample using the delta-delta C_t method.

4.3.3 Western blot

Cell samples were lysed in Pierce™ RIPA Buffer (Thermo Scientific) and the protein concentration of the samples was determined with Pierce™ BCA protein assay kit (Thermo Scientific). 4X Laemmli sample buffer (BIO-RAD), 2-mercaptoethanol (Sigma® Life Science) and Pierce™ RIPA Buffer were added to protein extracts. 15 µg of proteins were loaded into precast 4–20% Mini-PROTEAN TGX gels (BIO-RAD) and electrophoresed first at 60V for 20 min, and then at 100V for 120 min. The proteins were transferred onto 0.2 µm PVDF membrane (Trans-Blot Turbo Transfer Pack, BIO-RAD) using the Trans-Blot turbo transfer system (BIO-RAD) at 1.3 A and 25 V for 7 min. The membranes were blocked at RT for 5 min in EveryBlot Blocking Buffer (BIO-RAD) and incubated overnight at 4°C with anti-xCT (1:1000; Abcam Cat# ab307601, RRID not available) or anti-xCT (1:1000; Abcam Cat# ab175186, RRID:AB_2722749) primary antibodies diluted in EveryBlot Blocking Buffer or anti-xCT (1:500; Cell Signalling Technology, Cat# 12691, RRID:AB_2687474) primary antibody diluted in 5% non-fat milk in TBS-T. Vinculin (1:1000; Sigma-Aldrich Cat# V9131, RRID:AB_477629) was used as a loading control. The membranes were washed with TBS-T (5 × 5 min) and then incubated at RT for 1 hour with IRDye® 680RD (1:2000; LI-COR Biosciences, Cat# 926-68072, RRID:AB_10953628) and IRDye® 800CW (1:2000; LI-COR Biosciences, Cat# 926-32213, RRID:AB_621848) fluorescent secondary antibodies

diluted in EveryBlot Blocking Buffer with 0.02% SDS. The membranes were washed with TBS-T (6 × 5 min) before fluorescent signal detection with LI-COR Odyssey® CLx Imaging System (LI-COR Biosciences). The fluorescent signals were analysed with Empiria Studio 2.3 software (LI-COR Biosciences) and the signal intensities were normalized to housekeeping protein.

4.4 Immunohistochemistry

4.4.1 p16 (Studies I–III)

FFPE blocks were cut into 6 µm sections. Immunohistochemical staining of p16 (Roche/Ventana clone E6H4) was performed in a Ventana Bench-Mark XT staining automate (Ventana Medical Systems, Inc., Oro Valley, AZ, USA) in the laboratory of clinical pathology. Two independent investigators analyzed the immunohistochemical staining, and differences were conferred until consensus was reached. p16 immunostaining was graded positive if at least 70% of cells displayed strong nuclear and cytoplasmic staining intensity.

4.4.2 xCT (Study III)

FFPE samples were acquired from pathology archives through Auria Biobank. Final TMA blocks of duplicate 0.6 mm cores were made in TMA Grand Master (3D Histech) according to annotations on scanned HE slides. Samples of normal liver were included in each block for orientation.

xCT expression was determined by immunohistochemical staining with a recombinant monoclonal rabbit antibody (1:3000 dilution, catalogue no. ab307601, Abcam). Two independent authors (S.T. and L.N.) analysed the immunohistochemical staining, and differences were conferred until consensus was reached. xCT expression was scored in a semiquantitative manner based on the intensity of the staining on a scale of 0–3. Dichotomous cutoffs were applied for statistical analysis.

4.5 Statistical analyses

All statistical analyses were conducted using SPSS 27 software (SPSS, IBM, Armonk, NY, USA). *p* values less than 0.05 were considered to indicate statistical significance**.**

4.5.1 Study I

Overall survival and disease-free survival were plotted using Kaplan-Meier method.

4.5.2 Study II

Overall survival, disease-specific, and disease-free survival curves were plotted using the Kaplan-Meier method. The log rank test was run to determine the significance of differences in survival distribution between patient groups. Binomial logistic regression was performed to ascertain the effects of age, sex, smoking, alcohol consumption, tumor site, TNM class, stage, and treatment on the likelihood of patients being included in the TMA. Two-sample t tests and chi-square tests were used to evaluate differences between p16-positive and p16-negative OPSCC patients. The multivariable Cox proportional hazards method was applied to adjust the survival effect of p16 and treatment type on age, smoking, alcohol use, T-class, and nodal positivity. Hazard ratios (HR) with 95% confidence intervals (CIs) and p values were reported.

4.5.3 Study III

Logistic regression analysis was used to evaluate differences in the frequency of patients with high and low xCT expression in different patient groups. Survival curves were plotted using the Kaplan–Meier method and compared using the Cox proportional hazards model, which was also applied as a uni- and multivariate analysis tool to evaluate the survival effect of xCT and p16 in OPSCC patients. Backward stepwise regression, including all variables in Table 1 of the original article (except for site, treatment and sex), using 3-year DSS, the likelihood method, and exclusion p -value of 0.10 was used to identify variables included in the multivariate model. HRs with 95% CIs and p values were reported.

4.6 Ethical considerations

All studies were approved by Regional Ethics Committee of Turku University and conducted following the rules of the Declaration of Helsinki of 1975, revised in 2013. The use of human tissue samples in study I was approved by National Supervisory Authority for Welfare and Health (V47408/4017 and V47856/2018) and Auria Biobank scientific board (AB17-8403). For studies II and III, use of human tissue was approved by National Supervisory Authority for Welfare and Health (V/39706/2019) and Auria Biobank scientific board (AB19-6863).

5 Results

5.1 Study I

5.1.1 Survival and patterns of recurrence according to p16 status

Out of all 508 patients diagnosed with HNSCC in 2010–2015 in Southwestern Finland, 25% (n=127) had locoregional recurrence which was defined as locoregional relapse at least three months after completing cisplatin- or cetuximab-enhanced RT for the primary tumour. The p16 status was known for 72 patients. The 5-year OAS rates were 47% and 71%, and the 5-year DFS rates were 48% and 82% in p16-negative and p16-positive patients, respectively.

Altogether 25 patients fulfilled all inclusion criteria for further evaluation of locoregional recurrence. In these 25 patients, a total of 31 locoregional recurrences were detected. The number of locoregional recurrent tumours per patient at the timepoint when recurrence was first detected was one in 21, two in 3 and four in 1 patient, respectively. Eighteen patients (72%) had local recurrence only and two patients (8%) had neck recurrence only. The remaining five patients (20%) had a combination of local and neck recurrences, and distant metastases as shown in **Figure 9**. Out of all 31 recurrent tumours, 7 (23%) were detected in patients whose primary tumour was p16-positive and 24 (77%) were detected in patients whose primary tumour was p16-negative. No relapses in neck were found among originally p16 positive patients.

The median time from end of RT to the PET/CT or PET/MRI scan diagnostic for local or locoregional recurrence was 9 months for p16-negative patients and 14 months for p16-positive patients. The median time for p16-negative in-field recurrences was 6 months compared to that of 9 months for p16-positive in-field recurrences.

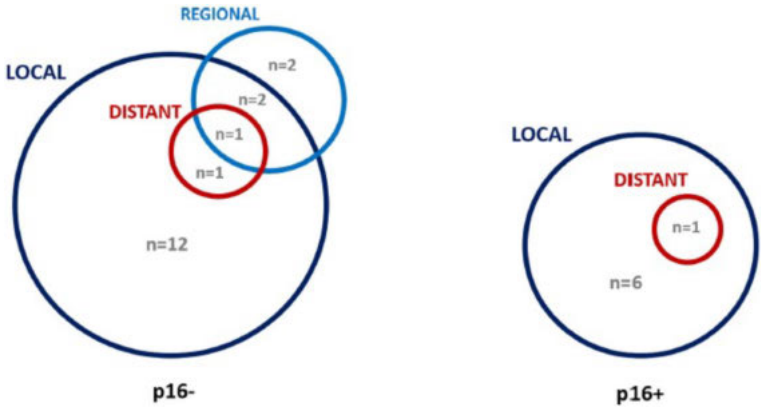

Figure 9. Breakdown of recurrence combinations in 25 patients according to p16 status of the primary tumour. None of the p16-positive cases relapsed in the neck. Adopted from publication I under the Creative Commons CC-BY-NC-ND license.

5.1.2 Recurrence in relation to high-risk treatment volume

Among 31 locoregional recurrences, 14 (45%) were classified as in-field, 5 (16%) as a marginal miss, and 12 (39%) as a true miss. As per calculations based on co-registered hybrid PET-scans and treatment planning CTs, mean radiation doses previously received by the volume of recurrence were estimated to be 67 Gy (range 62–71 Gy); 61 Gy (range 58–66); and 47 Gy (16–56 Gy), for in-field, marginal, and true miss recurrences, respectively. Among patients with a p16-positive primary tumour 4 in-field, 2 marginal, and 1 true miss recurrences were found. The recurrent tumour classified as true miss was found to be p16-negative in contrast to the original tumour. In contrast, p16-negative patients had 10 in-field, 3 marginal, and 11 true miss recurrences. The mean ± 95% confidence intervals for overlap percentages of high-risk treatment volume and recurrence volume were 58 (16–100)% in p16-positive and 52 (33–72)% in p16-negative patients.

5.1.3 Recurrence in relation to treatment modality and characteristics

Out of 14 in-field recurrences 7 had received definitive CRT, 2 had received preoperative CRT, and 5 had received postoperative CRT. Proportions of different primary treatments in each recurrence class are shown in **Table 8**. There was no clear association between modality of primary treatment (definitive or adjuvant CRT) and recurrence class. Twenty-three patients received weekly low dose (40 mg/m^2) cisplatin and two patients who could not receive cisplatin because of intercurrent morbidity received weekly cetuximab (250 mg/m^2). Median cumulative dose of cisplatin was 240 mg/m^2 (range, 120–240). The cumulative dose was 1900 mg/m^2 for both patients who received cetuximab. One patient who received cetuximab had a p16-positive primary tumour and the other one's primary

tumour was p16-negative. The median number of RT fractions was 33 (range 30–35). Median number of fractions did not differ between p16 subgroups. Median duration of RT was 46 days (range 43–53) in the p16-positive group and 48 days (range 43–60) in the p16-negative group. **Figure 10** presents an example of a patient who had a p16-positive primary tumour and an in-field recurrence afterwards.

Table 8. Primary tumour treatment modalities and recurrence classes of 31 recurrent tumours that occurred in 25 patients. Modified from publication I.

	In-field		Marginal miss		True miss	
Treatment	N	row %	N	row %	N	row %
Definitive crt	7	58%	3	25%	2	17
Preoperative crt	2	67%	1	33%	0	0%
Postoperative crt	5	33%	2	13%	8	53%

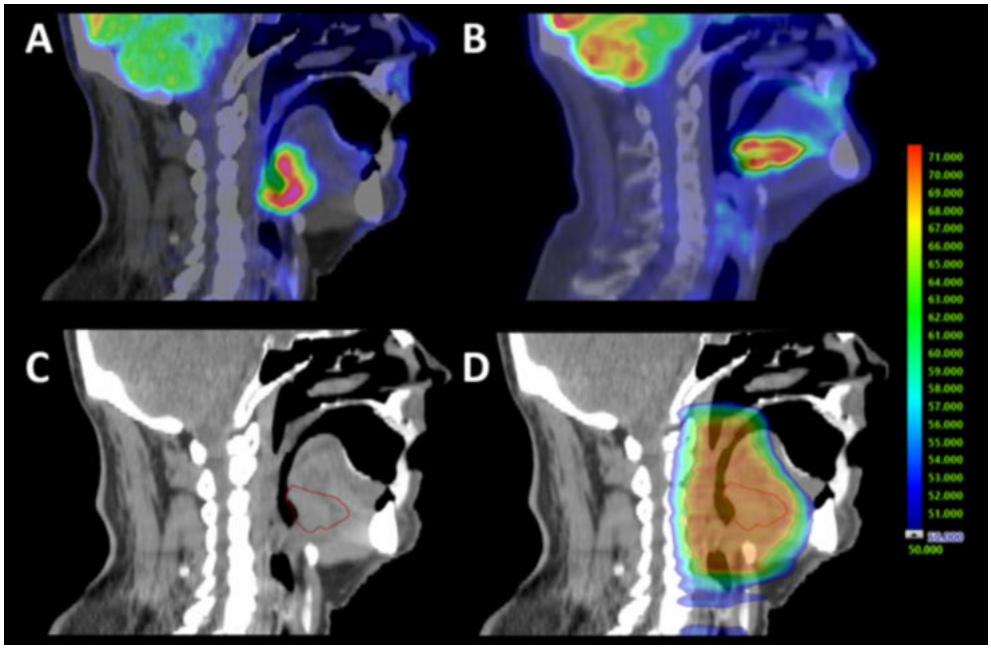

Figure 10. Image **A**: A 63-year non-smoking and abstinent man presented with a large left side tonsillar squamous cell carcinoma without cervical lymph node involvement that was p16-positive and confirmed HPV16-positive by DNA polymerase chain reaction (PCR). On audiometry, bilateral high frequency hearing loss as found. The patient received therefore cetuximab-enhanced radiotherapy (RT) to 70 Gy in the high-risk area. Two years after the end of the bio-RT, a recurrence in the base of tongue (**B**) and multiple lung metastases were detected in positron emission tomography (PET) combined to computed tomography (CT). Image **C** illustrates the local recurrence in PET (red contour). Image **D** presents the recurrent tumour superimposed on the treatment planning CT with dose wash locating the recurrent tumour within the original high-risk treatment volume (dark red contour). This recurrent tumour was classified as an in-field recurrence. Please note the different position of the mobile tongue in image **B** because of the use of mouthpiece during RT. Adopted from publication I under the Creative Commons CC-BY-NC-ND license.

5.2 Study II

5.2.1 Epidemiology of HNSCC in Southwest Finland 2005–2015

The population of Southwest Finland increased from 683,000 to 697,000 during 2005–2015. (*Statistical Databases: Statistics Finland*). The annual HNSCC incidence rates per 100,000 population varied between 10.05 (2008) and 16.72 (2013). The absolute number of newly diagnosed HNSCC cases in 2015 (n = 109) was 34.6% higher than that in 2005 (n = 81), as shown in **Figure 11**. Among the 1033 HNSCC patients, the median age of patients with a newly diagnosed HNSCC was 65 (range 23–95). Descriptive statistics of patient characteristics are shown in **Table 9**. In addition, the survival effect of selected variables was calculated and reported based on a prognostic model previously described by Denissoff et al., 2022. The mean and median follow-up times were 38.6 and 49.3 months, respectively. The five-year OAS, DSS, and DFS were 55.1%, 69.4%, and 60.6%, respectively. Lymph node metastases were detected in 38.2% (n = 395) of HNSCC patients.

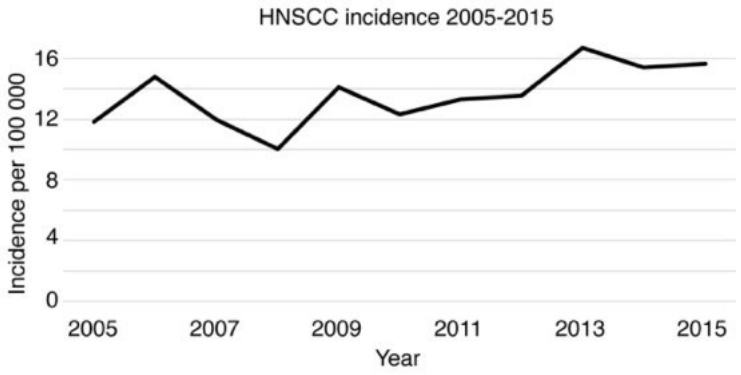

Figure 11. Incidence of HNSCC in Southwestern Finland between 2005 and 2015. Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

Regarding cancer therapy, the majority of HNSCC patients received either surgery only (35%) or combined treatment, including surgery and RT or surgery and CRT (36%). The remaining HNSCC patients received either definitive RT (8.2%), CRT (15.4%), or palliative therapy (5.3%).

Table 9 Characteristics of patients diagnosed with a new head and neck squamous cell carcinoma in Southwestern Finland between 2005–2015. Survival effects were analysed using a multivariable Cox proportional hazards model. Results include hazard ratios (HR), 95% confidence intervals (CI), and p-values. Tobacco use was defined as daily smoking at time of diagnosis. Alcohol use was defined as 10 doses or more a week at time of diagnosis. RT, radiotherapy; CRT, chemoradiotherapy. Adopted from publication II, under the Creative Commons CC BY 4.0 license.

Characteristic	n	%	Survival effect HR (95% CI)	*p*-value
Gender			not included	
Male	679	65.7		
Female	354	34.3		
Age			1.03 (1.02–1.05) /year	< 0.001
< 65	487	47.1		
> 65	546	52.9		
Tumor site			not included	
Oral cavity	505	48.9		
Oropharynx	193	18.7		
Larynx	184	17.8		
Hypopharynx	40	3.9		
Other	111	10.8		
T-class				
T1–2	676	65.4	1	-
T3–4	357	34.6	2.45 (1.84–3.24)	< 0.001
N-class				
N0	638	61.8	1	-
N+	395	38.2	1.79 (1.33–2.40)	< 0.001
Stage			not included	
0–II	481	46.6		
III–IV	552	53.4		
Gradus			not included	
G1	321	32.9		
G2	435	44.6		
G3	219	22.5		
Treatment			not included	
Surgery only	362	35.0		
Definitive RT	85	8.2		
Definitive CRT	159	15.4		
Surgery + CRT/RT	372	36.0		
Palliative treatment	55	5.3		
Tobacco use				
No	570	55.2	1	-
Yes	463	44.8	1.46 (1.07–1.99)	0.017
Alcohol use				
No	784	75.9	1	-
Yes	249	24.1	1.36 (1.00–1.85)	0.047

Patients who received RT or CRT either as definitive or adjuvant treatment were included for further analysis along with patients who were treated by surgery only. Patients in the RT group had worse OAS than patients treated by CRT (HR 0.57;

95% CI 0.41–0.81; p value 0.002) and surgery only (HR 0.63; 95% CI 0.43–0.93; p value 0.019). The survival effects were analysed using a multivariate Cox proportional hazards model adjusting for age, T class, nodal positivity, and consumption of alcohol and tobacco.

The most common tumour site was the oral cavity (n = 505), followed by cancers of the oropharynx (n = 193). The absolute number of patients diagnosed with squamous cell carcinoma of the oral cavity (OCSCC) in 2015 (n = 59) was 28.3% higher than that in 2005 (n = 46). Furthermore, the number of oropharyngeal SCC (OPSCC) patients rose by 100.0% during the same period (n = 11 in 2005 and n = 22 in 2015). Meanwhile, the number of patients with a new SCC of the larynx (LSCC) was 42.1% lower in 2015 (n = 11) than in 2005 (n = 19).

The existence of known risk factors, such as age, sex, and consumption of tobacco and alcohol, was also analysed. The site-specific presence of risk factors is presented in **Table 10**. A heavy smoking history of twenty or more pack years (PYs) was most common in patients with LSCC (83.2%, n = 153/184) and hypopharyngeal cancer (HPSCC, 72.5%, n = 29/40). Additionally, current use of alcohol was most common in patients with HPSCC (37.5%, n = 15/40). The association with male sex was notable in LSCC and OPSCC, with 86.4% (n = 159/184) and 77.8% (n = 150/193) of patients being males, respectively. Patients with SCC of the oropharynx were remarkably younger by the time of diagnosis compared to other locations, with only 31.6% (n = 61/193) of OPSCC patients being 65 years or older. On the other hand, 61.8% (n = 312/505) and 60.0% (n = 24/40) of patients with SCC of oral cavity and hypopharynx, respectively, were over 65 years old at the time of HNSCC diagnosis.

Table 10. Presence of known risk factors in different primary tumor sites of the head and neck region. Pack year (PY). Alcohol use was defined as 10 doses or more a week at the time of diagnosis. Adopted from publication II, under the Creative Commons CC BY 4.0 li-cense.

	Smoking ≥ 20 PY (%)	Alcohol use (%)	Gender Male (%)	Age ≥ 65 years (%)
Oral cavity	40.2	18.0	52.5	61.8
Oropharynx	63.2	32.1	77.8	31.6
Larynx	83.2	32.1	86.4	48.9
Hypopharynx	72.5	37.5	65.0	60.0
Other	38.7	19.8	71.2	53.2

5.2.2 Epidemiology of oropharyngeal squamous cell carcinoma

The site-specific and locoregional analyses showed that the highest increase in new HNSCCs from 2005 to 2015 existed in OPSCC. The HNSCC cohort included 193

OPSCC patients, of whom 77.8% (n = 150) were males. The median age of OPSCC patients was 60 years, and 63.2% (n = 122) had a smoking history of at least 20 PY. Moreover, 47.7% (n = 92) of the patients were previous alcohol consumers, and 32.1% (n = 62) were current consumers. The incidence of OPSCC varied from 1.46 (2007) to 3.74 (2014) per 100,000. The annual OPSCC incidence during 2005–2015 is illustrated in **Figure 12**.

In the OPSCC cancer treatment algorithm, RT and CRT are the main treatment options. Of 193 OPSCC patients, 7.8% (n = 15) received definitive RT, and 23.3% (n = 45) received definitive CRT as treatment. Most patients (52.8%, n = 102) were treated with a combination of surgery and CRT, while a minority (3.6%, n = 7) of patients received a combination of surgery and RT. In addition, the proportion of patients treated by surgery only was 3.6% (n = 7). Palliative care or no treatment at all was offered to 8.8% (n = 17) of patients.

The five-year OAS, DSS, and DFS of patients with OPSCC were 54.4%, 66.3%, and 61.7%, respectively. Treatment outcomes were compared between CRT (n = 147) and RT (n = 22) treated patients. Patients who received RT or CRT as an adjuvant treatment were included along with patients who received definitive RT or CRT. Patients treated with CRT had better OS ($p < 0.001$), DSS ($p = 0.002$), and DFS ($p = 0.008$). OS was 63.9% in the CRT group and 31.8% in the RT group, DSS was 75.5% in the CRT group and 50.0% in the RT group, and DFS was 71.4% in the CRT group and 50.0% in the RT group. The group of patients treated by surgery only was not analysed due to its small size.

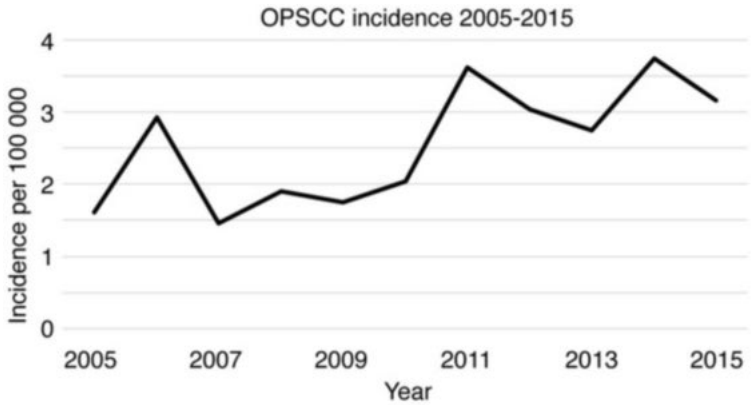

Figure 12. Incidence of oropharyngeal squamous cell carcinoma (OPSCC) in Southwestern Finland in 2005–2015. Adopted from publication II, under the Creative Commons CC BY 4.0 li-cense.

5.2.3 Establishment of a population-validated tissue microarray

Of 1033 patients, 685 (66.3%) had a tumour sample available for the population-validated TMA (PVTMA). Clinical data of TMA patients were compared to the background population of all HNSCC patients treated in the Southwest Finland region from 2005–2015. The representativeness of the TMA cohort against the background HNSCC population is presented in **Table 12**. The established TMA was confirmed to be representative in terms of age, sex, alcohol and tobacco consumption, T-class, and stage, while an uneven distribution of N-class was observed. However, the difference in patients presenting with nodal metastasis was rather modest (38% in background population vs. 43% in PVTMA).

Table 11. Univariate (left panel) and multivariate (right panel) analyses of tissue microarray (TMA) inclusion bias, including odds ratios (ORs), 95% confidence intervals (CIs), and *p* values. Results from binomial logistic modelling. Alcohol use was defined as 10 doses or more per week at the time of diagnosis. Adopted from publication II under the Creative Commons CC BY 4.0 license.

	Total		TMA		TMA inclusion		TMA inclusion	
	N	%	N	%	OR (95 % CI)	*P*	OR (95 % CI)	*P*
Gender								
Male	679	66	438	64	0.79 (0.60–1.04)	0.089	0.77 (0.56–1.05)	0.103
Female	354	34	247	36	1	-	1	-
Age								
< 65	487	47	334	49	1.21 (0.94–1.57)	0.145	1.02 (0.75–1.38)	0.904
≥ 65	546	53	351	51	1	-	1	-
Smoker								
< 20 pack years	483	47	311	45	0.85 (0.66–1.10)	0.221	0.86 (0.62–1.18)	0.340
≥ 20 pack years	550	53	374	55	1	-	1	-
Alcohol								
No	784	76	513	75	0.85 (0.62–1.15)	0.290	0.97 (0.68–1.39)	0.877
Yes	249	24	172	25	1	-	1	-
Tumor site								
Oral cavity	505	49	352	51	1	-	1	-
Oropharynx	193	19	146	21	1.35 (0.92–1.97)	0.121	0.94 (0.60–1.49)	0.795
Larynx	184	18	109	16	0.63 (0.45–0.90)	0.010	0.69 (0.44–1.07)	0.097
Hypopharynx	40	4	30	4	1.30 (0.62–2.73)	0.482	0.92 (0.41–2.06)	0.836
Other sites	111	10	48	7	0.33 (0.22–0.50)	<0.001	0.23 (0.14–0.38)	<0.001

	Total N	%	TMA N	%	TMA inclusion OR (95 % CI)	*P*	TMA inclusion OR (95 % CI)	*P*
T-class								
T1–2	676	64	428	62	0.67 (0.51–0.89)	0.005	0.85 (0.51–1.39)	0.510
T3–4	357	36	257	38	1	-	1	-
N-class								
N0	638	62	387	57	0.50 (0.38–0.66)	<0.001	0.49 (0.30–0.81)	0.005
N+	395	38	298	43	1	-	1	-
Stage								
I–II	481	47	287	42	0.57 (0.44–0.74)	<0.001	0.94 (0.48–1.82)	0.853
III–IV	552	53	398	58	1	-	1	-
Treatment								
Surgery only	362	35	220	32	1	-	1	-
RT	85	8	45	7	0.73 (0.45–1.17)	0.187	0.66 (0.38–1.16)	0.146
CRT	159	15	108	16	1.37 (0.92–2.03)	0.120	1.25 (0.74–2.13)	0.408
RT + surgery	85	8	66	10	2.24 (1.29–3.90)	0.004	2.10 (1.17–3.78)	0.013
CRT + surgery	287	28	209	30	1.73 (1.24–2.42)	0.001	1.15 (0.73–1.38)	0.559
Palliative	55	5	37	5	1.33 (0.73–2.42)	0.357	0.98 (0.48–1.99)	0.951

Most importantly, there was no statistically significant difference in OS (p = 0.200), DSS (p = 0.146), or DFS (p = 0.125) between the PVTMA and background HNSCC populations **Figure 13**. Thus, the established PVTMA can be considered to represent HNSCC patients treated in the Southwest Finland region from 2005–2015.

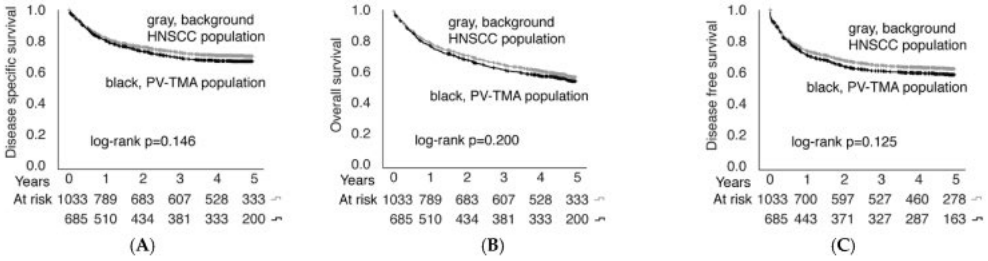

Figure 13. Overall survival (**A**), disease-specific survival (**B**) and disease-free survival (**C**) comparison between the population-validated tissue microarray (PV-TMA) and the background cohort. Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

5.2.4 p16 analyses

In p16 immunohistochemical staining, 593 of 685 patients in the PVTMA cohort had analysable p16 staining. Representative examples of immunohistochemical staining are presented in **Figure 14**. A total of 493 patients (83.1%) had p16-negative disease, while 100 patients (16.9%) had p16-positive disease. Of these 100 p16-positive diseases, 72 originated in the oropharynx. **Table 12** depicts the p16 results by primary tumour site. Notably, p16-positivity was very low in non-OPSCC regions.

Table 12. p16 staining results by primary tumour location. The group named "other" comprised of tumours of the nasal cavity, nasopharynx, sinuses, and tumours of unknown primary location. Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

	P16+		P16-	
Site	**N**	**row %**	**N**	**row %**
Oral cavity	14	5	278	95
Oropharynx	72	53	64	47
Larynx	5	5	89	95
Hypopharynx	1	4	24	96
Other	8	17	38	83

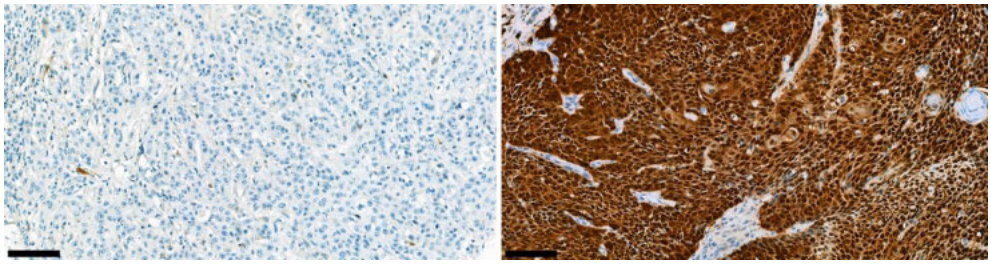

Figure 14. Representative immunohistochemical figures of negative (left) and positive (right) p16 staining. Black scale bars: 100 µm. Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

Regarding OPSCC patients, 52.9% (n = 72) were p16-positive, and 47.1% (n = 64) were p16-negative. In terms of sex, 55.2% of males and 45.2% of females were p16-positive. Furthermore, 80.6% of all p16-positive OPSCC patients were males. The median age of p16-positive patients was 60 years, while the median age of p16-negative patients was slightly higher, 62.5 years at the time of diagnosis. However, the difference was not statistically significant (p = 0.125) in this subgroup of the cohort. p16-positive diseases were more likely to spread to lymph nodes than their p16-negative counterparts (p = 0.032). However, T classification was lower in p16-positive primary tumours (p = 0.031).

In p16-positive OPSCC patients, smoking history was markedly rare ($p < 0.001$), as only 40.3% ($n = 29/72$) had a smoking history of 20 PY or more in comparison to 85.9% ($n = 55/64$) of p16-negative patients. In addition, p16-positive patients used less alcohol, with 19.4% ($n = 14/72$) being current and 29.2% ($n = 21/72$) being former consumers of alcohol. In this context, alcohol consumption was defined as 10 or more doses a week. In p16-negative patients, 45.3% ($n = 29/64$) had current and 65.6% ($n = 42/64$) had previous alcohol consumption. p values reached <0.001 in terms of both current and previous alcohol consumption. The incidence of p16-positive OPSCCs varied between 0.29 (2007) and 1.88 (2011) per 100,000 population. The absolute number of newly diagnosed annual p16-positive OPSCCs rose from 11 patients in 2005 to 22 patients in 2015, as illustrated in **Figure 16**. In OPSCC, p16-positivity in OPSCC was associated with better OS ($p < 0.001$), DSS ($p < 0.001$), and DFS ($p < 0.001$), as shown in **Figure 15**.

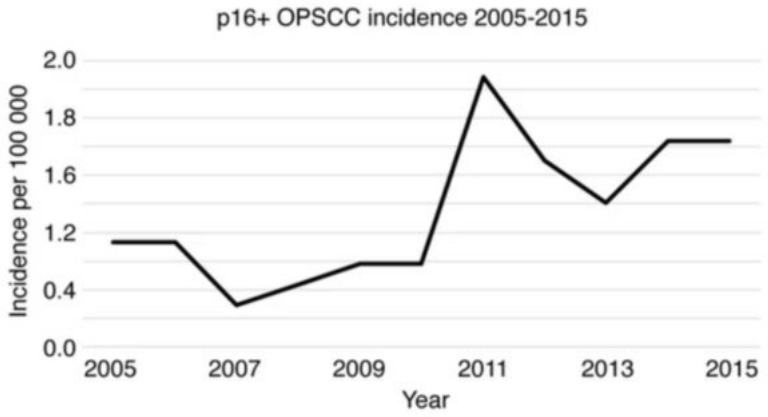

Figure 15. Incidence of p16-positive oropharyngeal squamous cell carcinoma (OPSCC) in the population-validated TMA in 2005–2015. Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

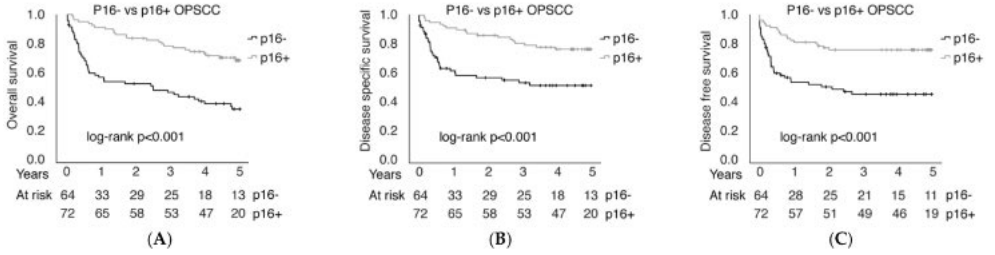

Figure 16. Overall survival (**A**), disease-specific survival (**B**) and disease-free survival (**C**) comparison between p16-neagtive and p16-positive oropharyngeal squamous cell carcinoma (OPSCC). Adopted from Publication II, under the Creative Commons CC BY 4.0 license.

In other SCCs, excluding tumours of the oropharynx, p16 positivity did not correlate with better OS (p = 0.264) or DSS (p = 0.095). However, p16-positive patients with tumours in sites other than the oropharynx had better DFS (p = 0.031). Comparing RT to CRT treatments, both p16-positive and p16-negative patients benefited from combining chemotherapy with RT, as OS was better in the CRT cohort vs. the RT group in both p16-positive (71.1% vs. 57.1%) and p16-negative (46.2% vs. 7.7%) patients. However, the difference reached statistical significance only in p16-negative OPSCC (p = 0.009 in p16-negative and p = 0.237 in p16-positive patients).

To conclude, p16-positive patients had better OS, regardless of treatment modality (HR 0.64; 95% CI 0.43–0.95; p value 0.028). The survival effect was analysed using a multivariable Cox proportional hazards model adjusting for T-class, nodal positivity, consumption of alcohol and tobacco, patient age, and treatment type.

5.3 Study III

5.3.1 Anti-xCT antibody validation

The RNA silencing (siRNA) technique was utilized to evaluate the specificity of three commercial anti-xCT antibodies (ab307601, ab175186, and CST #12691) in two different HNSCC cell lines, FaDu and Cal33. Using ab307601, successful xCT silencing was confirmed by RT–qPCR, as xCT mRNA expression was reduced by approximately 80% in Cal33 cells and by 58% in FaDu cells compared to that in the corresponding non-targeting (NT) siRNA cells (**Figures 17A-B)**. Western blotting revealed, a clear siRNA-mediated downregulation of xCT protein expression in both FaDu and Cal33 cells (**Figure 17C).** No effect on protein expression was detected in cells treated with nontargeting xCT siRNA, demonstrating that ab307601 is specific for xCT detection. Two additional xCT antibodies were unable to show specificity against the xCT protein as demonstrated in **Figure 18**. As the ab307601 antibody was the only one showing specificity against xCT protein, it was selected for immunohistochemical stainings. Representative examples of the staining results are presented in **Figure 17E**.

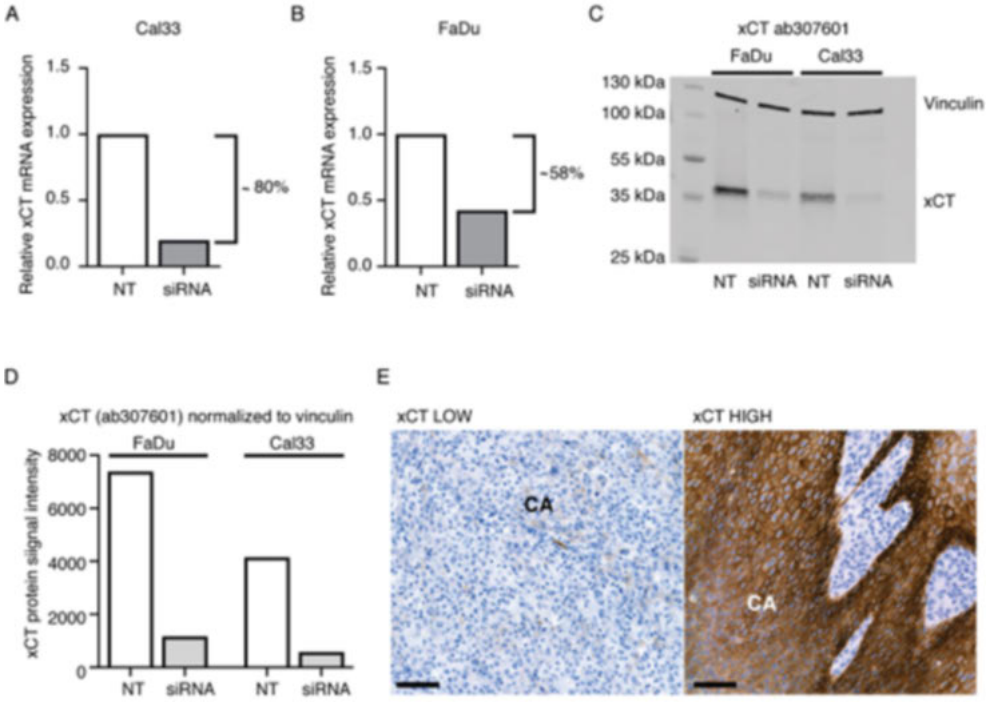

Figure 17. Validation of the anti-xCT antibody. Specificity of the ab307601 was tested using the siRNA knockdown method followed by real-time quantitative PCR (RT-qPCR). Two head and neck squamous cell carcinoma (HNSCC) cell lines, Cal33 and FaDu were used. RNA silencing resulted in downregulation of xCT both at mRNA level (**A** and **B**) and in protein signal intensity (**C** and **D**) in cells treated with xCT-targeting siRNA in relation to cells treated with non-targeting siRNA (NT-siRNA). Downregulation of xCT in siRNA lanes 2 and 4 (**C**) demonstrates the specificity of ab307601. Changes in xCT expression were calculated using the delta-delta Ct method. **E** and **F** represent xCT immunohistochemistry (IHC) stainings with ab307601 antibody. Staining intensities were classified as low and high, respectively. Black scale bars: 50 µm. CA: carcinoma. Adopted from manuscript III.

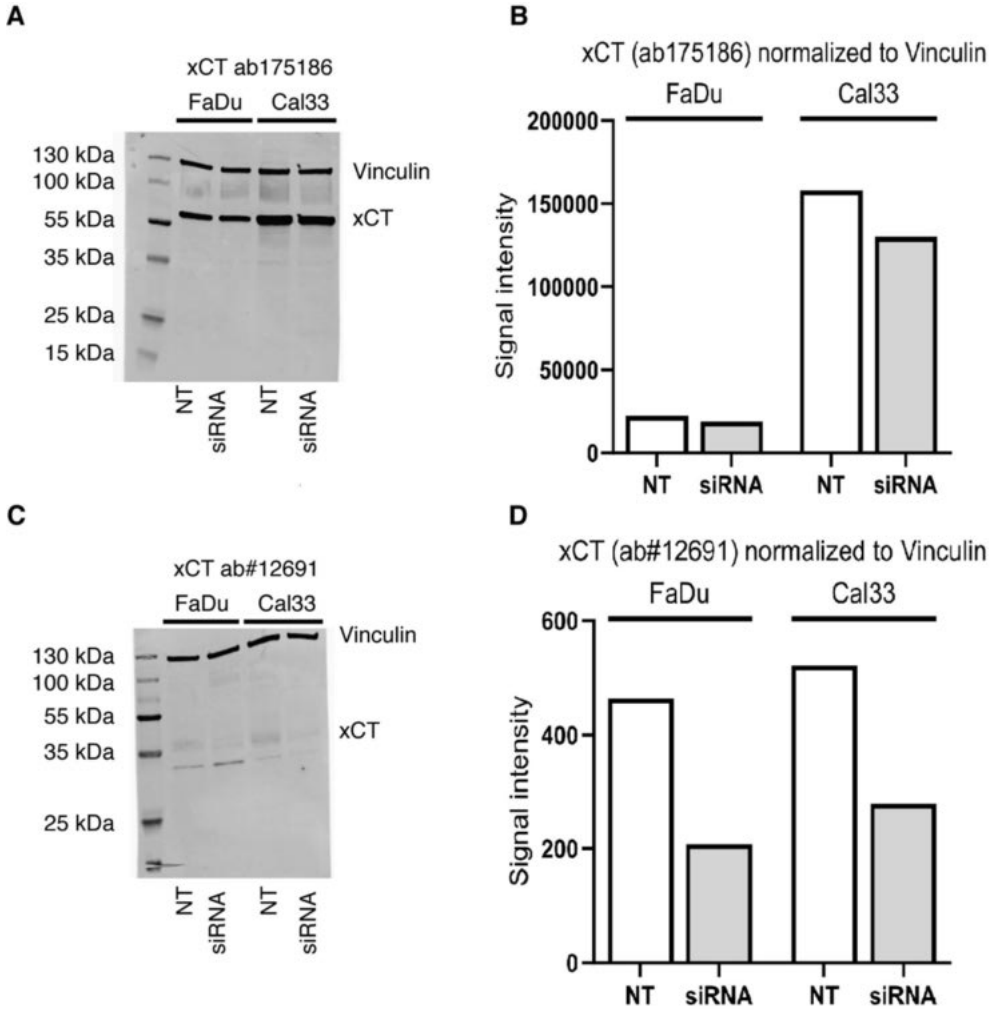

Figure 18. Evaluation of the specificity of two other anti-xCT antibodies. Ab175186 and CST #12691 were tested using the siRNA knockdown method. Two head and neck squamous cell carcinoma (HNSCC) cell lines, FaDu and Cal33 were used. **A** and **C**: Non-targeting (NT) siRNA samples of FaDu and Cal33 were loaded in lanes 1 and 3. xCT-targeting siRNA samples were loaded in lanes 2 and 4, respectively. The ab175186 antibody was unable to distinguish xCT siRNA cells from non-targeting siRNA cells (**B**). The CST #12691 antibody showed almost no signal for xCT in Cal33 cells, whereas the xCT signal was even stronger in xCT-targeting siRNA cells compared to non-targeting siRNA in FaDu cells (**D**). Adopted from manuscript III.

5.3.2 Patient characteristics

The characteristics of the patients are presented in **Table 13**. The median age at the time of diagnosis was 65 (range 27–95). Most patients received surgery only (32.5%; n=190) or combined treatment modalities (35.9%; n=210), including surgery and

chemoradiotherapy (CRT) or radiotherapy (RT). The remaining patients received definitive CRT (17.9%; n=105), definitive RT (7.5%; n=44), or palliative treatment (6.2%; n=36). During follow-up (median 57 months; range 1–144 months), 242 patients (41.4%) experienced disease recurrence. Of the 585 patients, 32.1% (n=188) died from HNSCC, and 20.0% (n=117) died from comorbidities.

5.3.3 Expression of xCT in HNSCC subsites and association to clinicopathological features

High expression levels of xCT were observed in 44.3% (n=259) and low levels in 55.7% (n=326) of the patients. The proportion of tumours with high xCT expression was greatest in the larynx (52.9%; n=46/87), followed by the hypopharynx (52.0%; n=13/25), the oral cavity (46.3%; n=133/287), and the oropharynx (38.4%; n=53/138). The associations of xCT expression with clinicopathological features is shown in Table 1. A high T-classification (T3–4) of the primary tumour, daily smoking, and alcohol use of more than 10 units per week at the time of HNSCC diagnosis were associated with high xCT expression. No significant differences were found between xCT expression and age, sex, or cervical lymph node metastasis.

5.3.4 xCT as a prognostic biomarker in HNSCC

To evaluate whether xCT is a prognostic factor for HNSCC, survival estimates were plotted. The five-year OAS, DSS, and DFS of the whole patient cohort were 52.8%, 68.7%, and 59.3%, respectively. xCT was not associated with significantly worse 5-year survival (OAS: HR 1.24; 95% CI 0.98–1.57; p=0.075. DSS: HR1.20; 95% CI 0.90–1.60; p=0.221. DFS: HR 1.23; 95% CI 0.96–1.59; p=0.105) when all patients were included.

Thereafter, site-specific analyses were performed for oral cavity, oropharynx, and larynx. As shown in **Figure 19**, the most significant association with xCT expression and survival was detected in OPSCC patients, in which high xCT was significantly associated with worse OAS (HR 2.71; 95% CI 1.67–4.39; p <0.001), DSS (HR: 2.58; 95% CI 1.47–4.54; p = 0.001), and DFS (HR: 2.69; 95% CI 1.55–4.64; p <0.001).

Table 13. Relationship between xCT expression and clinicopathological parameters. Alcohol use was defined as 10 doses or more a week and smoking as daily smoking at the time of diagnosis. LNM, lymph node metastasis; HR, hazard ratio; CI, confidence interval; CUP, cancer of unknown primary. *Sinonasal areas including the nasopharynx. **Surgery and CRT or RT. Adapted from manuscript III.

	Total		**Low xCT**		**High xCT**		**Logistic regression**	
	n	%	n	%	n	%	HR (95% CI)	***p* value**
Age								
< 65	273	46.7	153	46.9	120	46.3	1	-
≥ 65	312	53.3	173	53.1	139	53.7	1.02 (0.74–1.42)	**0.801**
Sex								
Female	210	35.9	121	37.1	89	34.4	1	-
Male	375	64.1	205	62.9	170	65.6	1.13 (0.80–1.59)	**0.491**
T class								
T1–2	366	62.8	217	66.8	149	57.8	1	-
T3–4	217	37.2	108	33.2	109	42.2	1.47 (1.05–2.06)	**0.026**
LNM								
N0	326	55.7	173	53.1	153	59.1	1	-
N+	259	44.3	153	46.9	106	40.9	0.78 (0.56–1.09)	**0.147**
Smoking								
No	299	52.0	196	60.7	103	40.4	1	-
Yes	276	48.0	128	39.3	148	59.6	2.28 (1.63–3.19)	**<0.001**
Alcohol use								
No	423	74.0	254	78.6	169	67.9	1	-
Yes	149	26.0	169	21.4	80	32.1	1.74 (1.20–2.54)	**0.004**
Site								
Oral cavity	287	49.1	154	47.2	133	51.4	1	**-**
Oropharynx	138	23.6	85	26.1	53	20.5	0.72 (0.48–1.09)	**0.123**
Larynx	87	14.9	41	12.6	46	17.8	1.30 (0.80–2.10)	**0.286**
Hypopharynx	25	4.3	12	3.7	13	5.0	1.25 (0.55–2.84)	**0.587**
Sinonasal areas*	32	5.5	19	5.8	13	5.0	0.79 (0.38–1.67)	**0.539**
CUP	16	2.7	15	4.6	1	0.4	0.08 (0.01–0.60)	**0.014**
TREATMENT								
SURGERY	190	32.5	103	31.6	87	33.6	1	**-**
CRT	105	17.9	59	18.1	46	17.8	0.92 (0.57–1.49)	**0.744**
RT	44	7.5	25	7.7	19	7.3	0.90 (0.46–1.74)	**0.754**
Combined**	210	35.9	123	37.7	87	33.6	0.84 (0.56–1.24)	**0.380**
Palliative	36	6.2	16	4.9	20	7.7	1.48 (0.72–3.03)	**0.284**
p16								
Positive	98	17.2	79	24.9	19	7.5	0.24 (0.14–0.42)	**<0.001**
Negative	473	82.8	238	75.1	235	92.5	1	-

Next, uni- and multivariate analyses were conducted to further elaborate the prognostic role of xCT. The survival effect of xCT seemed to be most present during the first three years. Thus, multivariate analyses for 3-year survival were conducted. In OPSCC, in a model adjusting for age, T-class, nodal positivity, and tobacco

consumption, high xCT was shown to be an independent prognostic factor for worse 3-year OAS, DSS, and DFS, as demonstrated in **Table 14**. Five-year survival effects of xCT on OAS (HR: 1.57; 95% CI 0.89–2.79, p=0.121) and DSS (HR: 1.78; 95% CI 0.92–3.43; p=0.085) remained nonsignificant in the multivariate model. Nevertheless, the 5-year survival effect on DFS (HR: 1.95; 95% CI 1.04–3.65; p=0.037) was statistically significant.

In oral cavity squamous cell carcinoma (OCSCC), high xCT was not a significant prognostic factor in univariate analyses, as demonstrated in Supplement Table 2 of the original article. However, in a multivariate model adjusting for age, T-class, and nodal positivity, high xCT was associated with significantly better 3-year OAS (HR: 0.57; 95% CI 0.38–0.86; p=0.007) and DFS (HR: 0.63; 95% CI 0.42–0.93; p=0.022). In laryngeal squamous cell carcinoma, xCT was not a significant factor in either uni- or multivariate analyses as shown in Supplement Table 3 of the original article.

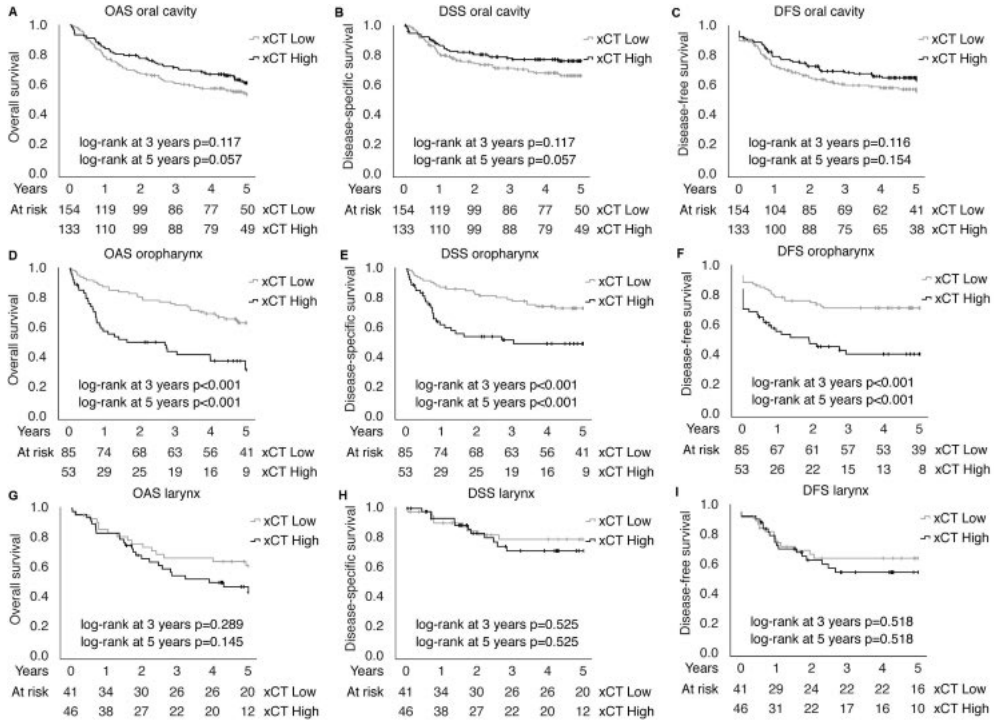

Figure 19. Site-specific survival analyses in relation to xCT expression. Prognostic trends with hazard ratios (HR) and 95% confidence intervals (CI) for survival in squamous cell carcinoma of oral cavity (**A-C**), oropharynx (**D-F**), and larynx (**G-I**). The results indicate the diverse role of xCT in tumours of different primary sites. Statistical significance was calculated using Cox proportional hazards model. OAS, overall survival; DSS, disease-specific survival; DFS, disease-free survival. Adapted from manuscript III.

Table 14. Multivariate 3-year survival analysis of oropharyngeal squamous cell carcinoma (OPSCC) patients. Hazard ratios (HRs), confidence intervals (CIs), and *p* values were reported. Tobacco use was defined as daily smoking at the time of diagnosis. OAS, overall survival; DSS, disease-specific survival; DFS, disease-free survival. Results from Cox proportional hazards model. Adapted from manuscript III.

	3-year OAS		**3-year DSS**		**3-year DFS**	
	Survival effect HR (95% CI)	*P* value	Survival effect HR (95% CI)	*P* value	Survival effect HR (95% CI)	*P* value
Age						
< 65	1	-	1	-	1	-
≥ 65	1.41 (0.81–2.46)	0.231	1.71 (0.94–3.10)	0.080	1.93 (1.12–3.31)	0.017
T-class						
T1-2	1	-	1	-	1	-
T3-4	2.21 (1.16–4.21)	0.016	2.60 (1.25–5.40)	0.011	2.13 (1.13–4.02)	0.020
N-class						
N0	1	-	1	-	1	-
N+	1.27 (0.69–2.34)	0.446	1.92 (0.93–3.98)	0.079	2.28 (1.15–4.51)	0.018
Tobacco use						
No	1	-	1	-	1	-
Yes	3.25 (1.50–7.05)	0.003	3.19 (1.38–7.38)	0.007	2.84 (1.37–5.88)	0.005
xCT						
Low	1	-	1	-	1	-
High	1.94 (1.03–3.67)	0.040	1.93 (0.98–3.80)	0.057	1.85 (1.00–3.41)	0.051

5.3.5 xCT outperforms p16 in 3-year survival prognostication

As the prognostic value of xCT was the highest in OPSCC patients, we first evaluated the benefits of combining p16 and xCT staining. p16 status was available for 99.3% (n=137/138) of OPSCCs. p16-positive tumours had remarkedly lower xCT expression (HR: 0.24; 95% CI 0.14–0.42; p<0.001) than did their p16-negative counterparts. Logistic regression was performed to evaluate the correlation between xCT and p16. The logistic regression model was significant (p<0.001), and it explained 34.2% (Nagelkerke R^2) of the variance. p16 stratification did not improve the prognostic resolution of xCT, as demonstrated in **Figure 20**.

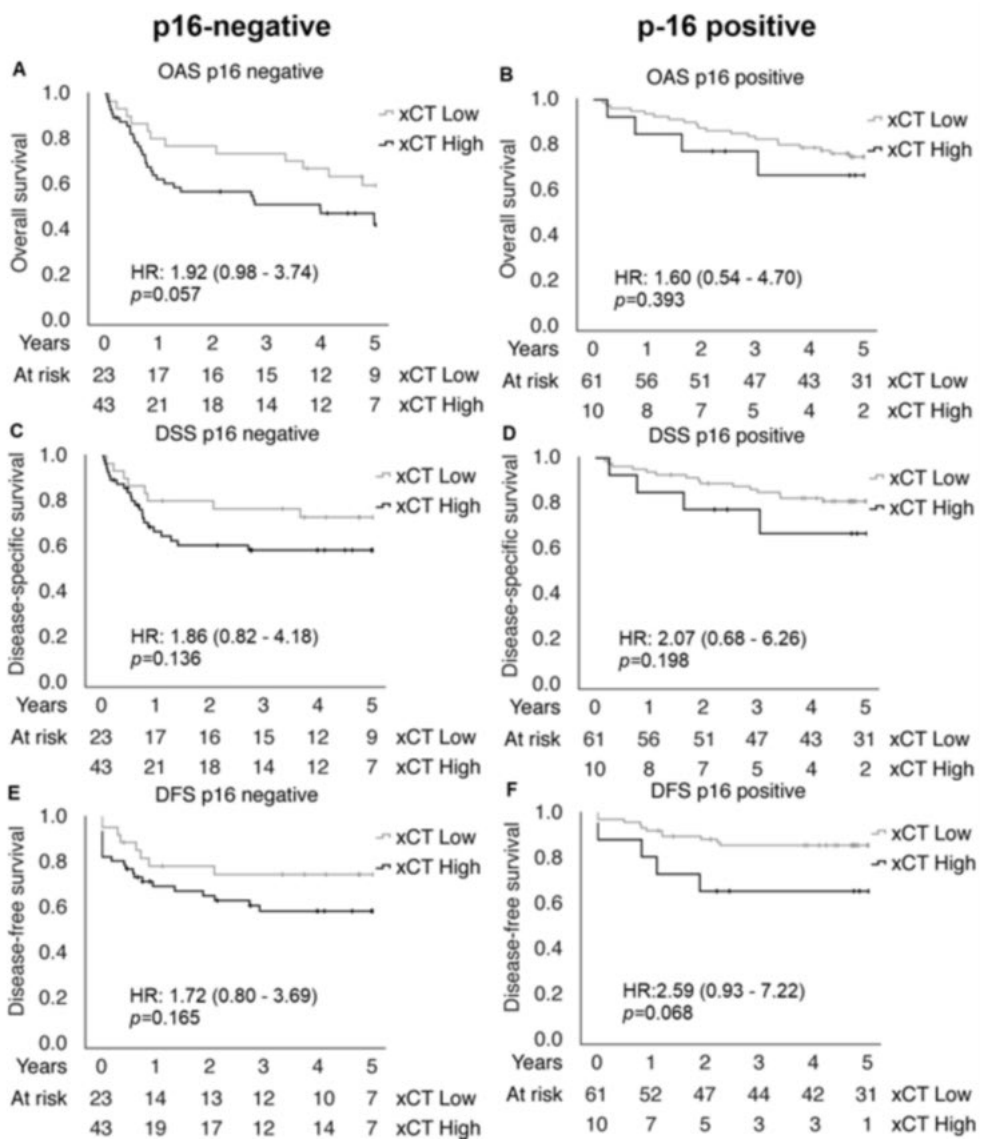

Figure 20. Prognostic trends in oropharyngeal squamous cell carcinoma according to p16 status. Statistical significance was calculated using Cox proportional hazard model. HR, hazard ratio; CI, confidence interval; OAS, overall survival; DSS, disease-free survival; DFS, disease-free survival. Adapted from manuscript III.

To evaluate whether xCT could bring additional clinical value, xCT was compared to p16, the only established biomarker in newly diagnosed OPSCC. First, the survival effects of p16 were calculated with a similar multivariate model that was utilized for xCT in the previous chapter. After adjusting for age, T class, nodal positivity, and tobacco consumption, the 3-year survival effects of p16 (OAS: HR

1.99; 95%CI 0.95–4.14; p=0.067. DSS: HR 1.85; 95%CI 0.83–4.11; p=0.131. DFS: HR 1.60; 95%CI 0.78–3.31; p=0.203), were weaker than those reported for xCT in the previous chapter.

Second, both xCT and p16 were entered into the same multivariate model, which included age, T class, nodal positivity, and tobacco consumption, using backward stepwise regression and an exclusion *p* -value of 0.10. This procedure resulted in the exclusion of p16 (OAS *p*=0.178, DSS *p*=0.290, and DFS *p*=0.489). However, xCT was included in the model (3-year OAS *p*=0.040, DSS *p*=0.057, and DFS *p*=0.052).

Finally, p16 and xCT were combined into a product variable with two categories: 1) $p16^{negative}$ and xCT^{high} or 2) any other combination. The product variable, xCT, and p16 were again entered into the previously described multivariate model using backward stepwise regression. As a result, in 3-year OAS xCT (p=0.904) was excluded first, followed by p16 (*p*=0.648) in the next step. The product variable was included in the model (HR: 2.51; 95% CI 1.28–4.92; *p*=0.017). For 3-year DSS, xCT was excluded first (*p*=0.762), followed by p16 (*p*=0.803). Again, the product variable was included (HR: 2.42; 95% CI 1.18–4.93; *p*=0.015). In contrast, for 3-year DFS, the product variable was excluded first (*p*=0.765), followed by p16 (*p*=0.489). However, xCT was included (HR: 1.94; 95% CI 1.04–3.62; *p*=0.038).

Analogous multivariate models were constructed for 5-year survival. Firstly, effects of p16 on 5-year survival were calculated in a multivariate model adjusting for age, T-class, nodal positivity, and tobacco consumption. 5-year survival effects of p16 on DSS (HR 1.43; 95%CI 0.67–3.02; *p*=0.354) and DFS (HR 1.60; 95%CI 0.78–3.31; *p*=0.203) were weaker than those reported for xCT. The 5-year survival effects of p16 (HR 1.61; 95% CI 0.85 – 3.06; *p*=0.146) and xCT (HR 1.57; 95% CI 0.89–2.79; *p*= 0.121) on OAS were close to equal.

Secondly, both p16 and xCT were entered into the multivariate model, including age, T-class, nodal positivity, and tobacco consumption, using backward stepwise regression and exclusion p-value of 0.10. In 5-year OAS, this resulted in the exclusion of p16 (p=0.257), followed by xCT (p=0.120) in the next step. In 5-year DSS, p16 was excluded (p=0.582). While xCT was included in the model (p=0.085). In 5-year DFS, p16 was excluded (p=0.489). While xCT was included in the model (p=0.038).

Finally, the product variable of xCT and p16, was entered into the previously described multivariate model along with xCT and p16, using backward stepwise regression. As a result, in 5-year OAS, xCT was excluded first (p=0.994), followed by p16 in the next step (p=0.631). While the product variable was included in the model (p=0.040). In 5-year DSS, p16 was excluded first (p=0.964), followed by xCT in the next step (p=0.675). While the product variable was included in the model (p=0.059). In 5-year DFS, the product variable was excluded first (p=0.765),

followed by p16 in the next step (p=0.489). While xCT was included in the model (p=0.038).

In summary, xCT was a better independent prognostic marker for 3-year survival than p16 in this cohort. However, for OAS and DSS, the best prognostic resolution was achieved when the results from both p16 and xCT stainings were combined. Analogous multivariate models were constructed for 5-year survival, with the same conclusion, suggesting that the product variable best predicts 5-year OAS and DSS. Nevertheless, xCT alone was the most predictive factor for both 3- and 5-year DFS.

5.3.6 xCT and treatment modality

We also evaluated the prognostic potential of xCT for patients receiving different treatment modalities. For this purpose, patients were divided into two groups based on whether they received radiotherapy as a part of their primary treatment (definitive CRT, definitive RT, or adjuvant therapy). A significant association was observed between high xCT expression and poor 5-year DFS (HR 1.46; 95% CI 1.01–2.10; p=0.042) in the radiotherapy group, as presented in **Figure 21**. Moreover, a similar association was not observed in the patient group that underwent surgery only.

Interestingly, the difference in DFS associated with xCT was present only in the RT group (HR: 2.14; 95 %CI: 1.01–4.49; p=0.046), as shown in **Figure 22**. The site-adjusted survival effect of xCT on DFS in the RT group was as follows: HR 2.28; 95% CI: 1.05–4.92; p=0.037).

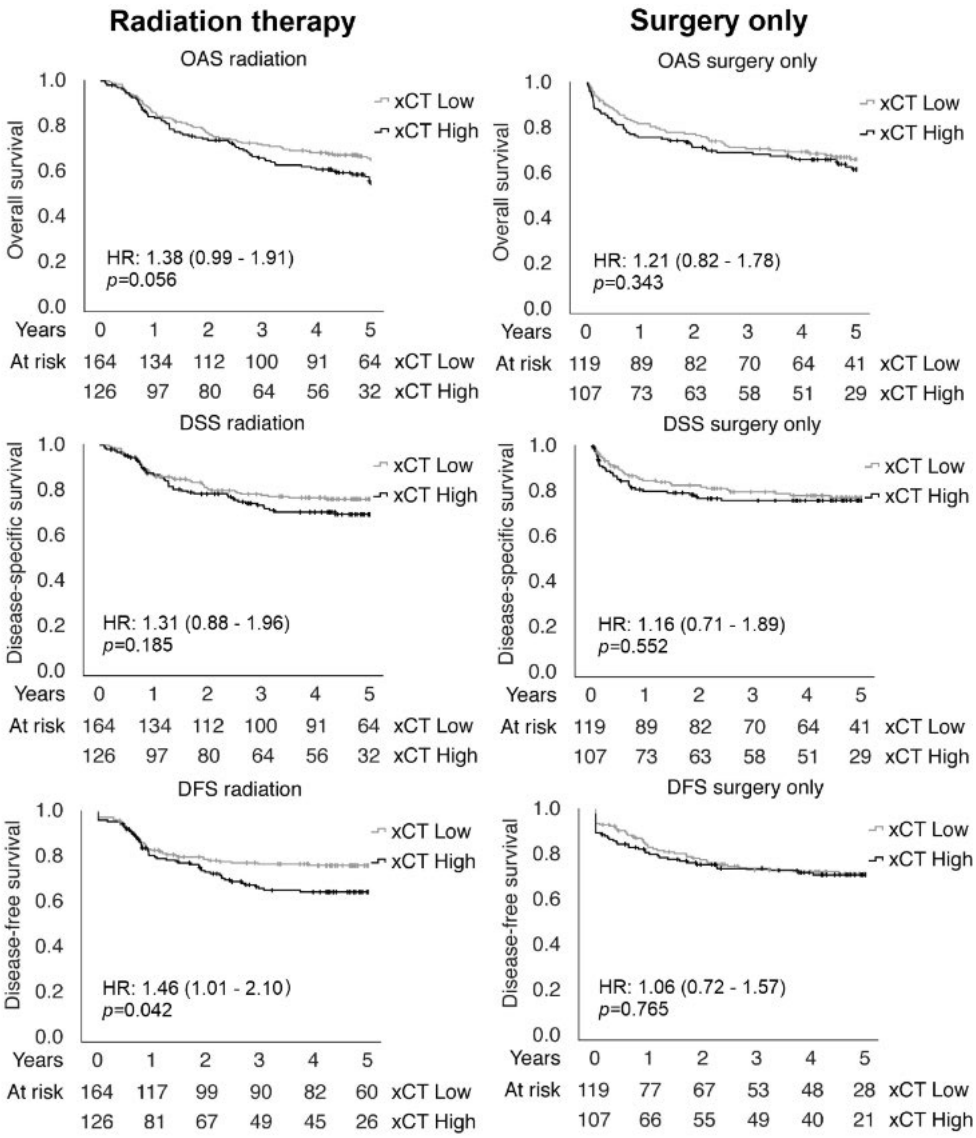

Figure 21. Prognostic trends according to treatment type. The radiation therapy group is defined by patients who received radiation therapy as a part of their treatment (definitive radiation therapy, definitive chemoradiation therapy, or in combination to surgery). Hazard ratios (HR), 95% confidence intervals (95% CI), and statistical significance were calculated using Cox proportional hazards model. Overall survival (OAS); disease-specific survival (DSS); disease-free survival (DFS). Adopted from manuscript III.

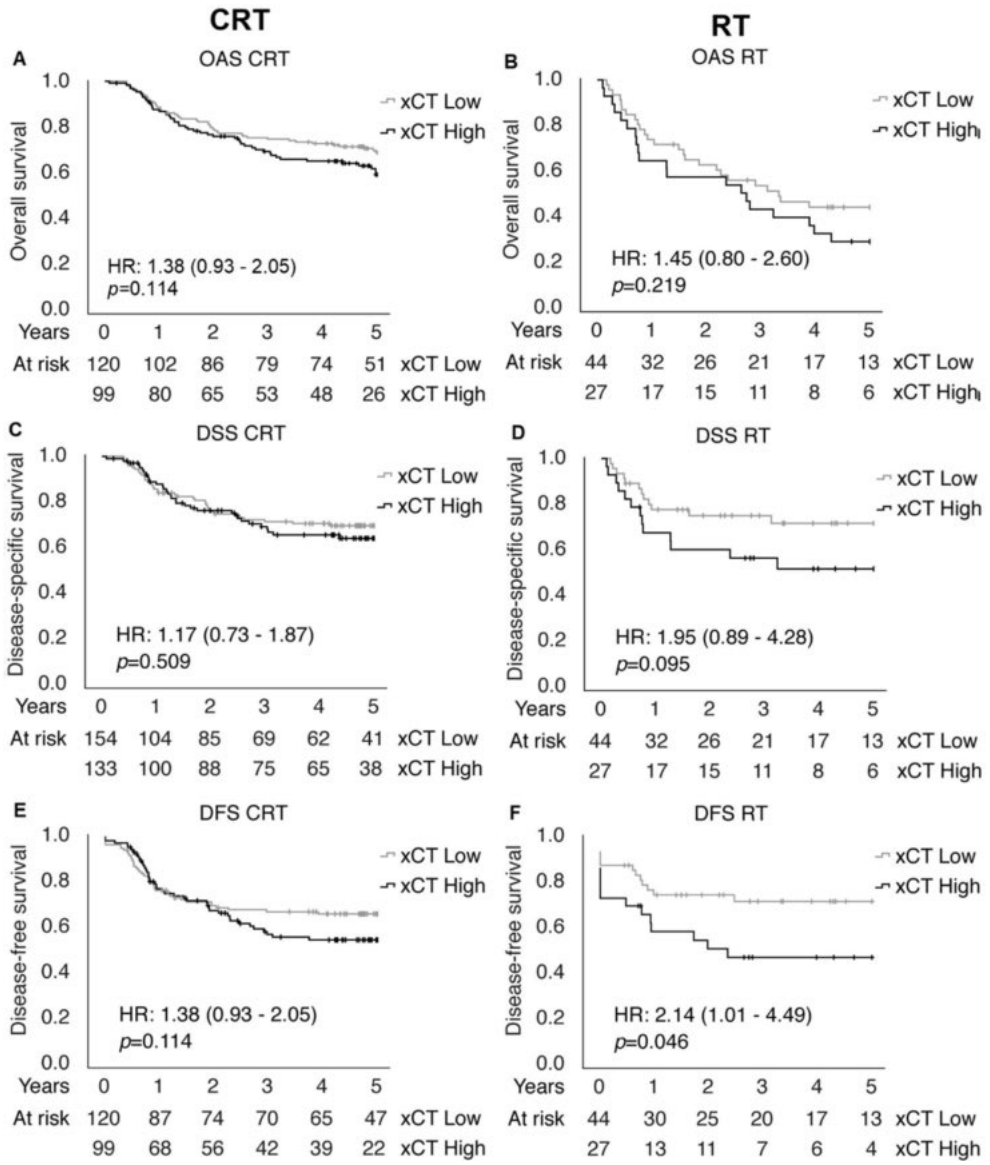

Figure 22. Survival in patients receiving chemoradiation therapy (CRT) and radiation therapy (RT) as a part of their first-line cancer treatment. Hazard ratios (HR), 95% confidence intervals (95% CI), and statistical significance were calculated using Cox proportional hazard model. Overall survival (OAS); disease-specific survival (DSS); disease-free survival (DFS).

6 Discussion

6.1 HNSCC in Southwest Finland 2005–2015

In study II, the population-based cohort, comprising of all 1033 patients diagnosed with a new HNSCC in Southwestern Finland in 2005–2015, showed an increase in the incidence of SCCs of the oropharynx and the oral cavity. In contrast, the incidence of laryngeal SCC was decreasing. The absolute number of newly diagnosed HNSCC cases in 2015 (n = 109) was 34.6% higher than that in 2005 (n = 81). These findings are consistent with current literature and data provided by the NORDCAN database. (Chaturvedi et al., 2011; Gillison et al., 2015; Johnson et al., 2020; *Nordcan 2.0*) The 5-year OAS, DSS, and DFS in the study II cohort were 55%, 69%, and 61%, respectively. Previously, Routila et al. have reported the 5-year OAS (53%) and DSS (68%) of the HNSCC patients diagnosed in Southwestern Finland in 2005–2010, i.e. the earlier half of the population included in study II. Moreover, they compared observed survival rates with the data of Eurocare-5 study for Northern Europe and found survival rates in the Southwestern Finland region to be higher especially in elderly patients and hypopharyngeal cancer. (Routila et al., 2021) When comparing survival rates of the 2005–2010 and 2005–2015 populations diagnosed with HNSCC in Southwestern Finland, a modest improvement in survival can be observed acknowledging that the latter material also includes the patients of the first dataset.

The indisputable impact of known prognostic factors such as patient age, T class, lymph nodal involvement was also shown in study II, as each of these factors was associated with a worse prognosis. Furthermore, alcohol and tobacco are well-known risk factors for HNSCC. (Denissoff et al., 2022; Maier et al., 1992) This was also evident in our HNSCC cohort. Twenty-four percent of HNSCC patients had moderate or excessive alcohol consumption at the time of diagnosis, while the same is true for 13% of the Finnish working-age population. (Warpenius & Mäkelä, 2020) Tobacco consumption was also markedly common in the HNSCC cohort, as 68% of patients smoked daily at the time of diagnosis, whereas only 11% of the average Finnish working-age population smoked in 2022. (*Tobacco statistics 2022* - Finnish institute for health and welfare) Although smoking was less common among patients whose tumour was associated with HPV, 40.3 % of patients with a p16-positive

OPSCC were also daily smokers by the time of diagnosis. This indicates the existence of tumours with mixed aetiology which presents a challenge for clinical decision making, especially in the context of de-escalation.

6.2 p16 as a biomarker in HNSCC

The difficulty of choosing the optimal cancer treatment method for HNSCC is due to the lack of clinically validated biomarkers. Much expectation has been set on HPV detection and p16 staining for OPSCC cancer treatment de-escalation. In study II, p16 positivity was mainly encountered in the oropharynx and p16 proved to be a significant independent factor for improved survival in this site. However, the results do not truly support the de-escalation strategy in p16-positive patients because, in all studied settings, the prognosis was better in the CRT group than in RT-treated patients. Also several trials have demonstrated a decrease in the prognostic benefit of p16 when treatment deintensification strategies are implemented. (Chera & Amdur, 2018; Gillison et al., 2019; X. J. D. Lu et al., 2022; H. Mehanna et al., 2019; Wagner et al., 2020) The results from publication II, that are in concordance with previous data, together with results from publication I, discussed in chapter 6.4, strongly suggested that successful de-escalation of p16-positive OPSCC patients would likely require novel biomarkers alongside p16 to predict cancer sensitivity to radio- and chemoradiotherapy.

When oropharyngeal cancers were excluded in study II, the impact of p16 expression on OAS or DSS was not statistically significant in other HNSCCs. However, this analysis was complicated by the fact that a very significant portion of HNSCC tumours occurring outside the oropharynx were p16 negative (83–95% depending on the location of the tumour). Furthermore, the results of study II showed a very strong locoregional expression profile of p16 (only 4–5% of OSCC, LSCC, and HPSCC were p16 positive); therefore, it would be most cost-effective to focus routine testing of p16 in clinical practice for only OPSCC. Moreover, the strong locoregional specificity of p16 expression supports the hypothesis that, the location of the primary tumour may play a significant role in the functionality of a HNSCC biomarker. (Kang et al., 2015; K. Y. Kim et al., 2014; Kokko et al., 2011)

6.3 xCT as a biomarker in HNSCC

xCT promotes tumour cell growth partly through inhibition of ferroptosis, a form of programmed cell death that has been intensively researched in recent years. Furthermore, xCT has ferroptosis-independent functions in promoting tumour development, such as maintaining redox homeostasis, as reviewed by Koppula et al., 2020; W. Lin et al., 2020. xCT has also been considered a novel prognostic

biomarker in HNSCC. (J. R. Lee et al., 2018; M. Li et al., 2022) Regarding primary tumour sites, the role of xCT in HNSCC has been studied mainly in tumours of the oral cavity with conflicting results. (J. R. Lee et al., 2018; Toyoda et al., 2014)

In study III, xCT expression of HNSCC tumours originating from different sites was evaluated in an extensive population-based cohort. Our findings suggested that xCT is a prognostic factor in OPSCC, a tumour site not well presented in previous xCT-related studies. Moreover, xCT outperformed p16 in predicting survival in most settings. However, the best prognostic resolution for OAS and DSS was achieved when p16 and xCT staining were combined, while xCT alone was the strongest biomarker for predicting DFS. In contrast to some of the previous studies, xCT was not found to be prognostic for survival in SCC of the oral cavity or of the larynx. (J. R. Lee et al., 2018; Z. Ma et al., 2017) These disparities may be due to differences in the qualities of the antibodies and the evaluation practices used to measure xCT expression. These are limitations inherent to immunohistochemical techniques and need to be addressed in the present study as well. Problems concerning xCT antibodies, including the debated molecular weight of the protein and batch-to-batch fluctuation of antibody specificity, have been previously addressed by Van Liefferinge et al., 2016. Therefore, confirming the specificity of the anti-xCT antibody was specifically emphasized in the current thesis by validation via siRNA experiments in two well established HNSCC cell lines, FaDu and Cal33.

Results from study III also confirmed that p16-negative tumours have higher expression levels of xCT which is consistent with the findings of Hémon et al., 2020. Moreover, a previous study found xCT expression to be significantly elevated in tumours with p53 mutations, that are common in HPV-negative diseases. However, upregulation of xCT occurred also in tumours with wild type p53, suggesting that other factors also influence xCT expression in cancer. (Jiang et al., 2015) Furthermore, we observed that xCT expression was associated with increased T class, as also reported in previous studies assessing xCT in HNSCC. (J. R. Lee et al., 2018; Z. Ma et al., 2017; Toyoda et al., 2014) In terms of epidemiological risk factors, we found that xCT expression was markedly greater in patients who smoked daily at the time of diagnosis. This finding is in accordance with a previous in vitro study demonstrating that smoking could induce xCT expression in oral cancer cells. (Nagaraj et al., 2006) Moreover, xCT has been reported to be inducible by hypoxia in a HIF1-dependent manner. (H. Lu et al., 2015) However, to our knowledge, study III was the first study to demonstrate the association between high xCT expression and smoking in a clinical setting.

6.4 Response to radiotherapy

6.4.1 Recurrence in relation to high-risk treatment volume, p16 status, and treatment modality

The information on the location of recurrent tumours in relation to radiation fields could have an impact on optimization of dose planning while using modern, highly conformal techniques or even de-escalated radiotherapy protocols. In study I, patterns of HNSCC recurrence in and outside of high-risk treatment volume planning target volumes were retrospectively investigated. An additional interest was to detect putative differences in p16-positive and p16-negative tumours based on the higher radiosensitivity and tendency to present with extensive nodal involvement in neck of the former.

Four of the patients had a multifocal recurrence pattern previously described also by Geretschläger et al., 2012. Recurrent tumours in study I were classified as in-field (45%), marginal miss (16%), and true miss (39%). The proportions of recurrences were fairly similar to those presented by Chen et al. (40%, 41%, and 18% respectively) in their study on tumour recurrence in 50 patients with HPV-positive oropharyngeal squamous cell cancer. (A. M. Chen et al., 2017) The higher amount of true miss recurrences in study I could be explained by the larger variety of primary tumour sites of our study and inclusion of p16-negative cases. Considering the results of study I and those reported by Chen et al., together one could argue that a notable portion of patients with p16-positive recurrent tumours relapse in-field or as marginal misses in relation to the high-risk volume. In contrast to the results of study I, Geretschläger et al. reported a lower proportion of recurrences occurring within the high-risk treatment volume. The p16 status was not reported but since no oropharyngeal cancers were included it is likely that the majority of all tumours were p16-negative. (Geretschläger et al., 2012)

In line with existing literature, 5-year OAS and DFS rates of study I demonstrated better overall prognosis for p16-positive HNSCC. However, a p16-positive subpopulation whose disease recurred rapidly in a median of 9 months within the high-risk treatment volume was also described. This is in concordance with time interval of 10 months reported by Chen et al. in their study of p16-positive OPSCC. (A. M. Chen et al., 2017) In study I, four out of seven p16-positive patients had a recurrent tumour with 95% or more of the recurrence volume overlapping with the high-risk treatment volume. These findings implicate the heterogenous treatment response among a group of diseases that are generally considered curable by RT. While true miss recurrence in adequately planned and treated p16-positive patients seems to be rare, focus should be on early assessment of local resistance and introduction of adaptive or radiosensitizing approaches during CRT.

Although half of the patients who had an in-field recurrence in study I had received definitive CRT as a primary treatment, no significant relation was observed between primary treatment modality and recurrence class. This result is similar to the findings of Johansen et al. who did not include information about p16 status in their findings but, based on tumour sites, included mostly p16 negative patients. (Johansen et al., 2017)

6.4.2 Radiotherapy and xCT

Regarding different treatment modalities in study III, xCT was found to be most predictive in HNSCC patients who received radiotherapy. Previously, Lei et al. demonstrated that xCT expression promotes radioresistance through inhibiting ferroptosis. (Lei et al., 2020) Furthermore, Ye et al. reported the administration of ferroptosis inducers to enhance the antitumor effect of radiation. (Ye et al., 2020) These findings are in line with results of study III and might explain the remarkably poor survival of patients with high xCT expression in the RT group. We hypothesized that in the CRT group, chemotherapy partially aided in overcoming radioresistance. Thus, xCT might predict the need for concurrent chemotherapy alongside radiation therapy to overcome radioresistance.

xCT inhibition has gained interest as a potential adjuvant therapy for radiotherapy. (Feng et al., 2022; Sarowar et al., 2022; Yang et al., 2021) Roh et al. also demonstrated that both genetic silencing of the SLC7A11 gene and pharmacological inhibition of xCT by sulfasalazine significantly sensitize cisplatin-resistant head and neck cancer cells by inducing ferroptosis. (Roh et al., 2016) Moreover, Wang et al. suggested that targeting ferroptosis-associated metabolism in cancer cells could improve the efficacy of immunotherapy. (W. Wang et al., 2019)

6.5 Study strengths and limitations

Limitations of study I included the retrospective design, small size, and heterogeneity of the patient cohort and treatment modalities. The proportion of p16-positive patients was unfortunately small since p16 was not routinely analysed during the study period in 2010–2015. Finally, not all patients had PET/CT or PET/MRI as part of their diagnostic work-up in the follow-up phase. Furthermore, co-registration of 3D-imaging sets acquired several months or years apart is prone to compromised spatial accuracy. On the other hand, metabolic imaging for treatment planning, IMRT and concurrent cisplatin/cetuximab were standard approaches in all patients rendering the findings of study I applicable for current practice.

The main strength of studies II and III was the representative population-based patient collection that included all new HNSCC patients diagnosed and treated in the southwest Finland area, covering approximately one-sixth of Finland's HNSCC patients, within an 11-year period. Inclusion bias caused by health insurance or socioeconomic status-related issues was also avoided, as all HNSCC patients in need of oncological treatment were referred to tertiary referral centres according to the national treatment guidelines. Due to this arrangement, all patients were given the opportunity to receive the most beneficial treatment. Thus, the cohort represented real-life patient material, which increases the applicability of the results in clinical decision-making. In addition, a comprehensive electronic medical record system provided an effective patient follow-up regimen, with follow-up rates up to 97% and 81% at 3 and 5 years, respectively. A further strength of study III was the thorough validation of the antibody used for IHC.

Studies II and III also highlighted the important role of different primary tumour sites in HNSCC biomarker studies. However, the variety of sites covered in these studies can also be considered a limitation. Although study III involved HNSCC samples from 585 patients and it was the largest xCT-related HNSCC study thus far, the site-specific subgroups were relatively small. Therefore, larger, site-specific and OPSCC focused studies are warranted to clarify the role of xCT expression in the heterogeneous disease entity of HNSCC. In study II, there were certain limitations in comparing survival in different treatment groups, as treatment modalities were not randomized. Instead, the choice of treatment was affected by patients' comorbidities and capacity to tolerate certain therapies, such as chemotherapy.

6.6 Future perspectives

The challenges underlying the lack of biomarkers in HNSCC include substantial problems in translating biomarker findings into clinical practice. This may be partly explained by the difficulty of designing biomarker studies. Population-based and validated patient and tissue datasets may be one approach to overcome these challenges. Furthermore, a standardised methodology to detect HPV would increase the reproducibility of study results and bring clarity to studies investigating treatment de-escalation for HPV-related HNSCC. The new separate staging system for p16-positive OPSCC in the UICC/AJCC 8th edition has improved prognostic discrimination compared with the seventh edition, when applied to retrospective cohorts. (Van Gysen et al., 2019; Würdemann et al., 2017) However, prospective studies are still needed to validate the eighth edition. In contrast to HPV-positive OPSCC, current staging of nasopharyngeal carcinoma is still based on anatomy, without incorporation of EBV status. Baseline plasma EBV DNA levels have been shown to improve hazard discrimination for EBV-positive nasopharyngeal

carcinoma. (Guo et al., 2019; V. H. F. Lee et al., 2019) Thus, EBV status will likely be considered for future editions of the UICC/AJCC staging manuals. Furthermore, EBV has been studied as a biomarker for screening, treatment selection, and follow-up in nasopharyngeal carcinoma, particularly in endemic areas, as reviewed by Su et al., 2023.

Particularly interesting phenomena in the future of HNSCC epidemiology will be the consequences of HPV vaccination. Recent studies have indicated that the prevalence of vaccine type oral HPV (types 6, 11, 16, and 18) is significantly lower in vaccinated adults than in unvaccinated adults. (Chaturvedi et al., 2018; Herrero et al., 2013; Hirth et al., 2017) The results of study II advocate the importance of vaccinating males against HPV, as 80.6% of all our p16-positive OPSCC patients were males. The HPV vaccine was introduced in the Finnish national vaccination program in 2013 for girls and in 2020 for boys, and it is offered for 10- to 12-year-old citizens. Future studies are needed to fully evaluate the effect of vaccines on the prevalence of HPV-positive oropharyngeal cancers.

As the current TNM- and p16-based treatment stratification often fails to capture the biologic heterogeneity of HNSCCs, the search for prognostic and predictive biomarkers continues. Among their many potential purposes in HNSCC, biomarkers predictive of immunotherapy response would be particularly valuable, as only a proportion (15–20%) of patients responds to immunotherapy. Furthermore, PD-1 immune checkpoint inhibitors are currently being investigated also for primary tumour treatment in adjuvant and neoadjuvant settings. Concerning radiotherapy, recent studies have introduced the concept of imaging biomarkers that could asses underlying tumour heterogeneity, identify areas within the tumour that are less sensitive to radiation, and thereby allow for biologically focused target delineation and dose calculation, as reviewed by Beaton et al., 2019. Dose painting is a novel radiotherapy approach developed to improve local tumour control by producing optimized non-uniform dose distribution by using information from functional imaging. The two main strategies for dose painting are dose painting by contours (DPBC) and dose painting by numbers (DPBN). In DPBC, a dose boost is applied to a subvolume of the tumour by a certain threshold of a biomarker. Whereas in DPBN, dose prescription to each voxel is determined by the voxel value in functional images, as reviewed by Pang et al., 2022.

Regarding future biomarkers, advancements in digital genomic technologies have enabled the detection of circulating tumour DNA from clinical specimens such as blood samples. Circulating tumour DNA provides a landscape of the mutational status of the cancer and may provide prognostic and predictive information. Methods detecting biomarkers, such as circulating tumour DNA and microRNAs, in body fluids through different technologies have been commonly named as liquid biopsies. The advantages of liquid biopsies include repeatability and minimal invasiveness.

Thus, liquid biopsies hold a promise especially for detection of recurrence and follow-up monitoring of patients. (Galot & Machiels, 2020; Silvoniemi et al., 2023) Genomic profiling of tumours themselves may also become a part of clinical practice during the next decades. However, the relevance of this approach to HNSCC management is yet to be demonstrated, given the predominance of mutations in tumour suppressor genes, high mutational burden, questions concerning the clinical significance of these genetic alterations, and the distorted tumour microenvironment.

Finally, growing understanding of the altered cell death pathways in HNSCC may yield to the discovery of novel biomarkers along with targeted therapies. An example of this is Xevinapant, an antagonist of IAPs (inhibitors of apoptosis proteins) that is thought to enhance the antitumour effects of chemotherapy and radiotherapy by restoring cancer cell sensitivity to apoptosis. An ongoing phase III study is currently investigating Xevinapant as an adjuvant therapy alongside standard-of-care CRT in unresected locally advanced HNSCC. In addition to apoptosis, current scientific consensus describes around 10 other types of programmed cell death, such as ferroptosis, that affect tumorigenesis and modulate therapeutic response, as reviewed by Raudenská et al., 2021. These alternative forms of cell death may provide new, so far undiscovered biomarkers and therapeutic windows.

7 Conclusions

Study I:

1. Many true and marginal misses in patients with p16-negative tumours suggest that even meticulous treatment planning with multimodality imaging may fail to detect all clinically significant disease. Therefore, validation of new imaging protocols in addition to ^{18}F FDG-PET-CT/-MRI is encouraged for better delineation of the gross tumour volume and radioresistant subvolumes especially in the setting of highly conformal, modern irradiation techniques.
2. HNSCCs respond heterogeneously to RT. A small subset of p16-positive diseases relapse within the high-risk treatment volume despite the common view of their high radiosensitivity and better prognosis.

Study II:

3. The incidence of OPSCC, p16-positive OPSCC, and HNSCCs in general was increasing during the years 2005–2015 in Southwestern Finland.
4. It would be most cost-efficient in clinical practice to focus routine testing of p16 only for OPSCC.
5. In OPSCC, p16 is an independent prognostic factor when adjusted for age, treatment modality, T class, nodal positivity, and consumption of alcohol and tobacco. However, successful treatment de-escalation of patients with p16-positive OPSCC still likely requires further biomarkers predictive of RT and CRT response.

Study III:

6. The expression and prognostic value of xCT varies markedly among different primary tumour sites.

   In OPSCC, xCT is a powerful prognostic factor that outperformed p16 in 3-year survival prognostication. The results encourage for further studies on therapeutic targeting of xCT to overcome radioresistance.

Acknowledgements

This research project was performed at the Department of Oncology and Radiotherapy and the Department of Otorhinolaryngology – Head and Neck Surgery at Turku University and Turku University Hospital during the years 2016–2024. The studies were financially supported by Olga and Vilho Linnamo Foundation, Finnish Medical Foundation, Häme Regional Fund, Emil Aaltonen Foundation, Finnish Cancer Foundation, Moikoinen Cancer Research Foundation, Finnish ORL-HNS Research Foundation, and Turku University Hospital Research Funds. I express my gratitude to foundations and donors who make it possible to do science.

Along the road, I've received invaluable support from others and there are acknowledgements to be given. I'd like to begin with my supervisors. Thank you Heikki Minn for your cutting-edge expertise and rational, yet utterly humane view of life. Heikki Irjala, I admire your MacGyver-like skills and knowledge on head and neck surgery. Your cheerful and pragmatic approach has helped me through a lot. Sami Ventelä, your enthusiasm and excitement towards research is hard to resist. Thank you for many inspiring discussions. All the three of you share a sense of humor that kept me going during the hard moments of this project.

I'd like to sincerely thank my co-authors Sami Suilamo, Eero Kytö, Samuli Vaittinen, Mari Mylly, Teemu Huusko, Johannes Routila, Ilmo Leivo, Sanni Tuominen, Tove Grönroos, Maria Sundvall, and Petra Ketonen. Sami, you were the master of methodology in the first publication that would not have been possible without your expertise on medical physics and radiation oncology. Thank you Eero for collecting patient data for the first publication. Sanni, your wisdom on tumor biology and mastery of preclinical experiments were essential for the last manuscript. Thank you also for scoring hundreds of tissue samples with me along the years. Johannes, thank you for being the statistical master mind of the last two articles. Your research on biomarkers has led the way for me as well. I want to express my wholehearted gratitude to Tove for also being a truly supporting member of my supervisory committee.

I am thankful for Associate Professor Pernille Lassen and Professor Timo Paavonen, the pre-reviewers of this thesis manuscript. Your remarks were of great

value and made the book better. I also want to thank Professor Linda Marklund for accepting the invitation to be the opponent of the thesis defense.

I want to express my gratitude to colleagues and supervisors from my previous research groups at the Department of Anatomy and Cell Biology, at Turku University and at the Institute of Laboratory Medicine, Clinical Chemistry and Pathobiochemistry at Charité Universitätsmedizin Berlin. My special thanks go to Maria Alanne who set an example on being both a full-blooded scientist and a brilliant clinician. I am grateful for the Department of Oncology, especially Anneli Sainast and Jenni Peltonen, at Kymenlaakso Central Hospital for the possibility to gain clinical experience on oncology. Thank you to my current and former colleagues at Porvoo hospital for making everyday work worthwhile and for supporting my scientific endeavors.

I would have probably quit this project without the support of my friends. Thank you to my dear mates from medical school. Anna-Reetta, Sanna, and Elisa, my friends from health bioscience studies, it has been inspiring to follow your careers and exchange thoughts on research. Thank you to Kadotetut sielut. I am particularly grateful for my friends from Hämeenlinna, who I grew up with. Afterwards, I have been blessed with many meaningful acquaintances from across the globe. The last five years we have lived in Porvoo and there are several people who have made me feel at home. You are much appreciated.

Finally, I would like to thank my family. I owe my deepest gratitude and respect to my parents Tuire and Janne. You have cared for me and supported my education in every possible way. Also, the stories of my grandparents have inspired me a great deal. Thank you, my sisters Aliisa and Eliisa, for putting up with me throughout your whole lifes. In addition, I want to thank my parents-in-law, Anna-Kaisa and Kyösti, for your help in our everyday life. Aleksi, you deserve a gold medal (and a few beers) for your patience and support. As I started this project, we had just met. Now we have a family together. Thank you for being by my side all the way. Lastly, I want to thank my eager research assistants Konsta and Rauski for showing me what life is all about.

Porvoo, August 2024
Linda Nissi

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TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2024

ISBN 978-951-29-9771-8 (PRINT)
ISBN 978-951-29-9772-5 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)