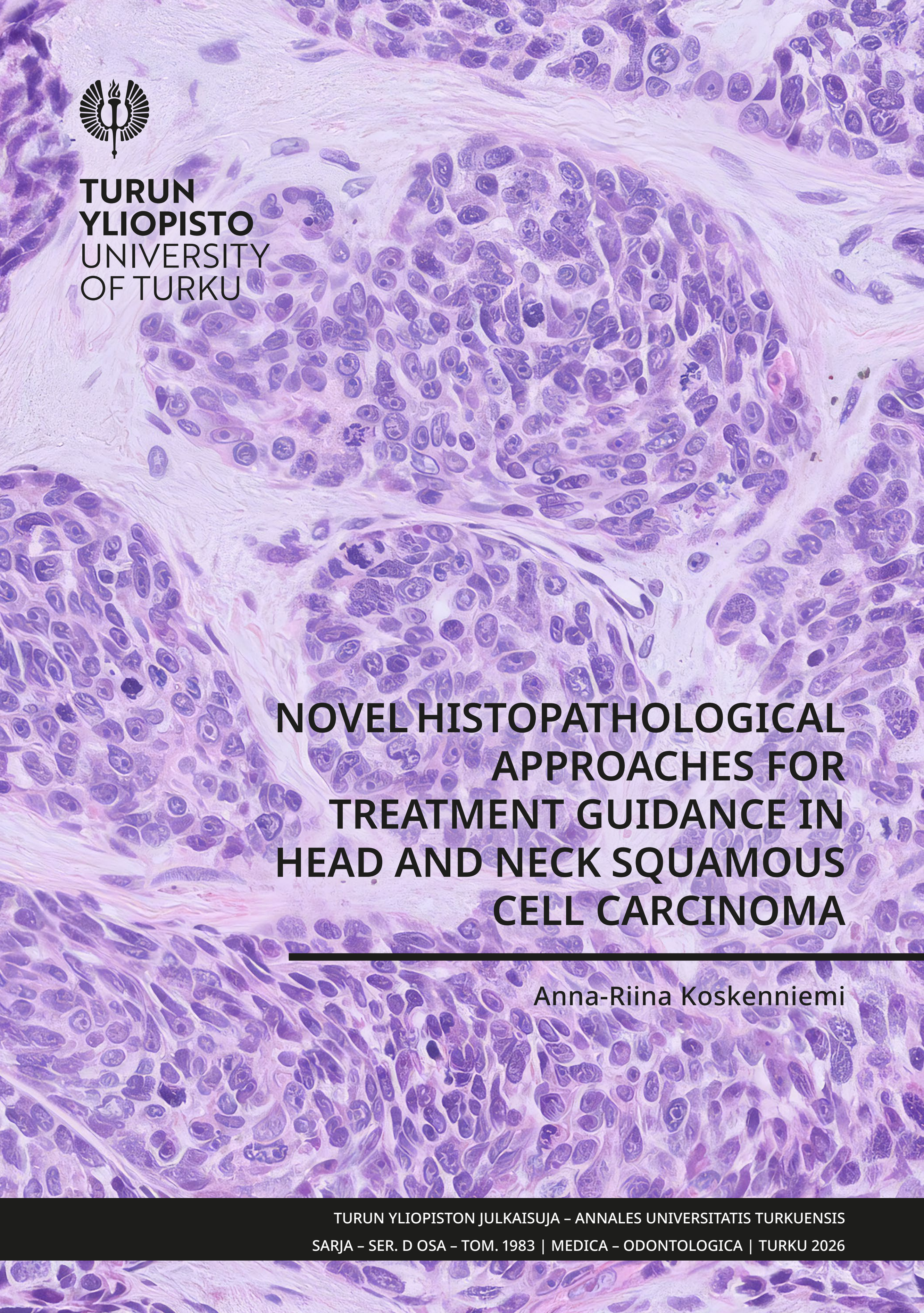

TURUN YLIOPISTO
UNIVERSITY OF TURKU

NOVEL HISTOPATHOLOGICAL APPROACHES FOR TREATMENT GUIDANCE IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Anna-Riina Koskenniemi

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a clinically and biologically heterogeneous malignancy that continues to pose major therapeutic challenges. As combination treatment strategies expand, practical biomarkers are needed to refine prognostication and guide treatment selection beyond conventional staging. This thesis identified histopathological and immunological biomarkers relevant to treatment guidance in HNSCC, with a particular focus on neoadjuvant radiotherapy and chemoradiotherapy (RT/CRT), and evaluated artificial intelligence (AI) for standardized morphologic assessment while investigating the clinical relevance of the cancer–germline antigen DEAD-box helicase 4 (DDX4).

Altogether, cancer tissues from 270 HNSCC patients were examined. In a retrospective neoadjuvant cohort with paired pre- and post-treatment specimens (n=53), analyses included immune profiling by immunohistochemistry (CD8, CD68, CD206, Clever-1, and PD-L1), deep learning–based quantification of pretreatment tumor necrosis, and post-treatment response assessment using a pragmatic two-tier combined metric (cHTR) that integrates regression measures with the histiocytic multinucleated giant cell reaction (MGC) as a novel component.

Pretreatment necrosis was a strong marker of poor outcome in neoadjuvant RT/CRT-treated HNSCC, and AI enabled reproducible, high-throughput necrosis quantification. The cHTR provided robust prognostic stratification, while higher baseline PD-L1 expression and increased intraepithelial $CD8^+$ TILs were associated with poorer cHTR. DDX4 formed tumor-associated germ-granule–like cytoplasmic ribonucleoprotein granules, and its loss altered gene regulation and attenuated malignant phenotypes, including impaired xenograft tumor formation. Notably, pretreatment DDX4 positivity predicted favorable cHTR in neoadjuvant RT/CRT-treated HNSCC. In conclusion, these findings may contribute to more individualized treatment strategies in HNSCC.

KEYWORDS: HNSCC, TIME, neoadjuvant chemoradiotherapy, tumor necrosis, predictive biomarkers, histopathological therapy response, cancer-germline antigens, DDX4.

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Pään ja kaulan alueen levyepiteelikarsinooma (HNSCC) on kliinisesti ja biologisesti heterogeeninen syöpä, ja edennyt tauti on hoidollisesti haastava. Yhdistelmähoitojen yleistyessä tarvitaan käytännöllisiä ja toistettavia hoitoa ohjaavia biomarkkereita. Tässä väitöskirjassa tavoitteena oli tunnistaa histopatologisia ja immunologisia biomarkkereita, joilla on merkitystä HNSCC:n hoidon ohjauksessa—erityisesti esiliitännäisen sädehoidon (RT) ja siihen yhdistetyn solunsalpaajahoidon (CRT) yhteydessä. Lisäksi tutkittiin syöpä–itulinja-antigeeni DEAD-box helikaasi 4:n (DDX4) merkitystä syövässä.

Työssä tutkittiin 270 HNSCC potilaan näytteitä. Näistä 53 potilaan kasvaimesta ennen ja jälkeen RT/CRT:n otetuista näytteistä tehtiin kasvaimen immuunimikroympäristön profilointi immunohistokemiaa (CD8, CD68, CD206, Clever-1, PD-L1) hyödyntäen sekä arvioitiin lähtötilanteen tuumorinekroosia, joka kvantifioitiin tekoälyyn perustuvalla menetelmällä. Hoidon jälkeinen vaste arvioitiin kasvaimen regressiota tarkastelemalla, ja kehitettiin käytännönläheinen kaksiportainen hoitovasteen arviointiluokitus (cHTR), joka yhdistää regressioarviointiin uutena komponenttina histiosytaarisen jättisolureaktion (MGC).

Lähtötilanteen kasvainnekroosi kertoi potilaan viisivuotisennusteen 34 prosenttiyksikön alenemisesta neoadjuvantti-RT/CRT hoidetuilla potilailla, ja tekoäly mahdollisti toistettavan nekroosin kvantifioinnin. cHTR tarjosi vahvan ennusteellisen riskiluokittelun, ja korkeampi lähtötilanteen PD-L1-ilmentyminen kasvainkudoksessa sekä lisääntyneet kasvainepiteelin $CD8^+$ T-solut ennustivat huonompaa hoitovastetta. DDX4 muodosti syöpäsoluissa itusolugranuloita muistuttavia ribonukleoproteiinirakeita, ja DDX4 geenin poisto syöpäsoluista muutti geenisäätelyä ja heikensi syöpäsolujen kykyä muodostaa kasvaimia solu- ja eläinmalleissa. Vaikka DDX4 oli HNSCC-potilailla yhteydessä huonompaan ennusteeseen, ennusti lähtötilanteen DDX4-ilmentyminen suotuisaa hoitovastetta RT/CRT-hoidetuilla HNSCC-potilailla. Tulokset tarjoavat kliinisesti merkityksellistä uutta tietoa, jota voidaan tulevaisuudessa hyödyntää HNSCC-potilaiden hoidonohjauksen kehittämisessä.

AVAINSANAT: Pään ja kaulan levyepiteelikarsinooma, kasvaimen mikroympäristö, kemosädehoito, hoitovasteen arviointi, syöpä-itulinja-antigeeni, DDX4

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AI	Artificial intelligence
AJCC	American Joint Committee on Cancer
CGA	Cancer-germline antigen
cHTR	Combined histopathological treatment response
CI	Confidence intervals
CPS	Combined positive score
CRT	Chemoradiotherapy
DAMP	Damage associated molecular pattern
DDX4	DEAD-box helicase 4
DOI	Depth of invasion
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
FDA	U.S. Food and Drug Administration
FFPE	Formalin fixed paraffin embedded
H&E	Hematoxylin-eosin
HNSCC	Head and squamous cell carcinoma
HPV	Human papilloma virus
HR	Hazard ratios
HTR	Histopathological treatment response
ICCR	International collaboration on Cancer Reporting
ICD	Immunogenic cell death
IF	Immunofluorescence
IHC	Immunohistochemistry, immunohistochemical
iTIL	Intraepithelial TIL
IVD	In vitro diagnostic
IVDR	In vitro diagnostic medical devices regulation
MGC	Multinucleated giant cells
MHC	Major histocompatibility complex
MPR	Major pathologic response
NCI	National Cancer Institute

NHGRI	National Human Genome Research Institute
OR	Odds ratios
OR	Overall survival
PC	Prostate cancer
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
pENE	Pathological extranodal extension
piRNA	PIWI-interacting RNA
PSA	Prostate-specific antigen
qRT-PCR	Quantitative real-time PCR
RNA-seq	RNA sequencing
RT	Radiotherapy
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SEER	The Surveillance, Epidemiology, and End Results
sTIL	Stromal TIL
TAM	Tumor-associated macrophage
TCGA	The Cancer Genome Atlas
TfH	Follicular helper T cells
TIL	Tumor infiltrating lymphocyte
TIME	Tumor immune microenvironment
TMA	Tissue microarray
TME	Tumor microenvironment
TNM	Tumor-node-metastasis classification
TPS	Tumor proportion score
Tregs	Regulatory T cells
TRG	Tumor regression grade
TYKS	Turku University Hospital
UICC	Union for International Cancer Control
WHO	World Health Organization

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 Koskenniemi, Anna-Riina; Huusko, Teemu; Routila, Johannes; Jalkanen, Sirpa; Hollmén, Maija; Vainio, Paula; Ventelä, Sami. Histological tumor necrosis predicts decreased survival after neoadjuvant chemotherapy in head and neck squamous cell carcinoma. Oral Oncol., 2025 Jun:165:107287. https://doi.org/10.1016/j.oraloncology.2025.107287

II Koskenniemi, Anna-Riina; Huusko, Teemu; Routila, Johannes; Vainio, Paula; Ventelä, Sami. A novel combined histopathological treatment response grading predicts survival after neoadjuvant chemoradiotherapy and associates with immune microenvironment in head and neck squamous cell carcinoma. Manuscript.

III Olotu, Opeyemi; Koskenniemi, Anna-Riina; Ma, Lin; Paramonov, Valeriy; Laasanen, Sini; Louramo, Elina; Bourgery, Matthieu; Lehtiniemi, Tiina; Laasanen, Samuli; Rivero-Müller, Adolfo; Löyttyniemi, Eliisa; Sahlgren, Cecilia; Westermarck, Jukka; Ventelä, Sami; Visakorpi, Tapio; Poutanen, Matti; Vainio, Paula; Mäkelä, Juho-Antti; Kotaja, Noora. Germline-specific RNA helicase DDX4 forms cytoplasmic granules in cancer cells and promotes tumor growth. Cell Rep. 2024 Jul 23;43(7):114430. doi:10.1016/j.celrep.2024.114430

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1 Introduction

Head and neck squamous cell carcinoma (HNSCC) remains a major global health burden with substantial morbidity and mortality despite advances in surgery, radiotherapy, chemotherapy, and targeted immunotherapies. Current treatment selection still relies largely on anatomic site, stage, and patient fitness, although patients with similar TNM (tumor-node-metastasis classification) categories often experience divergent outcomes—reflecting the biological heterogeneity of HNSCC and the limitations of conventional clinicopathological stratification. Histomorphology remains the cornerstone of tumor diagnostics, yet traditional three-tier histological grading in HNSCC has limited prognostic utility and no role in cancer therapy guidance. Similarly, while the tumor immune microenvironment (TIME) has emerged as a potential determinant of therapy response, clinically applicable immunological biomarkers are scarce. Digital pathology and AI-based image analysis offer new possibilities for objective quantification of histological features, yet clinically robust solutions are scarce and practical integration into diagnostic workflows is still in its infancy.

In these studies, three complementary lines of investigation were pursued, each examined in relation to the TIME. First, tumor necrosis, a readily assessable histomorphological feature, may capture key aspects of tumor aggressiveness and therapy response but is inconsistently quantified in routine diagnostics. AI-assisted necrosis quantification offers a pathway toward standardized and reproducible readouts from routine hematoxylin–eosin (H&E) slides. Second, in the era of neoadjuvant therapy, histological response assessment is gaining increasing importance. Post-treatment features such as the histiocytic multinucleated giant cell (MGC) reaction may reflect immune-mediated tissue remodeling and provide insight into therapeutic efficacy, yet remain under-characterized in HNSCC. Third, the germline-restricted RNA helicase DDX4, a cancer–germline antigen, represents an underexplored molecular axis that may interface with stress- and immune-relevant programs in cancer cells. Its clinical significance in HNSCC—and whether it refines prognosis or informs treatment selection—remains insufficiently defined.

Collectively, these studies aim to address the unmet need for reproducible, biologically grounded, and clinically interpretable histopathological biomarkers in

HNSCC. By integrating morphology-driven metrics, tumor immune microenvironment analysis, post-treatment immune remodeling, and germline-related biology (DDX4), the goal of this thesis is to advance context-aware pathology toward a framework where diagnostic readouts not only describe morphology but also inform prognosis and guide therapy selection in HNSCC.

2 Review of the Literature

2.1 Head and neck squamous cell carcinoma (HNSCC)

Head and neck squamous cell carcinoma (HNSCC) is a mucosal malignancy arising from squamous epithelium lining mucosa of the head and neck area. Globally, head and neck cancers ranks as the seventh most common cancer, and approximately 90% of all head and neck cancers are squamous cell carcinomas (Dunn et al., 2026). The head and neck region can be divided into five major anatomical subsites (Figure 1). Among these, the most common sites of HNSCC include the oral cavity, oropharynx, and larynx, whereas hypopharyngeal and nasopharyngeal carcinomas are comparatively rare (WHO Classification of Tumours Editorial Board, 2023).

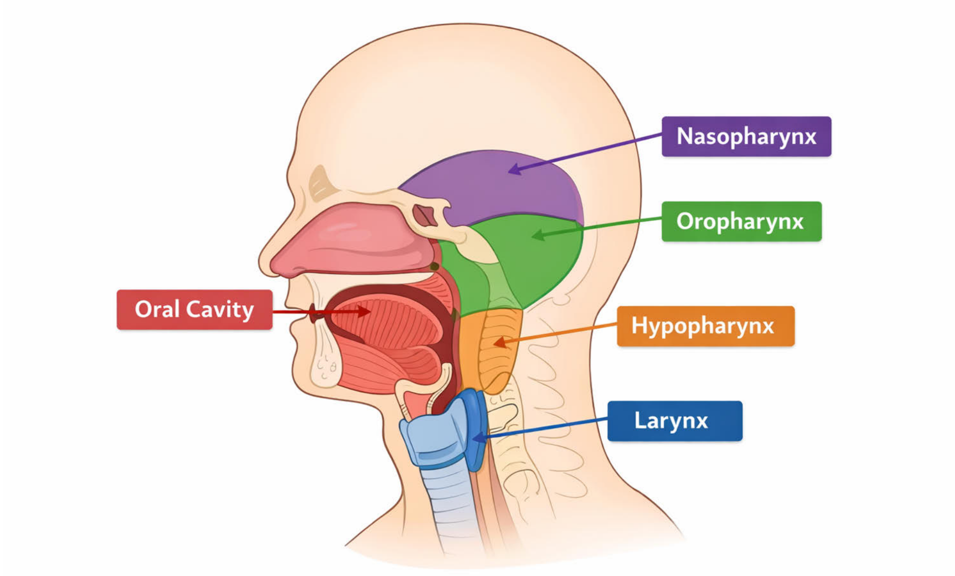


Figure 1. Major anatomical subsites of the head and neck region relevant to HNSCC. The figure shows five major anatomical subsites of the head and neck region relevant to HNSCC: the oral cavity, oropharynx, nasopharynx, hypopharynx, and larynx. Image created with ChatGPT (OpenAI) based on the author’s prompt.

The most important risk factors for HNSCC are tobacco smoking, alcohol consumption, and viral infections. Among these, high-risk human papillomavirus (HPV) is of particular importance, especially in oropharyngeal SCC, whereas Epstein–Barr virus (EBV) is less commonly implicated and is primarily associated with nasopharyngeal carcinoma. Additional risk factors include immunosuppression, chronic mucosal inflammation and occupational exposures. Genetic predispositions have also been described, but they are rare. (Mody et al., 2021). A characteristic phenomenon of mucosal carcinogenesis is field cancerization, whereby widespread epithelial injury and an increased mutational burden create a fertile ground for the development of multiple local tumors (Willenbrink et al., 2020).

Globally, incidence trends in HNSCC are heterogeneous and vary by anatomical subsite and geographic region. In many populations, the incidence of HPV-related oropharyngeal SCC has increased, whereas trends in other subsites have been more variable (Menezes et al., 2021). In Finland, increasing incidence has been reported particularly for oral cavity and oropharyngeal cancers (Figure 2) (Koskinen et al., 2022). As HPV vaccination coverage increases, HPV-positive oropharyngeal cancer is expected to become less common (Landy et al., 2023; Syrjänen & Rautava, 2017).

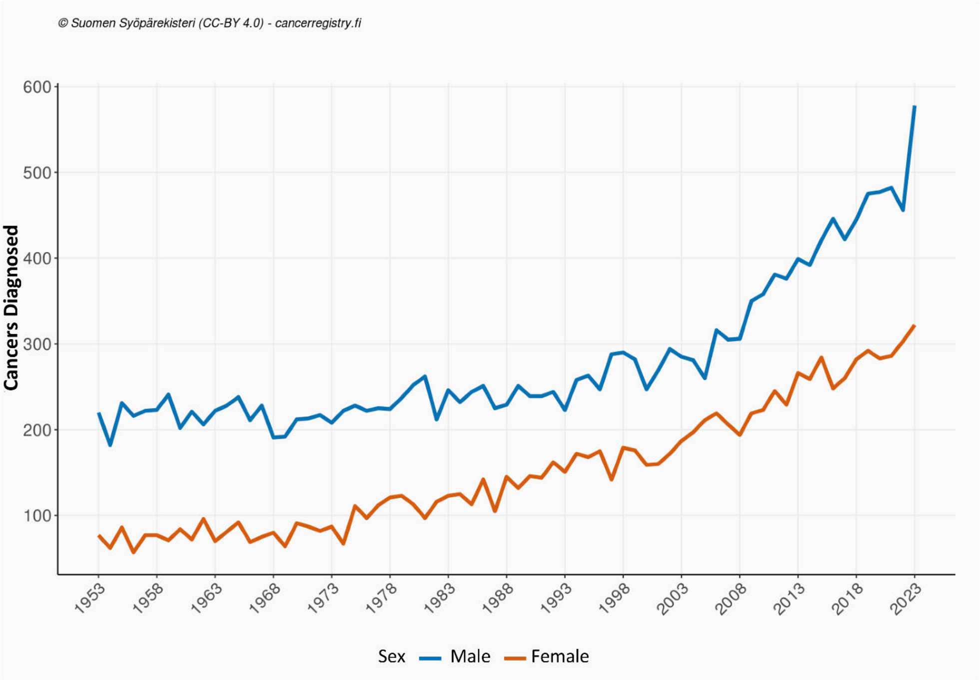


Figure 2. Incidence of oral and pharyngeal cancer in Finland. Modified from Finnish Cancer Registry, cancer statistics.

Prognosis of HNSCC is strongly stage dependent: early-stage disease (stage I–II) is generally associated with more favorable outcomes, whereas stage III–IV disease represents locoregionally advanced cancer and is associated with a higher risk of recurrence and death (Dunn et al., 2026). This stage-dependent pattern is also reflected in population-level SEER survival data. For oral cavity and pharyngeal cancers, the 5-year relative survival is 88.4% for localized disease, 69.4% for regional disease, and 36.9% for distant disease (Figure 3), while the corresponding figures for laryngeal cancer are 79.3%, 49.0%, and 35.2%, respectively (National Cancer Institute, 2026b, 2026a). Over the past 10–15 years, prognosis in HNSCC has improved in some subgroups, particularly in HPV-associated oropharyngeal SCC, whereas improvement has been more limited in other subsites such as the oral cavity and larynx despite the introduction of new treatment modalities (Dunn et al., 2026).

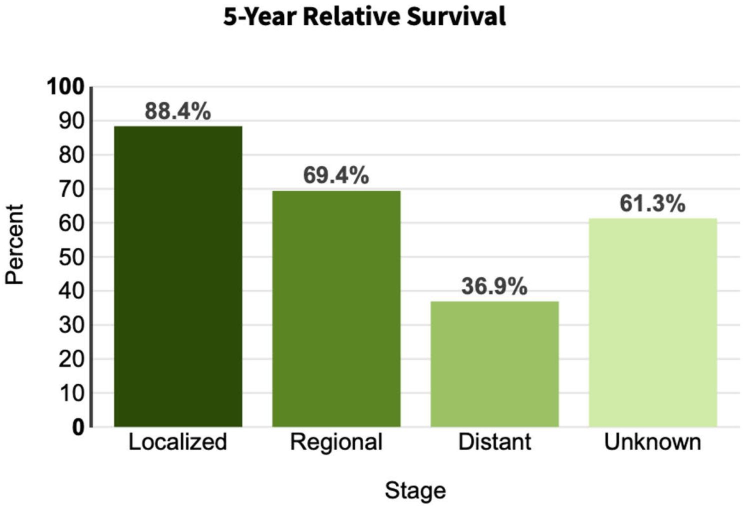


Figure 3. 5-year Relative Survival of oral and pharyngeal HNSCC. (SEER 21 (Excluding IL) 2015–2021, All Races, Both Sexes by SEER Combined Summary Stage). Source: The Surveillance, Epidemiology, and End Results (SEER) Cancer Stat Facts, National Cancer Institute.

For patients, HNSCC is a particularly challenging cancer. In large tumors, surgical resections are often mutilating, resulting in major functional (speech, swallowing) and aesthetic impairments, underscoring the importance of early detection and effective treatment. Key clinical challenge remains the lack of reliable prognostic and treatment-response predictive markers to guide treatment selection (Johnson et al., 2020). Increasing evidence indicates that the immunological contexture of HNSCC plays a crucial role in shaping treatment response. This has become an especially active area of investigation with the advent of immune checkpoint inhibitors and other immuno-oncological treatments (Ruffin et al., 2023).

2.1.1 WHO tumor classification

The World Health Organization (WHO) Classification of Tumours is the most widely used system for classifying different types of neoplasms. Pathologists rely on this classification in everyday diagnostic practice. The classification of head and neck tumors was last updated in 2023, introducing several changes, most notably the recognition of HPV-associated squamous cell carcinoma as a distinct carcinoma entity in the oropharynx (WHO Classification of Tumours Editorial Board, 2023).

Depending on the anatomical site, HNSCC is categorized into different subtypes according to HPV-association and histological subtype. The histological subtypes of HNSCC, which may overlap, include conventional (keratinizing / non-keratinizing), verrucous, basaloid, acantholytic, adenosquamous, lymphoepithelial, spindle cell / sarcomatoid, and papillary, the latter of which was upgraded to a distinct subtype in the most recent edition. In addition, in the oral cavity a new rare histological subtype, carcinoma cuniculatum, was recognized in the latest edition (WHO Classification of Tumours Editorial Board, 2023). Histological variants of HNSCC are shown in Figure 4 (reproduced from Gonçalves et al. 2025 with permission from Elsevier).

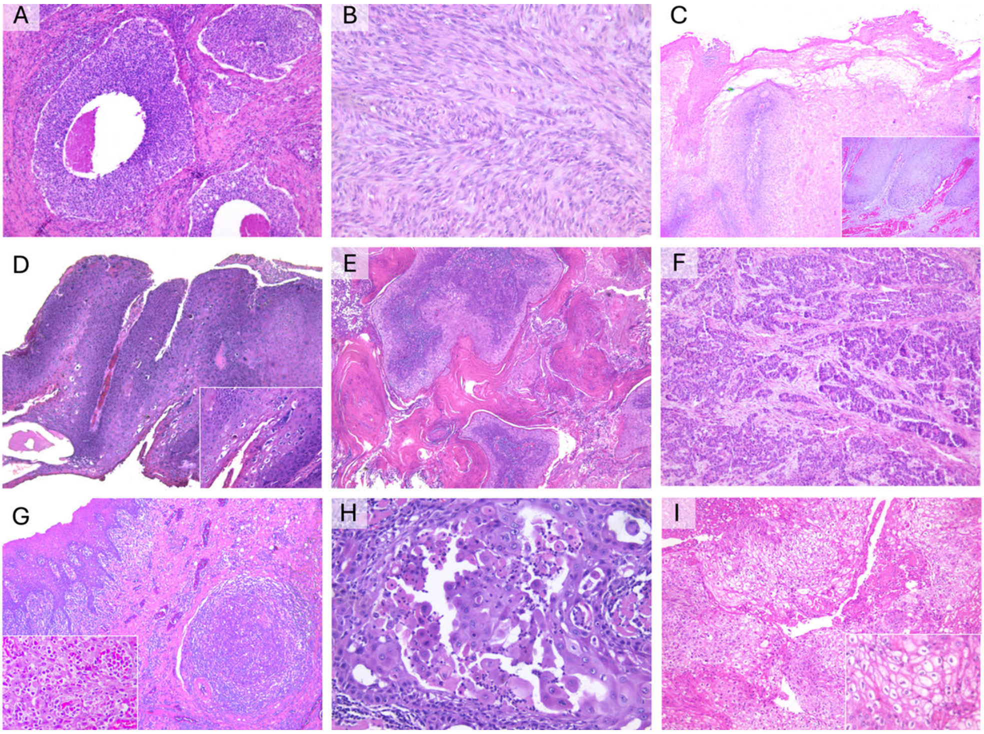


Figure 4. Histological variants of head and neck squamous cell carcinoma (SCC) according to the World Health Organization Classification. (**A**) Basaloid SCC, (**B**) Spindle cell SCC, (**C**) Verrucous SCC, (**D**) Papillary SCC, (**E**) Carcinoma cuniculatum, (**F**) Adenosquamous carcinoma, (**G**) Lymphoepithelial carcinoma, (**H**) Acantholytic SCC. Emergent histological subtypes. (I) Clear cell SCC. Figure reproduced from Gonçalves et al. 2025 with permission from Elsevier.

Histological morphology-based subtype may have prognostic and therapeutic implications. Certain histological variants are associated with worse prognosis, including spindle cell, basaloid, and adenosquamous carcinoma, acantholytic variant being controversial regarding to prognosis (Gonçalves et al., 2025). Verrucous carcinoma, despite its well-differentiated nature and low metastatic potential, generally shows poor responsiveness to radiotherapy and may even undergo more aggressive anaplastic transformation after irradiation (Shear & Pindborg, 1980).

In summary, histological subtype in HNSCC can carry diagnostic, prognostic, and therapeutic significance, underlining the importance of careful pathological evaluation.

2.1.2 Genomic alterations

In 2006, the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) launched The Cancer Genome Atlas (TCGA) (NCI, 2024). The project aimed to comprehensively characterize the genomic landscape of human cancers using emerging high-throughput technologies, including whole-exome and whole-genome sequencing, copy number analysis, RNA sequencing, methylation profiling, and proteomics. In a landmark TCGA study, it was demonstrated that HNSCC exhibits a complex and heterogeneous genomic landscape (Lawrence et al., 2015). Despite this heterogeneity, the clearest genomic distinction is between HPV-positive and HPV-negative tumors, particularly in the oropharynx.

In HNSCC, HPV-negative tumors are characterized by a higher mutational burden, frequent inactivation of tumor suppressors such as *TP53* and *CDKN2A*, and broad copy number alterations. Common changes include amplifications at oncogenic loci such as 3q26–28 and 11q13–22, as well as *EGFR* amplification, which together promote proliferation and invasiveness. By contrast, HPV-positive tumors show a less complex genomic profile, dominated by growth-promoting alterations such as *PIK3CA* mutations, *TRAF3* loss, and occasional *E2F1* amplification. Overall, this pattern is consistent with a more focused, virally driven oncogenic process, whereas HPV-negative tumors more often reflect carcinogen-associated field cancerization and broader genomic instability (Lawrence et al., 2015; Leemans et al., 2018).

Gene activity in cancer can be regulated through multiple mechanisms. Alterations may occur in the gene coding sequence itself (e.g., mutations or copy number alterations in functionally important genes), but gene transcription can also be modified through changes in transcription factors as well as epigenetic mechanisms. Even if genes and transcription operate normally, additional layers of regulation can occur after transcription, affecting the gene product at the post-transcriptional, translational, or post-translational level (Hanahan, 2022). Epigenetic mechanisms regulate which genes are active and which are silenced, and include DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs (Baylin & Jones, 2016).

The tumor suppressor gene *CDKN2A,* whose protein product is p16^INK4a, can be inactivated through multiple mechanisms—including homozygous deletion, point mutation, and epigenetic silencing via promoter hypermethylation—resulting in loss of p16 and deregulated cell-cycle entry (Asokan et al., 2014). In clinical practice, IHC staining for p16 in HNSCC is used for a different purpose than to show the loss of p16: as a surrogate marker of HPV infection. This is because HPV-mediated activation of the viral oncogenes *E6/E7* functionally inactivates the RB1 tumor suppressor protein, resulting in compensatory p16 overexpression (Lewis et al., 2018). In oropharyngeal SCC, p16 immunohistochemistry (IHC) is a highly sensitive but only moderately specific surrogate marker of HPV-driven disease, with reported sensitivity around 94% and specificity around 83% in meta-analysis (Prigge et al., 2017).

The major genomic alterations in HNSCC are summarized in Table 1, based on TCGA and subsequent studies (Hammerman et al., 2015; Lawrence et al., 2015; Leemans et al., 2018; Seiwert et al., 2015).

Although the genomic alterations in HNSCC are now relatively well characterized, their clinical implementation in patient management remains challenging due to the complexity of the field. Despite the increasing amount of knowledge, this has not yet translated into substantial improvements in overall survival. As further insights are gained into the interplay between genomic, epigenetic and immunological features and their association to treatment response, it is likely that these aspects will increasingly be considered in staging and treatment stratification. Importantly, the distinction between HPV-positive and HPV-negative disease has already been incorporated into the latest TNM classification, and selected limited molecular features can already guide treatment decisions. These aspects will be further discussed in the chapter on targeted therapies.

Table 1. The main genomic alterations in head and neck squamous cell carcinoma based on The Cancer Genome Atlas (TCGA) and subsequent studies (Hammerman et al., 2015; Lawrence et al., 2015; Leemans et al., 2018; Seiwert et al., 2015).

Feature	**HPV-positive HNSCC**	**HPV-negative HNSCC**
Etiology	HPV infection (oropharynx)	Tobacco, alcohol
Mutation burden	Low	High
Key tumor suppressor pathways	Functional inactivation of p53 and Rb via HPV E6/E7 (leading to compensatory p16 over expression)	*TP53* and *CDKN2A*
Growth factor signaling	*EGFR* events rare	*EGFR* amplification /overexpression common (but kinase-domain mutations rare)
Copy number alterations (CNAs)	Fewer broad CNAs overall Amplifications: *E2F1* (subset) Deletions: *TRAF3* (subset)	Amplifications: *CCND1*, 3q26/28 (*PIK3CA/TP63/SOX2*), 11q13/22 (*CTTN, FADD, YAP1*), *EGFR* (7p11.2) Deletions: 9p21 (*CDKN2A/B*), 3p, 8p
PI3K/AKT/ mTOR signaling pathway	*PIK3CA* mutations; *PTEN* loss relatively more frequent	*PIK3CA* amplifications, occasional mutations; *PTEN* loss uncommon
Epigenetic regulation	HPV E6/E7 alters DNA methylation machinery; *TRAF3* and other genes epigenetically silenced	*CDKN2A* silencing; *NSD1* inactivation; *PTEN* silencing; global DNA hypomethylation
Other recurrent driver genes	*FGFR3* mutations/fusions	*FAT1, NOTCH1, CASP8*
Prognosis	Better, especially in oropharyngeal cancers	Worse, more aggressive disease

HPV: human papilloma virus, HNSCC: head and neck squamous cell carcinoma, CNAs: copy number alterations.

2.1.3 Tumor immune microenvironment (TIME)

The tumor microenvironment (TME) encompasses all non-malignant elements surrounding the neoplastic parenchyma, including stromal cells (e.g., cancer-associated fibroblasts), extracellular matrix, immune cells, blood and lymphatic vessels, and soluble mediators. This so-called tumor stroma plays a central role in processes such as invasion and metastasis (Hinshaw & Shevde, 2019). Whereas earlier cancer research focused predominantly on the malignant cells themselves, current approaches increasingly emphasize the interplay between malignant cells and their surrounding microenvironment.

In HNSCC, the tumor immune microenvironment (TIME), the immunological component of the TME, comprises cytotoxic and helper T cells, regulatory T cells (Tregs), B cells/plasma cells, dendritic cells, macrophages, myeloid-derived suppressor cells, natural killer cells, neutrophils and mast cells (Cillo et al., 2020; Ferris, 2015). The TIME influences tumor biology, patient outcomes, and therapy response; it is not only the number of cells that matters, but also their spatial organization, functional states, and the immune mediators they express (Binnewies et al., 2018; Mandal et al., 2016).

The TIME may act as both a facilitator and a barrier to therapy, which underscores the growing interest in its clinical relevance (Binnewies et al., 2018). In HNSCC recent research has focused on themes such as the heterogeneity of immune cell populations, the impact of HPV status on immune responses (still not fully understood), mechanisms of immune evasion, and the development of novel immunotherapeutic strategies.

In this study, the TIME was investigated with a focus on tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), and PD-L1 expression (immune evasion). Following sections will provide background on their role in HNSCC.

2.1.3.1 Tumor-infiltrating lymphocytes (TIL)

Tumor-infiltrating lymphocytes (TILs) can be localized both interspersed among the malignant tumor parenchymal cells (intraepithelial TILs, iTILs) and within the surrounding stromal compartment (stromal TILs, sTILs) (Figure 5) (Salgado et al., 2015). In HNSCC, tumor-infiltrating lymphocytes (TILs) have frequently been shown to carry biological significance in tumor progression as well as prognostic and predictive value with regard to treatment response (Almangush, Jouhi, et al., 2022; Balermpas et al., 2014; Borsetto et al., 2021; Torri et al., 2024). However, a universally adopted, HNSCC-specific standardized approach for TIL scoring is still lacking, contributing to interstudy variability and limited reproducibility of study results when assessing the significance of TILs (Almangush, De Keukeleire, et al., 2022; Hendry et al., 2017).

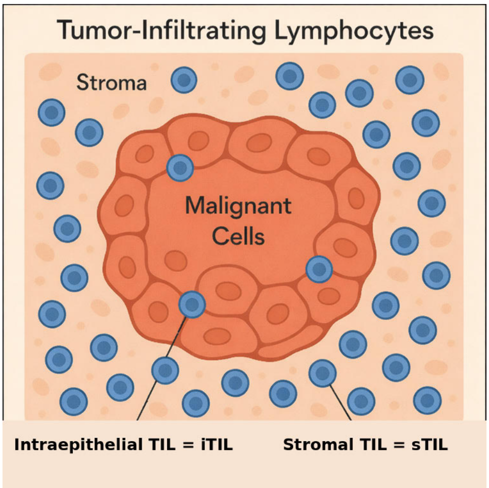


Figure 5. Tumor-infiltrating lymphocytes (TILs) within malignant epithelium and surrounding stroma. Schematic illustration showing the localization of intraepithelial TILs (within tumor cell nests) and stromal TILs (within the peritumoral stroma). Both compartments contribute to the overall TIME, but their prognostic and predictive significance may differ. Image created with ChatGPT (OpenAI) based on the author's prompt.

The most important TIL populations are T lymphocytes, particularly CD8$^+$ cytotoxic T cells and CD4$^+$ helper T cells. CD8$^+$ cytotoxic T cells, often referred to as "killer T cells," are part of the adaptive immune system. CD8 is a surface molecule (coreceptor) that recognizes MHC class I molecules. When a CD8$^+$ lymphocyte encounters a cell presenting foreign antigens on MHC I, it becomes activated and differentiates into a cytotoxic effector cell (Abbas et al., 2025). Upon activation, it can directly eliminate target cells through mechanisms such as the perforin–granzyme pathway or Fas–FasL interactions (Kägi et al., 1994). Typical targets include virus-infected cells and malignant cells. As a result of continuous antigenic stimulation, T cells may become exhausted and gradually lose their functional capacity. In this state, their gene expression and epigenetic profiles are altered, distinguishing them from normal effector T cell populations (Blank et al., 2019). Because CD8$^+$ TILs are capable of directly destroying malignant cells, their abundance has frequently been associated with favorable prognosis in HNSCC (Contrera et al., 2024; Rodrigo et al., 2021). The activity of CD8$^+$ T cells is tightly regulated, and cancer cells are adept at evading their cytotoxic effects by diverse mechanisms. Indeed, immune evasion was recognized as one of the fundamental hallmarks of cancer in 2011 (Hanahan & Weinberg, 2011).

Among CD4$^+$ helper T cells, the most relevant subsets in the TIME are follicular helper T cells (Tfh), Th17 cells characterized by IL-17 secretion, and Tregs typically defined by FoxP3 expression. CD4$^+$ helper T cells function as key orchestrators of the immune response by shaping the activity of other lymphocyte populations through cytokine secretion and cellular interactions. Tfh cells promote antitumor immunity by facilitating B-cell activation and the development of tertiary lymphoid structures, while both Tfh and Th17 subsets contribute to the recruitment of cytotoxic CD8$^+$ T cells and other effector cells to the tumor site. (Cillo et al., 2020).

In contrast, Treg cells exert immunosuppressive effects that may enable tumor immune evasion and are frequently associated with unfavorable prognosis and resistance to checkpoint inhibitors (A. Zhang et al., 2024). Interestingly, in some HPV-positive HNSCCs the prognostic association has been the opposite, which may be explained by the ability of Treg cells to suppress inflammation that otherwise promotes tumor progression (Y. Wang et al., 2024). This highlights the notion that individual immune markers are seldom consistently associated with favorable or unfavorable outcomes; rather, the function of immune cells is largely context-dependent and critically shaped by their interplay within the TME.

2.1.3.2 Tumor-associated macrophages (TAM)

Macrophages are lymphoid-derived cells that develop from hematopoietic stem cells in the bone marrow through the myeloid lineage. They have multiple functions, of which the most relevant in the cancer biology context are the phagocytosis of pathogens and dead cells, antigen presentation to other lymphoid cells, and the secretion of cytokines to regulate inflammation (Abbas et al., 2025).

TAMs can be broadly divided into two subgroups: M1 and M2 macrophages. M1 macrophages promote inflammation through the secretion of proinflammatory mediators and contribute to tumor elimination, whereas M2 macrophages are immunosuppressive, promote angiogenesis, and can support tumor growth (J. Liu et al., 2021; Shapouri-Moghaddam et al., 2018). Importantly, macrophage polarization is not a static state but rather a highly plastic process that is continuously shaped by signals from the TME.

In HNSCC, M2-type TAMs associate with adverse prognosis and resistance to conventional treatment modalities (notably radiotherapy/chemoradiotherapy, RT/CRT) and can contribute to immunotherapy resistance via myeloid-driven immunosuppression (Fu et al., 2020; A. T. Kumar et al., 2019). RT/CRT can increase TAM-infiltration and bias polarization toward an M2-like, immunosuppressive phenotype—observed in oral cavity models and clinical specimens—and this may contribute to resistance to RT/CRT (Beach et al., 2022; Okubo et al., 2016).

Therapeutic strategies targeting TAMs are under active development and shifting the M1/M2 balance toward a pro-inflammatory, M1-dominated phenotype through macrophage reprogramming approaches is considered a promising strategy. Another approach is the inhibition of M2-polarized TAMs using specific agents (Brancewicz & Kucharzewska, 2025; B. Li et al., 2020; Mantovani et al., 2022).

CD68 is widely used as a pan-macrophage marker, while CD163 and CD206 are commonly employed to identify M2-polarized TAMs (Betjes et al., 1991; Jayasingam et al., 2020). Clever-1 (Stabilin-1) is an immunosuppressive scavenger receptor expressed on TAMs; functionally, it acts as a myeloid checkpoint, and its expression has been linked to poor prognosis in several cancers. Clever-1 promotes an immunosuppressive microenvironment by inhibiting T-cell activation and supporting tumor growth and metastasis (Hollmén et al., 2020; Karikoski et al., 2014; Mantovani & Bonecchi, 2019).

A relatively understudied manifestation of TAMs in cancer is multinucleated giant cells (MGCs), which form when large numbers of activated macrophages accumulate in tissue (Milde et al., 2015). In pathology, MGCs are a well-recognized phenomenon in the context of foreign body reaction and chronic inflammation, and the process is known to be driven by a cytokine milieu, persistent exposure to necrotic debris or treatment induced tissue damage (Ahmadzadeh et al., 2022). In cancer diagnostics, however, little attention has been paid to MGCs, as they have generally been regarded as a reactive change secondary to tissue damage, without clinical relevance. In squamous cell carcinomas, MGCs have traditionally been interpreted to reflect a foreign body reaction to keratin deposits in well-differentiated tumors (de Medeiros et al., 2022). There are indications that a MGC reaction in treatment-naive esophageal SCC is associated with improved prognosis (H. Wang et al., 2021).

In 2024, a study reported that MGC reactions were more frequent in HNSCC treated with chemo-induction therapy compared to treatment-naive tumors (Gessain et al., 2024). Importantly, this was the first HNSCC study to demonstrate that MGC carries a favorable prognostic value not only in treatment-naive tumors but also after RT/CRT. However, dedicated studies directly linking post-RT/CRT MGC to the immunologic TME appear to be limited across carcinomas, including HNSCC, despite broader evidence that RT reshapes myeloid compartments (Boorsma Bergerud et al., 2024; S. Liu et al., 2023).

2.1.3.3 PD-1 / PD-L1

In the regulation of tissue immune responses, a central role is played by the inhibitory surface receptor programmed death-1 (PD-1) expressed on immune cells, and its ligand, programmed death-ligand 1 (PD-L1). When PD-L1 binds to PD-1, T-cell activation is suppressed and the immune response is attenuated. In normal tissues, PD-L1 can be expressed by antigen-presenting immune cells (e.g., macrophages), by other immune cells (e.g., activated T cells), and by certain non-immune tissue cells, such as trophoblasts in the placenta, where it prevents the maternal immune system from attacking the fetus (Boussiotis, 2016).

In cancer, PD-1/PD-L1 axis is critical because cancer cells exploit this physiological pathway of immune regulation to evade host immune surveillance. This occurs through the overexpression of PD-L1 on the surface of tumor cells themselves or on immune cells within the TME (Boussiotis, 2016). Importantly, PD-L1 expression in tumors does not necessarily indicate poor prognosis; in fact, an opposite association has been reported in oropharyngeal, oral cavity, and laryngeal HNSCC (Polesel et al., 2021; Kogashiwa et al., 2017; Vassilakopoulou et al., 2016). This may reflect the fact that these tumors often harbor a more abundant population of active T cells which tumor cells attempt to suppress, representing an immunologically “hot” tumor phenotype (S.-W. Chen et al., 2019; Sanchez-Canteli et al., 2020).

2.1.3.4 Interaction and clinical implication

Interpreting specific immune cells in isolation is not feasible, as they continuously interact with one another, with stromal components of the TME, and with the malignant cells themselves. The TIME differs not only between patients with the same cancer type but also across distinct regions within a single tumor, reflecting substantial intratumoral heterogeneity. Moreover, the immunological milieu is dynamic rather than static—a mediator-driven system that is continually remodeled by multiple factors, including therapeutic interventions (S. Guo et al., 2023). These interactions ultimately shape whether a tumor is immunologically “hot” or “cold,” with direct implications for treatment responsiveness (Duan et al., 2020)

In recent years, several key themes have emerged in studies of the HNSCC TIME. Clinically, two questions are paramount: how the TIME influences responses to oncological therapies, and conversely, how oncological therapies remodel the TIME (Z. Guo et al., 2025). A deeper understanding of these bidirectional interactions also provides the basis for a second major line of investigation: how to maximize the synergy between immuno-oncologic and targeted therapies in individual patients (Binnewies et al., 2018).

2.1.4 Cancer cell death

Cancer cells may die as a consequence of host immune responses, anticancer therapies, or intrinsic microenvironmental stresses such as hypoxia and nutrient deprivation due to insufficient vascularization (Z. Chen et al., 2023). In surgery, cancer cells are physically removed from the body, but in all other therapeutic modalities, cancer cell death is the intended outcome. Cytotoxic agents have been in use since the 1940s, yet the precise mechanisms underlying chemotherapy-induced cell death are still not fully understood. Traditionally, it was assumed that cytotoxic cancer therapies directly kill tumor cells. (Bracci et al., 2014). More recent evidence has shown that the efficacy of conventional therapies also partly relies on the induction of host immune responses, which contribute to tumor cell death (Zitvogel et al., 2008).

Several modes of cancer cell death are recognized, the best-known being apoptosis, necrosis, and necroptosis (Galluzzi et al., 2018). Apoptosis is a programmed, energy-dependent process in which the cell eliminates itself without eliciting a significant inflammatory response. Morphologically, the cell shrinks, the nucleus condenses, and DNA is fragmented. The nuclear debris is packaged into small membrane-bound vesicles, called apoptotic bodies, through membrane blebbing. These are subsequently recognized and cleared by macrophages (Elmore, 2007). Thus, immune cells, in this case macrophages, are involved in the process even though overt inflammation does not occur. In HNSCC, apoptotic regulation is often disrupted (e.g., TP53 loss-of-function, BCL2 overexpression), allowing continued survival under conditions where the cell should normally die (Lawrence et al., 2015).

Necrosis, by contrast, is not a clean or programmed process but usually represents a stress reaction to tissue damage, leading to cell destruction and subsequent inflammation. Characteristic features of necrosis include cellular swelling, rupture of the plasma membrane, and release of cytoplasmic and nuclear material, which attracts immune cells. Several types of necrosis are recognized, of which the most relevant in the context of cancer biology is coagulative necrosis (V. Kumar et al., 2020). In treatment-naive HNSCC, rapid tumor growth often exceeds vascular supply, leading to hypoxia and nutrient deprivation. As a result, tumor cells die and remain in the tissue for some time as ghost-like, anucleate necrotic cells (Beasley et al., 2001).

Necroptosis, identified in the early 2000s, is a programmed form of necrosis that cannot be distinguished histologically from necrosis, since the cell likewise swells and disintegrates, triggering inflammation (Degterev et al., 2005; Galluzzi, Kepp, et al., 2017). Unlike accidental necrosis, necroptosis is a biochemically regulated and predictable process. In HNSCC it has been estimated that about half of histologic necrosis would present necroptosis and it could be a potential cancer promoter (J. Li et al., 2020).

2.1.4.1 Immunogenic cell death (ICD)

Immunogenic cell death (ICD) refers to a process that not only leads to cell death but also activates the host immune system through damage-associated molecular patterns (DAMPs) released by dying cells. These signals recruit antigen-presenting immune cells, namely dendritic cells, which then present antigens to T cells. ICD is therefore not a distinct form of cell death but rather a functional concept that can occur in association with different death modalities (apoptosis, necrosis, necroptosis). From a histological perspective, it is not possible to determine whether cell death is immunogenic but DAMP release most commonly takes place in the context of necroptosis (Galluzzi et al., 2020).

In cancer, ICD transforms a dying tumor cell into a sort of “vaccine,” releasing and exposing intracellular signals that render the tumor visible to the immune system (Galluzzi, Buqué, et al., 2017). In practice, ICD is particularly important in the therapeutic setting. When tumor cells die in response to treatments such as radiotherapy, DAMPs are released from cancer cells, thereby initiating an adaptive immune response and inducing ICD. The effect of ICD may depend on how effectively the TME, and the immune system are able to exploit these signals (e.g., dendritic cell activation and T-cell priming). If an effective immune response fails to occur, the consequence may be chronic inflammation, which in turn can promote tumor growth. (Golden & Apetoh, 2015; Kroemer et al., 2013).

Recent studies demonstrate that ICD is mechanistically and clinically relevant also in HNSCC (Economopoulou et al., 2019; J. Li et al., 2020). It has been proposed that harnessing ICD in HNSCC could help to overcome the challenge of chemoresistance and improve responses to immunotherapy (X. Zhang et al., 2023).

2.1.5 Prognostic factors and staging

The prognosis of cancer largely depends on the TNM classification, which considers prognostic features of the primary tumor, nodal status, and distant metastases. The system was originally developed by the French surgeon Pierre Denoix at Institut Gustave Roussy in between 1943–1952 (Denoix, 1946). Today, TNM is maintained by the Union for International Cancer Control (UICC) in collaboration with the American Joint Committee on Cancer (AJCC). Based on the TNM classification, a cancer is assigned a four-stage grouping, which has strong prognostic value and guides treatment decisions.

The most recent update, TNM 9th edition, was published in 2025, replacing the previous TNM 8th edition from 2017 (Brierley et al., 2025). For HNSCC, there were no major changes in the latest edition. However, the definition of pathological extranodal extension (pENE) was clarified and its role strengthened, and importantly, pENE is now incorporated into staging also for HPV-associated oropharyngeal carcinoma.

Prognostic features of the primary tumor (T category) incorporated into the TNM classification primarily reflect the extent of invasion. In oral cavity squamous cell carcinoma, depth of invasion (DOI; cutoffs 5 and 10 mm) was incorporated into T classification because DOI has a strong and independent association with nodal metastasis and survival, as demonstrated in large international cohort studies

(Ebrahimi et al., 2014)**.** Tumor size contributes to the T category in the oral cavity, oropharynx, and hypopharynx, consistent with TNM consensus criteria informed by extensive clinicopathological evidence linking tumor extent to outcome. In the larynx, the T category is instead determined by subsite, involvement of one or both vocal cords, and local invasion rather than size, reflecting the site-specific anatomical and functional determinants emphasized in TNM staging.

In addition to TNM classification, prognosis is influenced by tumor-related factors and patient performance status, as well as by health-system and social factors such as timely access to treatment (Graboyes et al., 2019; Johnson et al., 2020). The 9th edition introduces prognostic factor grids that integrate anatomical staging with biological, pathological, host- and treatment-related determinants. Surgical margin status and performance status are consistent key factors, while site-specific elements include lymphovascular invasion (oral cavity), HPV genotype (oropharynx), plasma EBV DNA (nasopharynx), and vocal cord mobility (larynx) (Ang et al., 2010; Comer & al, 2023; Lo et al., 1999; Amin et al., 2017). It is noteworthy that the essential prognostic factors do not encompass immunological features, such as TILs or other microenvironmental features. The prognostic factors according to TNM 9th edition are summarized in Table 2.

Table 2. The prognostic factors in head and neck squamous cell carcinoma according to TNM classification, 9th 2025 (Brierley et al., 2025).

Site	Essential factors	Additional factors
Oral cavity	TNM, resection margin, lymphovascular invasion, performance status, smoking during RT[1]	Tumor budding, tumor hypoxia, lymph node ratio, perineural invasion, worst pattern of invasion, PD-L1, tumor grade, age, betel/areca nut chewing
Oropharynx	TNM, resection margin, performance status, smoking during RT	HPV genotype, tumor volume, hypoxia, PD-L1, age, comorbidity, number of involved nodes (HPV-independent)
Nasopharynx	TNM, histological type, age, performance status, comorbidities	Plasma EBV DNA, tumor volume, site of metastases, SUVmax, PD-L1
Larynx & hypopharynx	TNM, vocal cord mobility, resection margin, performance status, smoking during RT	Number of involved nodes, number of cartilage involvement, regions/subsites involved, tumor volume, hypoxia, PD-L1, age, comorbidity
Nasal cavity & paranasal sinuses	TNM, surgical resection margin, performance status	Histotype, PD-L1, HPV/p16 status, age
Unknown primary (cervical nodes)	Histology, N category, M category, HPV/p16 status or EBV DNA status, immunosuppression	Tumor differentiation or grade, nodal location, PD-L1, gender, hemoglobin, smoking

[1] RT: radiotherapy, TNM: tumor-node-metastasis staging, EBV: Epstein-Barr virus, DNA: deoxyribonucleic acid, PD-L1: programmed death ligand 1, HPV: Human papilloma virus, SUVmax: maximum standardized uptake value in positron emission tomography imaging.

Tumor grading is one of the oldest histopathological tools, traditionally based on microscopic assessment of how aggressive a tumor appears and thus reflecting its biological behavior (Broders, 1921). In HNSCC, grading relies on the degree of resemblance to normal squamous epithelium, considering keratinization, cytological atypia, nuclear features, mitotic activity, and stromal reaction, broadly following Broders' criteria (Broders, 1920, 1921). Despite its historical use, grading in HNSCC appears to have limited prognostic utility. The WHO 5th edition does not recognize it as a reliable prognosticator, and in TNM 9th it is mentioned only in oral cavity and unknown primary carcinomas as an additional, non-essential factor (Table 2). (WHO Classification of Tumours Editorial Board, 2023; Brierley et al., 2025). In contrast to several other cancers, notably prostate carcinoma where histological grading (Gleason grading) has fundamental prognostic value, grading in HNSCC does not influence staging or treatment decisions. Overall, its clinical utility remains limited. (Epstein et al., 2016).

The evolution of prognostic factor grids in HNSCC has not followed the path many expected: rather than embracing molecular markers, the 9th edition of the TNM classification has underscored the continuing importance of histopathology. Traditional parameters remain central, and even new histopathological features such as tumor budding and worst pattern of invasion have been incorporated (Table 2) (Almangush et al., 2015; Mäkitie et al., 2019). Meanwhile, most molecular pathology markers highlighted in the 2017 "new and promising" category were omitted from the 2025 edition, resulting in a contraction rather than expansion of molecular factors (Brierley et al., 2017, 2025). The only major exception is PD-L1, now listed as a notable prognostic factor in all head and neck sites, although in routine practice it serves primarily as a predictive biomarker for response to immune checkpoint inhibition.

Current tools cannot reliably distinguish patients who require treatment intensification from those who might benefit from de-intensified regimens with reduced long-term morbidity. Rapid advances in molecular profiling, biomarker discovery, and imaging technologies have intensified efforts to develop integrated prognostic models (Leemans et al., 2018; Zeng et al., 2020). Yet, translation of such tools into routine practice has been slow, and validated, widely applicable biomarkers remain scarce. This gap hampers progress toward truly personalized therapy in HNSCC.

2.1.6 Treatment

The curative therapeutic options for HNSCC includes surgery, radiotherapy (RT), chemotherapy (CT), chemoradiotherapy (CRT) and targeted therapies including immuno-oncological agents. The choice of treatment depends on the tumor site and stage. Localized, non-advanced disease is typically managed with surgery or RT, whereas locally advanced disease often requires multimodality treatment. Cisplatin-based CRT remains the traditional first-line standard for many HNSCC patients. For recurrent or metastatic disease, targeted therapies may also be considered (National Comprehensive Cancer Network, 2025).

The overarching aim of these treatment modalities is to improve patient outcomes. However, challenges remain with the decreased survival rate of HNSCC, and the long-term treatment-related toxicities such as dysphagia, xerostomia, and osteoradionecrosis, underscoring the need for more precise therapeutic stratification. With the advent of novel therapeutic approaches, the determination of the optimal modality, sequencing, and combination of cancer treatments for individual HNSCC patients is currently in transition (Machiels et al., 2020). A persistent challenge across all modalities is the lack of reliable predictive biomarkers to guide treatment selection (Leemans et al., 2018).

2.1.6.1 Radio- and chemoradiotherapy

RT has been used for more than a century, with therapeutic X-ray applications emerging shortly after the discovery of X-rays in 1895–1896. Its tumoricidal effect stems from ionizing-radiation–induced DNA damage that cancer cells are relatively inefficient at repairing. Rapidly proliferating cells are particularly susceptible to such damage. If this damage persists, cells may undergo apoptosis, mitotic catastrophe, or permanent growth arrest (senescence). (Eriksson & Stigbrand, 2010; Sia et al., 2020). In addition, radiation can elicit ICD, characterized by the release or exposure of DAMPs that promote antigen presentation and antitumor T-cell priming. Beyond its direct effects on malignant cells, radiation also remodels the TME. (Citrin, 2017; Zhu et al., 2022).

Chemotherapy denotes pharmacologic cancer treatment that disrupts cell division and DNA function. In HNSCC, the most widely used agent is the platinum compound cisplatin, which forms intra- and interstrand DNA crosslinks, thereby blocking replication and transcription and inducing lethal DNA damage. Because repair pathways are often compromised in cancer, this injury culminates in apoptosis, necrosis, or mitotic catastrophe. (Jordan & Carmo-Fonseca, 2000; Roos & Kaina, 2013). Cisplatin also radiosensitizes tumor cells—by impairing DNA repair and amplifying radiation-induced damage—providing the rationale for cisplatin-based CRT (Sharma & Wilson, 1999).

Preclinical and translational studies increasingly delineate how oncologic therapies bidirectionally modulate the TIME: RT/CRT can be immunostimulatory (e.g., ICD, increased antigen presentation, enhanced T-cell infiltration) but also immunosuppressive (e.g., lymphopenia, expansion of regulatory T cells) (Citrin, 2017). In line with these observations, RT is increasingly discussed as an immunomodulatory adjuvant rather than purely a local modality. The prognostic and therapeutic implications of these immunologic countervailing effects remain incompletely understood, underscoring the need for deeper characterization of RT/CRT-induced immune remodeling in HNSCC.

The most significant limitation of RT/CRT, however, is the absence of robust predictive biomarkers for the treatment response to identify cancer patients who will benefit from RT/CRT treatment (Sobti et al., 2025). It is well established that verrucous squamous cell carcinoma of the oral cavity is relatively radioresistant, whereas HPV-positive SCC of the oropharynx is generally radiosensitive; beyond these, no clinically validated biomarkers reliably predict RT/CRT response in routine practice (Özcan-Wahlbrink et al., 2019; Shear & Pindborg, 1980).

2.1.6.2 Targeted therapies and predictive markers

Currently, targeted therapy options in HNSCC include the epidermal growth factor receptor (EGFR) inhibitor and immune checkpoint inhibitors. Their clinical use has so far been restricted to advanced or recurrent disease, and they have not replaced conventional cisplatin-based CRT, largely due to the absence of robust predictive biomarkers to guide treatment selection.

Cetuximab, an EGFR inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2006, was the first targeted agent introduced for HNSCC (Bonner et al., 2006). Cetuximab is thought to exert its antitumor effect through dual mechanisms: by blocking EGFR-mediated signaling pathways and by activating antibody-dependent cellular cytotoxicity (ADCC). In ADCC, antibodies are designed to selectively bind malignant cells, thereby recruiting immune effector cells for targeted cytolysis (Sun et al., 2021). However, in HNSCC no reliable predictive biomarker for cetuximab response has been identified to date, which has limited its broader clinical utility.

Immunotherapeutic options for HNSCC include the PD-1 inhibitors pembrolizumab (FDA approved in 2016 for second-line treatment and in 2019 for first-line therapy) and nivolumab (FDA approved in 2016) (Ferris et al., 2016; Chow et al., 2016; Burtness et al., 2019). Both belong to the class of immune checkpoint inhibitors and function by blocking the PD-1 receptor. Their pharmacodynamic mechanism involves releasing the inhibitory “brake” exploited by tumor cells to evade immune-mediated cytotoxicity, thereby restoring antitumor immune activity. In addition, PD-1 blockade can reinvigorate exhausted T cells and partially restore their effector function (Ai et al., 2020).

For pembrolizumab, clinical indication requires IHC demonstration of programmed death-ligand 1 (PD-L1) expression in either tumor cells or tumor-infiltrating immune cells (lymphocytes, macrophages). To standardize clinical trial protocols, two scoring systems for PD-L1 IHC were developed: the tumor proportion score (TPS) and the combined positive score (CPS) (Figure 6). Both systems were validated in regulatory trials and subsequently adopted by the FDA as official criteria for therapeutic indications (Burtness et al., 2019). Nevertheless, PD-L1 testing remains complex and partly subjective (Crosta et al., 2021). Not all patients meeting the criteria respond to treatment, while some who could benefit are excluded by current thresholds (Burtness et al., 2019). Thus, more accurate predictive biomarkers are needed to optimize patient selection for immuno-oncological therapies.

TPS (Tumor Proportion Score)

$$\text{TPS} = \frac{(\text{\#PD-L1–positive tumor cells})}{\text{total number of viable tumor cells}} \times 100$$

CPS (Combined Positive Score)

$$\text{CPS} = \frac{(\text{\#PD-L1–positive tumor cells} + \text{\#PD-L1–positive lymphocytes} + \text{\#PD-L1–positive macrophages})}{\text{total number of viable tumor cells}} \times 100$$

Figure 6. FDA-approved scoring systems for immunohistochemical PD-L1 staining.

Several other targeted therapies are under investigation in HNSCC. These include additional immune checkpoint inhibitors (such as PD-L1 and CTLA-4 inhibitors), HER2-directed agents (e.g., monoclonal antibodies like trastuzumab or antibody–drug conjugates for HER2-amplified tumors), VEGFR/angiogenesis inhibitors, compounds targeting the PI3K/AKT/mTOR signaling pathway, and FGFR inhibitor (Y. Liu et al., 2024).

2.1.6.3 Neoadjuvant therapies and combinatory approaches

Oncological treatments administered after surgery are referred to as adjuvant therapies, whereas those given before surgery are called neoadjuvant therapies. The aims of neoadjuvant treatment are to shrink the tumor, facilitate surgical resection, and eradicate potential micrometastases at an early stage (Contrera et al., 2025). Cancers in which neoadjuvant therapy constitutes the standard of care include selected sarcomas, breast cancer, esophageal cancer, and rectal cancer; commonly used neoadjuvant treatment modalities in these settings are RT, CT, and CRT. More recently, immuno-oncological therapies have also been applied in the neoadjuvant setting. Given the immunomodulatory effects of RT/CRT, there is growing interest in combining these modalities with immune checkpoint inhibitors. Mechanistically, ICD-driven systemic antitumor immunity after RT provides a plausible basis for synergy between CRT and immunotherapy, for which recent combination studies have reported encouraging results (Economopoulou et al., 2019).

In HNSCC, neoadjuvant approaches were explored decades ago, but consistent survival benefits were not demonstrated, and these strategies therefore did not enter routine practice (Pignon et al., 2009). With the advent of immuno-oncological agents, interest in neoadjuvant therapy—using conventional modalities (RT/CRT), immune checkpoint inhibitors, cetuximab, or their combinations—has re-emerged (Contrera et al., 2025). Several studies and phase II–III trials are currently underway to evaluate the efficacy and safety of neoadjuvant regimens in HNSCC (Kimura et al., 2025; C. Liu et al., 2024; López et al., 2025; Sadeghi et al., 2025; Smussi et al., 2023; Wu et al., 2024; Z. Zhang et al., 2022). From this perspective, it remains important to continue rigorous investigation of the prognostic determinants and immunomodulatory effects of traditional oncological treatments alongside the expanding research on targeted and immuno-oncological therapies (Morel et al., 2024).

2.1.6.4 Treatment response evaluation

Treatment response in cancer can be assessed clinically, radiologically, histopathologically, or with biological markers. Clinical assessment relies on inspection and palpation of the tumor site, whereas radiological assessment compares imaging findings (computer tomography, magnetic resonance imaging, positron emission tomography) with the pre-treatment baseline and is widely used in practice. In HNSCC, an example of a biological marker is EBV DNA: circulating EBV DNA assays are used to monitor response in non-keratinizing nasopharyngeal carcinoma (Lin et al., 2004; Lv et al., 2019).

Histopathological treatment response (HTR) is evaluated from post-treatment biopsies or resection specimens by examining the tumor parenchyma and stroma for therapy-induced changes. For several cancer types, prognostically validated systems exist (e.g., Becker for gastric carcinoma; Modified Ryan for rectal adenocarcinoma), but no standardized method is available for HNSCC. In these carcinoma HTR grading systems, grading relies on the proportion of viable tumor relative to regressed areas (tumor regression grade, TRG). ((Becker et al., 2003; Ryan et al., 2005).

With increasing use of neoadjuvant therapy, major pathology organizations including the International Collaboration on Cancer Reporting (ICCR) and the Royal College of Pathologists of Australasia—recommend reporting histological tumor regression after neoadjuvant therapy also in HNSCC (International Collaboration on Cancer Reporting (ICCR), 2025a; The Royal College of Pathologists of Australasia, 2025). Several recent HNSCC studies employing immuno-oncologic regimens define.major pathologic response (MPR) as <10% residual viable tumor and non-PR (pathologic response) as ≥10%. While this two-tier system associates with improved overall and disease-free survival and local control, its reproducibility remains suboptimal (Cao et al., 2025).

Percentage-based approaches suffer from subjectivity and reproducibility issues. From a pathologist's perspective, limitations include uncertainty about the treatment-naive tumor size, sampling bias and spatial heterogeneity of response, sensitivity to the chosen cutoff, and interobserver variation—each constraining standardization (Janssen et al., 2022). A practical, harmonized and standardized HTR system for HNSCC would be therefore both timely and clinically valuable. Desirable features include demonstrated prognostic value for overall survival, disease-free survival, and local control; simplicity and ease of adoption; few categories; high reproducibility with low intra- and interobserver variability; cost-effectiveness; global applicability without ancillary or costly stains; and suitability for AI integration to minimize subjectivity (Deutsch et al., 2025).

2.1.7 Digital pathology and artificial intelligence

Over the past decade, histopathology has undergone a substantial digital transformation, shifting from conventional light microscopy towards the review of digitalized whole-slide images on computer displays. This transition requires high-resolution scanners, medical-grade monitors, and secure digital archiving. Digitization is not only foundational for efficient workflow management but also a prerequisite for implementing artificial intelligence (AI) in diagnostic pathology.

Turku University Hospital (TYKS) has been a national pioneer in implementing digital pathology in Finland. Validation of slide scanning on the Philips platform was completed in September 2021, after which digitization was rolled out across specimen types. By May 2022, large-format macro slides were included, and today nearly all histological specimens are scanned. Exceptions—still assessed with conventional microscopy—include frozen sections, immunofluorescence, fluorescence in situ hybridization (FISH), and cytological preparations.

The digital workflow has improved efficiency: in 2024, TYKS pathologists reviewed ~8% more slides per working day than before digitization (Exploration of Artificial Intelligence Applications for Improving Diagnostic Efficiency, 2024). These findings accord with broader reports of increased throughput and reduced turnaround times following digital adoption (Hanna et al., 2019).

Although AI tools are not yet in routine clinical use at TYKS, several CE-marked in vitro diagnostic (IVD) systems—compliant with the In Vitro Diagnostic Medical Devices Regulation (IVDR; EU 2017/746) and compatible with the Philips platform—are being evaluated. Barriers to adoption include limited funding as well as legal, regulatory, and governance uncertainties. While AI shows promise in image analysis, concerns persist regarding liability for diagnostic errors, algorithmic transparency and reproducibility, and the need for robust, multi-site validation. Any AI tool must also undergo local verification before clinical deployment, in line with national and EU requirements.

Even without AI, digital pathology has already transformed routine diagnostics. Tumor-related metrics—such as size, mitotic rate, depth of invasion, and surgical margin assessment—are more readily and reproducibly measured on screen than at the microscope. This is particularly relevant in complex head and neck resections, where precise anatomical orientation is critical. Digital slides have likewise enhanced multidisciplinary tumor boards: real-time display of key histopathological findings—such as margin status in relation to adjacent anatomical structures—supports more informed decision-making. Digitization also enables rapid national and international consultation, if data protection and patient confidentiality are rigorously maintained.

In head and neck pathology, AI could further improve accuracy by distinguishing for instance potentially malignant from reactive epithelial changes, differentiating low- from high-risk tumors, quantifying tumor-infiltrating lymphocytes, interpreting PD-L1, or more broadly characterizing the TME. Moreover, predicting therapeutic response or assessing post-therapeutic changes would have significant clinical utility. Emerging work suggests that AI may soon reduce ancillary IHC in selected contexts—for example, differentiating HPV-positive from HPV-negative HNSCC and reducing observer variability (Hieromnimon et al., 2025).

Looking ahead, AI systems are expected to integrate clinical, radiological, histopathological, molecular, and genomic data in support of precision oncology (Song et al., 2025). Nonetheless, unresolved issues around clinical accountability, reproducibility, certification, and sustainable funding continue to slow widespread adoption.

2.2 Cancer-germline antigens

Cancer-germline antigens (CGAs)—also called cancer–testis antigens—are proteins normally expressed only in germ cells but aberrantly expressed in cancer cells. They are absent from healthy somatic tissues. (Simpson et al., 2005). Many CGAs fulfill diverse cellular functions, including roles in RNA-regulatory processes, cell cycle control, and stress responses, which tumor cells can exploit to support their growth and survival (Gibbs & Whitehurst, 2018). The regulation of CGA expression is largely epigenetic, yet the precise reasons for their activation in tumors remain incompletely understood (C. Wang et al., 2016). Certain CGAs may confer a selective growth advantage to tumor cells—for example by promoting proliferation, invasion, or tolerance to DNA damage (Bruggeman et al., 2018; Gibbs & Whitehurst, 2018).

The best-studied CGAs include the (Melanoma Antigen Gene (MAGE) family and New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1), both reported in HNSCC (Laban et al., 2014). In routine diagnostics, the most widely adopted CGA is PRAME (Preferentially Expressed Antigen in Melanoma), used as an IHC marker to help distinguish malignant melanocytic tumors (melanomas) from benign melanocytic lesions (e.g., nevi).

In theory, a similar approach could be applied in HNSCC—for instance to differentiate potentially malignant lesions from reactive epithelial changes. Although some evidence supports this possibility, CGAs have not yet reached practical application in HNSCC diagnostics—nor broadly in other epithelial malignancies (Baran et al., 2019; Ries et al., 2012). The utility of CGAs as treatment-response predicting biomarkers remains insufficiently studied.

2.2.1 Cancer-germline antigens and immunity

CGAs are of particular immunological interest because they can be recognized as non-self by cytotoxic T lymphocytes. Germ cell differentiation occurs in an immune-privileged environment, and immune tolerance against germ cell–restricted antigens is therefore not established. (Bruggeman et al., 2018; Huijbers et al., 2012). For such recognition to occur, CGAs must be presented on major histocompatibility complex (MHC) molecules and thus made visible to immune cells. Cancer therapies that induce tumor cell death may facilitate this process by releasing intracellular CGAs into the tumor microenvironment, thereby enhancing antigen presentation and potentially stimulating immune responses against tumor cells (Golden & Apetoh, 2015).

In immuno-oncology, numerous strategies aim to activate or boost anti-tumor immunity. Owing to their inherent immunogenicity, CGAs could in principle serve as natural immune triggers, and they have therefore been explored as potential targets for cancer vaccines (Meng et al., 2021). However, clinical trials have so far not yielded major breakthroughs. One major challenge is how to expose CGAs effectively within the tumor milieu to allow efficient immune recognition and T-cell activation (Yang et al., 2021).

It has been proposed that simultaneous release of damage-associated molecular patterns (DAMPs) is required for robust immune activation. DAMPs such as calreticulin, extracellular ATP, and HMGB1 are normally sequestered within cells but are released during immunogenic cell death (ICD). (Krysko et al., 2012). As introduced in Section 2.1.4.1, these signals serve as “danger” cues that attract and activate dendritic cells and macrophages. The phagocytes can then engulf dying tumor cells and their released CGA proteins, process them, and present CGA-derived peptides on MHC class I molecules, leading to the activation of CGA-specific CD8$^+$ T cells capable of killing remaining tumor cells expressing these antigens (Figure 7) (Janssens et al., 2024).

Some studies suggest that RT or chemo-immunotherapy can enhance T-cell responses to CGAs, consistent with ICD-mediated antigen presentation. Conversely, CGAs have also been associated with CD8$^+$ T-cell evasion, underscoring the need for further research (Kortleve et al., 2022).

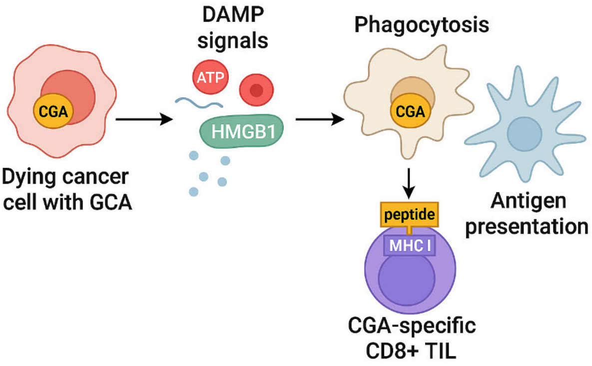


Figure 7. Schematic drawing of immunogenic cell death related to cancer-germline antigens. Image created with ChatGPT (OpenAI) based on the author's prompt.

2.2.2 DEAD-box helicase 4 (DDX4)

DEAD-box helicases constitute an enzyme protein family with important regulatory functions in RNA metabolism and immune response signaling pathways with context-dependent role in cancer cells (Fuller-Pace, 2013). Within this family, DEAD-box helicase 4 (DDX4) is unique in being germline-specific: it plays a central role in spermatogenesis as a key component of cytoplasmic germ granules, which are essential for RNA regulation and spermatogenesis (Tanaka et al., 2000; Lehtiniemi & Kotaja, 2018). Unlike other family members, DDX4 is not expressed in somatic cells, but it has been found to be aberrantly expressed in certain cancers, including ovarian and lung cancer as well as hematological malignancies, thereby classifying it as a CGA (W. Chen et al., 2018; Noyes et al., 2023; Schudrowitz et al., 2017).

In the germ cell lineage, DDX4 functions in close association with the PIWI–piRNA pathway. PIWI proteins bind PIWI-interacting RNAs (piRNAs), a class of small non-coding RNAs that are essential for transposon silencing and post-transcriptional gene regulation during spermatogenesis. Together with DDX4, PIWI proteins localize to cytoplasmic germ granules, where they form ribonucleoprotein complexes central to germline RNA homeostasis (Tanaka et al., 2000; Lehtiniemi & Kotaja, 2018). In somatic tissues, however, the PIWI–piRNA pathway is normally inactive, and its aberrant activation in cancer has been reported only in selected contexts and remains controversial.

While the germ granule functions of DDX4 in spermatogenesis are well characterized, its localization and role in cancer cells remain far less understood and DDX4 has been relatively little studied in cancer biology (Bruggeman et al., 2018). One reason for this may be that the DDX4 gene itself is rarely genetically altered in tumors; rather, its activation often occurs through epigenetic mechanisms that cannot be detected by conventional DNA-sequencing approaches such as whole-genome or whole-exome sequencing. Beyond protein-level analyses, such changes can be detected by methylome profiling, while transcriptome analysis may also reveal altered gene expression. However, germline-restricted genes such as DDX4 often exhibit short-lived transcripts that are tightly regulated at the post-transcriptional level. This likely contributes to their weak or transient detection in transcriptomic datasets despite protein-level evidence of expression. (Bruggeman et al., 2018). This likely contributes to underrepresentation of DDX4 in large-scale cancer studies.

In the limited number of cancer studies that have been published, DDX4 has consistently been linked to malignant phenotypes. In small cell lung cancer (SCLC), DDX4 promotes cell motility and confers resistance to platinum-based chemotherapy, with high expression correlating with inferior survival in large patient cohorts (Noyes et al., 2023). In non-serous ovarian carcinomas, DDX4-positive cells are enriched in advanced-stage tumors and display features of invasiveness, stemness, and chemoresistance (D'Oronzo et al., 2020). Functional studies in ovarian cancer cell lines further demonstrate that siRNA-mediated knockdown of DDX4 reduces invasion, while vitamin D treatment downregulates its expression and decreases proliferation (W. Chen et al., 2018). Moreover, DDX4 co-localizes with the cancer stem cell marker CD133 in ovarian tumors, suggesting a role in sustaining tumor-initiating cell populations (Kim et al., 2014). Beyond solid tumors, DDX4 has also been detected in multiple hematological malignancy cell lines, where its depletion impairs proliferation and migration, although clinical correlations remain limited (Schudrowitz et al., 2017).

Taken together, these findings suggest that aberrant DDX4 expression may promote tumor aggressiveness and stemness, yet its precise role and clinical relevance in cancer remain poorly understood.

3 Aims

The aim of this thesis was to find immunohistochemical and histopathological biomarkers that could be used in the treatment guidance of HNSCC. The specific aims were to:

1. Characterize the tumor immune microenvironment ($CD8^+$ TILs, TAMs, PD-L1) in paired pre-/post-neoadjuvant RT/CRT HNSCC samples and define therapy-induced changes.
2. Identify histopathological predictors of RT/CRT outcome in HNSCC and develop feasible methods for their evaluation.
3. Analyze prognostic histomorphological changes in HNSCCs after RT/CRT and develop a robust histopathological treatment response metric.
4. Determine the clinical relevance of DDX4, including its granule formation and functional roles in HNSCC with complementary analyses in other cancers, and test whether pre-treatment DDX4 expression predicts response to neoadjuvant RT/CRT in HNSCC.

By addressing these objectives, this thesis seeks to contribute to improved treatment stratification and prognostication in HNSCC, with the ultimate goal of improving the clinical management of patients with this disease.

4 Patients, Materials and Methods

4.1 Patients and tumors

The combination of retrospective population-based cohorts, institutional series, and commercial tumor tissue microarrays (TMAs) enabled integrated analyses of tumor histology, the TIME, and DDX4 expression across different treatment settings and cancer types. The cohorts used in each study are listed in Table 3.

Table 3. Summary of patient cohorts, settings and sample types used in Studies I-III and in additional analysis for thesis summary.

Cohort	Patients / Setting	Sample type	n	Study[1]
HNSCC cohort I	Neoadjuvant RT/CRT; paired pre-/post-treatment tumor samples	Whole section (paired surgical specimens)	53 oral (31) oroph (11) larynx (5) other (6)	Study I Study II Additional analysis (for thesis summary)
HNSCC validation cohort I	Oral cavity SCC patients treated with surgery, pre-treatment samples	Whole section (surgical specimens)	49 (oral)	Study I
HNSCC validation cohort II and III	Oropharyngeal and oral cavity SCC patients treated with definitive RT/CRT, pre-treatment samples	Whole section (surgical specimens)	51 (oroph) 71 (oral)	
HNSCC cohort II	HNSCC patients; diagnostic/surgical tumor samples	TMA	46 oral (22) oroph (14 hypoph (7) skin (3)	Study III Additional analysis (for thesis summary)
Prostate cancer cohort (PC)	Prostate adenocarcinoma; radical prostatectomy patients/tumor samples	TMA	164	Study III
Commercial TMA: Multiple organ TP481a	Multiple organ tumor tissue array (AJCC 7th)	TMA	48	
Commercial TMA: Soft tissue T242b	Soft tissue array (fibrosarcoma, leiomyosarcoma); duplicate cores	TMA	12 × 2	

TMA: tissue micraoarray, RT/CR: radio- or chemoradiotherapy, SCC: squamous cell carcinoma, AJCC: American Joint Committee on Cancer. Study I: Oral Oncology (2025), Study II: Submitted (2026), Study III: Cell Reports (2024), Additional analysis (for thesis summary): Unpublished data.

4.1.1 HNSCC (Studies I-III)

To investigate the TIME, cancer germline antigen DDX4 and treatment-related changes in HNSCC, two complementary patient cohorts were utilized. These cohorts were designed to explore both the effects of neoadjuvant oncological treatment (RT/CRT) on TIME and the tumor morphology (HNSCC cohort I in Study I-II) as well as expression of DDX4 in a broader clinical and biological context (HNSCC cohort II in Study III and additional study with HNSCC cohort I).

The HNSCC cohort I was drawn from a large, validated, retrospective, and population-based dataset comprising all newly diagnosed HNSCC cases (n = 1033) in Turku University Hospital region between 2005 and 2015. This cohort reflects a population of approximately 697,000 individuals and includes comprehensive clinical and pathological data. (Denissoff et al., 2022; Mylly et al., 2022; Routila et al., 2021, 2022). Among these patients, 618 had received RT or CRT, predominantly cisplatin-based regimens. A subset of 53 patients who underwent neoadjuvant RT/CRT and had matched tumor samples available both before treatment and within six months after treatment was included in HNSCC cohort I for an in-depth evaluation of treatment-induced changes in the TIME. Whole sections of formalin-fixed, paraffin-embedded (FFPE) tumor tissue, cut at 3.5–5 µm, were used.

To ensure robust evaluation, only patients with curative treatment intention and representative paired tissue samples and complete clinical information were included. All treatment decisions were made by a specialized multidisciplinary tumor board and tumor staging was reassessed using the 8th edition of the UICC/AJCC TNM classification. The majority of patients in this cohort had advanced-stage disease (stage III–IV, 89%) with regional lymph node involvement (81%), and most were treated with CRT (n = 42) or RT alone (n = 11). At five years after diagnosis, 47% of patients had developed recurrence, and 30% were alive. Additionally, a validation cohort of 171 patients was identified from the same population-based registry, including patients treated either with surgery alone (Validation cohort I, n = 49) or with definitive RT/CRT (Validation cohort II and III, n = 122) without neoadjuvant therapy. Only pre-treatment tumor samples from this validation cohort were included for H&E evaluation (Study I).

The HNSCC cohort II for Study III was assembled to enable evaluation of DDX4 expression in a clinically representative setting. 46 HNSCC patients diagnosed and treated at Turku University Hospital were included. In contrast to HNSCC cohort I with whole-section FFPE samples, cohort II employed a TMA built from 0.6 µm FFPE primary tissue cores.

Together, these two cohorts provide a robust framework for investigating both the treatment-related dynamics of the tumor immune environment and tumor histology as well as the potential role of DDX4 in HNSCC pathobiology.

4.1.2 Prostate adenocarcinoma (PC) and other cancers (Study III)

In addition to HNSCC samples, DDX4 expression was also evaluated in prostate and other human cancer types, which are described in more detail below. A cohort of 164 prostate cancer (PC) patients who underwent radical prostatectomy at Tampere University Hospital was included. The median age at diagnosis was 63 years. The mean and median follow-up times from diagnosis were 54 and 38 months, respectively. The median prostate-specific antigen (PSA) level at diagnosis was 10.8 μg/L. TMAs were constructed by extracting 0.6 mm cores from FFPE tumor blocks.

To broaden the cancer-type spectrum analyzed, commercially available TMAs were also utilized. These included: (1) A multiple organ tumor tissue array (TP481a, Tissuearray, USA) with 1.5 mm core diameters, containing normal tissues (breast, colon, and lung), along with breast invasive adenocarcinoma, colon adenocarcinoma, and lung adenocarcinoma samples. (2) A soft tissue TMA (T242b, Tissuearray, USA), also with 1.5 mm core diameters, containing fibrosarcoma and leiomyosarcoma samples. These additional cancer cohorts enabled cross-tumor evaluation of DDX4 expression across epithelial and mesenchymal malignancies.

Collectively, the patient materials provided a comprehensive framework for assessing tumor histology, TIME dynamics and DDX4 expression, thereby complementing the mechanistic insights gained from the experimental cancer models described in the following sections.

4.2 Histopathological methods

4.2.1 Histopathological grading (Studies I-III)

In HNSCC, histological grading was performed according to the three-tiered classification (I: well, II: moderately and III: poorly differentiated) based primarily on keratinization, cytological atypia, nuclear features and mitotic activity broadly following Broders' criteria (Broders, 1920, 1921). Tumor grading was performed in the routine diagnostic setting by pathologists and was not repeated for research purposes.

For prostatic adenocarcinoma, the globally used, updated Gleason grading system was applied (Epstein et al., 2016; Gleason, 1966). The routine diagnostic Gleason score assigned on the prostatectomy specimens and corresponding Gleason Grade Group (1–5) was reported. In addition, each TMA core was independently reviewed by two pathologists, and a Gleason grade pattern (3–5) was assigned for every core; any interpretive discrepancies were resolved by consensus.

4.2.2 Treatment-naive tumor necrosis in HNSCC (Study I)

Histological evaluation was conducted retrospectively using routine diagnostic FFPE treatment-naive tumor samples from each patient. Whole-tissue sections of 3.5–5 µm thickness were prepared and stained with hematoxylin and eosin (H&E) using the Ventana HE 600 system (Roche Diagnostics, Basel, Switzerland).

In treatment-naive HNSCC samples, intratumoral histologic coagulative necrosis was identified according to established diagnostic criteria defined as areas of confluent eosinophilic cell death with nuclear ghosting located within invasive carcinoma, explicitly excluding ulcerated regions (V. Kumar et al., 2020). To improve reproducibility and minimize interobserver variability, necrosis was assessed as a binary variable (present/absent).

All slides were reviewed by two pathologists blinded for the patient outcome. Any discrepancies in interpretation were resolved by consensus.

4.2.2.1 Artificial intelligence-based method

To develop the AI-based necrosis detection algorithm, digitized whole-slide images were uploaded to Aiforia Create (Aiforia Technologies, Helsinki, Finland), a commercially available cloud-based deep learning platform for histopathological image analysis. For this purpose, a set of 20 HNSCC H&E-stained slides from Study cohort I, with and without necrotic areas, were uploaded to a commercial cloud-based deep learning platform. H&E-stained slides were scanned using a Pannoramic 1000 digital slide scanner (3DHISTECH H-1141, Budapest, Hungary). The algorithm was trained in a supervised manner using pathologist-annotated regions of necrosis and viable tissue, and its performance was evaluated on an independent validation set.

4.2.3 Histopathological treatment response in HNSCC (Study II)

Post-treatment tumor diagnostic whole section samples were evaluated for key histopathological features indicative of treatment response, including tumor vitality, tumor regression grade (TRG), and the presence of CD68-positive histiocytic MGC reaction.

Tumor vitality was scored as *present* if any viable tumor cells were identified and *absent* if no viable tumor cells were observed in the specimen.

TRG assessment was performed using a semi-quantitative approach based on previously established regression grading systems. The method was originally introduced by Mandard et al. for esophageal SCC and later adapted into a three-tiered system by Ryan et al. for rectal adenocarcinoma (Mandard et al., 1994; Ryan et al., 2005). Tumor regression was classified as follows: 0: No response or only minor regression, 1: Partial regression with residual viable carcinoma and 2: Complete regression with no evidence of viable tumor cells. In cases of complete pathological response (TRG 2), the presence of immunologic or stromal reactive changes was required to confirm that the tissue sample was derived from the original tumor site.

MGC reaction was evaluated within the tumor bed and classified as *present* if any histiocytic giant cells were identified, and *absent* if none were seen. Assessment was carried out using H&E staining, but CD68-positivity was seen in all histiocytic giant cells.

4.3 Antibody based tissue staining

To explore both immune contexture and cancer–germline biology in patient samples, IHC and immunofluorescence (IF) analyses were performed using FFPE tissues. In HNSCC, paired pre-/post-treatment specimens (HNSCC cohort I) were assessed to characterize the TIME and treatment-induced changes, focusing on TILs, TAMs, expression of the immune checkpoint ligand PD-L1, and HPV association (p16 as a surrogate marker). In parallel, DDX4 protein expression was evaluated across HNSCC (Cohort I and II), prostate cancer, and additional epithelial and mesenchymal malignancies using TMAs, enabling cross-tumor assessment of its prevalence and clinical associations. Together, these immunostaining approaches provided a complementary view of the TME and the role of DDX4 in human cancers. Table 4 summarizes the antibodies, working dilutions, staining patterns, scoring methods, and cutoffs used for the immunostaining analyses and indicates the cohorts to which each antibody was applied.

Table 4. Antibodies, working dilutions, staining patterns, scoring methods, and cutoffs used for the immunostaining analyses, with cohorts applied to. Cohort details are presented in Table 3.

Antigen	**Cohort applied to**	**Antibody**	**Dilution**	**Staining**	**Scoring**	**Cutoffs**
CD8	HNSCC cohort I	Ventana SP57	1:800	cytoplasmic staining of cytotoxic T lymphocytes (TILs)	semi-quantitative; amount of positive intraepithelial TILs (iTIL) and stromal TILs (sTIL)	non or low (0-1) high (2)
CD68	HNSCC cohort I	Dako M0876	1:100	cytoplasmic and membranous staining of all TAMs	semi-quantitative; number of positive TAMs	non or low (0-1) high (2)
CD206	HNSCC cohort I	Cell Signaling	1:1000	cytoplasmic and membranous staining of a subset of M2 TAMs	semi-quantitative; number of positive TAMs	non or low (0-1) high (2)
Clever-1	HNSCC cohort I	Clone 2-7, in-house	supernatant, undiluted	cytoplasmic and membranous staining of a subset of M2 TAMs	semi-quantitative; number of positive TAMs	non or low (0-1) high (2)
PD-L1	HNSCC cohort I, II	Dako 22C3	1:40	membranous staining of vital tumor cells and any staining in TILs and TAMs	TPS and CPS[1]	TPS: 0-9%, 10-49%, ≥ 50% CPS: <1, <20, ≥ 20
p16	HNSCC cohort I	Roche CINtec® Histology kit, E6H4 ™	Ready-to-use	nuclear and cytoplasmic staining of tumor cells	semi-quantitative, amount of positive tumor cells	negative < 70% positive ≥ 70%
DDX4	HNSCC cohort I, II PC cohort Commercial TMAs	Abcam, ab13840	1:10	cytoplasmic granules in cancer cells	semi-quantitative, amount of positive cancer cells	negative (0) positive (1-2)

U.S. Food and Drug Administration. Approval For The PD-L1 IHC 22C3 PharmDx. Published 2019.

4.3.1 TIME in HNSCC (Studies I-II)

Representative FFPE tissue blocks obtained from the same HNSCC patients before and after neoadjuvant RT/CRT were selected for IHC staining to assess changes in the TIME. Serial whole-tissue sections were stained for CD8, CD68, CD206, Clever-1, PD-L1, and p16 with antibodies and dilutions listed in Table 4. Samples with unavailable or technically failed staining were excluded. All staining procedures were performed using the BenchMark ULTRA automated staining system (Ventana Medical Systems, Tucson, AZ), with tonsil, placenta, and ovarian adenocarcinoma tissues included as positive controls. IHC evaluation was carried out independently by two pathologists (ARK and PV), both blinded to disease recurrence and survival status during the five-year follow-up period. Discrepancies in interpretation were resolved by consensus.

CD8 was used to evaluate the density of tumor-infiltrating lymphocytes (TILs), reflecting cytotoxic T-cell activity within the TME. CD8 expression was evaluated separately for intraepithelial tumor-infiltrating lymphocytes (iTILs) and stromal tumor-infiltrating lymphocytes (sTILs), with sTILs defined as $CD8^+$ cells present within one-half of a 20× microscopic field from tumor cell clusters. CD68, CD206, and Clever-1 were included to assess TAMs and to distinguish between different macrophage phenotypes with potential tumor-promoting or tumor-suppressive functions. Macrophage markers were scored based on the average density of positively stained cells in proximity to viable or regressed tumor areas.

For both TILs and TAMs, a semi-quantitative three-tiered scoring system was applied: Pattern 0: No or sporadic positive cells, Pattern 1: Low number of positive cells, Pattern 2: High number of positive cells. For multivariable analyses, staining patterns 0 and 1 were grouped as low expression, while pattern 2 was considered high expression across all immune cell markers.

PD-L1 was analyzed as a marker of immune checkpoint expression, providing insight into mechanisms of tumor immune evasion and potential responsiveness to RT/CRT. PD-L1 expression was assessed using the FDA-approved scoring system, including both Tumor Proportion Score (TPS) and Combined Positive Score (CPS), according to the manufacturer's interpretation guidelines (Agilent Technologies, Inc., n.d.) , with cutoffs defined in Table 4.

p16 expression, used as a surrogate for HPV association, was scored according to WHO guidance: tumors were considered p16-positive and HPV-associated when ≥70% of tumor cells showed diffuse nuclear and cytoplasmic immunoreactivity; this threshold aligns with College of American Pathologists / American Society of Clinical Oncology (CAP/ASCO) recommendations (Fakhry et al., 2018; Lewis et al., 2018).

4.3.2 DDX4 in HNSCC and other cancers (Study III)

IHC was applied to study DDX4 in HNSCC cohort II TMA. FFPE sections were deparaffinized in xylene, rehydrated through graded ethanols, and subjected to heat-induced antigen retrieval in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0; 120 °C, 20 min). After cooling and PBST washes, sections were blocked (10% BSA/PBST, 1 h, RT), incubated with primary antibodies (overnight, 4 °C), quenched for endogenous peroxidase (1% H_2O_2, 20 min, RT), incubated with secondary antibodies (30 min, RT), and developed with DAB. Slides were counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, mounted (Pertex®), and scanned on a Pannoramic P1000 (3DHISTECH). FFPE sections of human testis tissue were used as the positive control.

DDX4 cytoplasmic granular staining in HNSCC cells was scored semi-quantitatively using a study-specific three-tier scale (negative, low, or high). For statistical analyses, low and high expression were combined and considered positive. All samples were evaluated independently by two pathologists blinded to five-year recurrence and survival outcomes, with discrepancies resolved by consensus.

Immunofluorescence was applied to prostate cancer cohort TMA. FFPE prostate cancer TMA tissue sections (4 µm) were deparaffinized, subjected to citrate-based antigen retrieval, and incubated with primary antibodies (Abcam, ab13840) overnight at +4 °C. Alexa Fluor 488/594-conjugated secondary antibodies (Life Technologies) and DAPI (Sigma-Aldrich) were used for visualization, and slides were mounted with ProLong™ Diamond Antifade Mountant (Life Technologies). Z-stack images were acquired with a 3i spinning disk confocal microscope (100×/1.4 NA oil objective, Intelligent Imaging Innovations, USA) and analyzed using SlideBook 6 and ImageJ (NIH).

DDX4 cytoplasmic granular immunofluorescence staining of cancer cells in prostate cancer samples was scored semi-quantitatively as negative, low, or high. For statistical DDX4 analyses, low and high expression were grouped as positive. All samples were evaluated independently by two pathologists blinded to patient outcome, with discrepancies resolved by consensus.

Other cancer TMAs (Multiple organ tumor TMA and sarcoma TMA) were analyzed for cytoplasmic granular expression of DDX4 (absent/present) using IHC.

In summary, the IHC and immunofluorescence approaches provided complementary insights into the TIME and DDX4 expression patterns across patient cohorts and tumor types. These tissue-level analyses were next complemented by molecular characterization of DDX4, aimed at elucidating its mechanistic role in cancer biology.

4.4 Molecular and Functional characterization of DDX4 (Study III)

Building on the histological, IHC and immunofluorescence findings, molecular analyses were undertaken to dissect the mechanistic role of germline-specific RNA helicase DDX4 in cancer. These approaches encompassed transcriptomic profiling, protein–protein interaction studies, and splicing analyses, providing a comprehensive view of how DDX4 contributes to tumor biology at the molecular level. Following methods were used to investigate the role of the DDX4 in cancer.

Prostate cancer (PC-3 human ATCC CRL-1435) and HNSCC (UT-SCC-14 human Cellosaurus CVCL_7810) cell lines were maintained in conventional monolayer culture, grown as three-dimensional spheroids in growth factor-reduced, phenol red-free Matrigel (BD Biosciences), and used to generate xenografts in immunocompromised mice. To induce translational stress, cells were treated with puromycin (Aviner, 2020).

DDX4 knockout (DDX4-null) clones were generated in both PC3 and UT-SCC-14 cells using CRISPR-Cas9–mediated targeted deletion of exon 11. Gene editing was validated by Sanger sequencing, genomic polymerase chain reaction (PCR), and western blotting, and rescue experiments were performed by transient overexpression of a DDX4–GFP fusion construct in knockout cells.

Functional characterization included proliferation and apoptosis assays in two-dimensional culture and spheroid assays in three-dimensional culture in both PC3 and UT-SCC-14 cells to assess growth, morphology, and invasiveness. In vivo tumorigenicity was evaluated using subcutaneous and orthotopic xenograft models in PC3 cells, and vimentin immunostaining (GeneTex, GTX40346) was used as a marker of invasive potential.

Transcriptomic analyses were performed using RNA sequencing (RNA-seq) of wild-type and DDX4-null PC3 cells cultured as monolayers and as xenograft tumors, followed by gene ontology and pathway analysis, as well as evaluation of alternative splicing events, including skipped exons, retained introns, and alternative splice sites.

Proteomic studies were carried out by immunoprecipitation of DDX4-containing complexes from PC3 xenografts followed by mass spectrometry to identify interacting proteins.

Finally, small RNA sequencing was performed on PC3 cells and tumors to investigate the potential involvement of small RNA species in DDX4-associated mechanisms. Selected RNA-seq findings were validated at the transcript level by quantitative real-time PCR (qRT-PCR) and at the protein level by western blotting. Together, these molecular approaches complemented the tissue-based analyses and experimental models, establishing a comprehensive framework to investigate the functional role and clinical relevance of DDX4 in human cancers, as presented in the Results section.

4.5 Statistical analysis (Studies I-III)

Patient characteristics, histopathological variables, and IHC results were analyzed using SPSS (v25.0, IBM, Armonk, USA), SAS (v9.4, SAS Institute Inc., Cary, NC, USA), or GraphPad Prism (v9.0). A significance level of 0.05 was applied throughout.

Changes in the TIME were assessed using the Wilcoxon signed-rank test, with Z and p values reported (Study I-II). For univariate survival analysis, Kaplan–Meier curves were used to estimate 5-year survival in HNSCC and progression-free survival in low-grade prostate adenocarcinoma (Study III), whereas Cox method was used to plot survival function in multivariable models (Studies I-II, HNSCC). For multivariable survival analyses in Studies I-II, age, T-class, nodal status, and alcohol history were included as covariates, based on a previously established prognostic model (Routila et al., 2021). Results are presented as hazard ratios (HR) and 95% confidence intervals (CI).

Logistic regression was applied to evaluate associations between immune markers and combined histopathological treatment response (cHTR), with odds ratios (OR), 95% CI, and p values reported (Study II). Associations between categorical variables were tested with Fisher's exact test, and PSA levels between DDX4 groups were compared using a two-sample t test after log transformation (Study III).

For the analysis of gene expression and cell, spheroid and tumor properties, data were compared using the Mann-Whitney U test, 2-tailed using GraphPad Prism (version 9.0) (Study III). Statistical significance in all analyses (Studies I-III) was set at a p-value of less than 0.05. Details on the statistical method used, sample size (n) and replicates are described in the Methods of the original publications**.**

4.6 Ethical issues

The collection and use of human tissue material were approved by the Finnish National Authority for Medicolegal Affairs (V/39706/2019), the Regional Ethics Committee of the University of Tampere and Turku (51/1803/2017), and the Auria Biobank Scientific Board (AB19-6863). FFPE samples were obtained from the pathology archives via University of Tampere and Auria Biobank, Turku University Hospital, Turku, Finland.

All animal experiments were performed in accordance with Finnish legislation and approved protocols of the Ethical Committee for Animal Experimentation at the University of Turku Central Animal Laboratory. Experiments were conducted in compliance with good laboratory practice under project license number 21485/2020 (Development and use of in vivo cancer models in research and drug development).

5 Results

Results are presented in four parts: (i) Patient and tumor cahracteristics, (ii) TIME and its treatment-induced alterations in HNSCC, (iii) histomorphological predictors and AI-based approach before and after RT/CRT, and (iv) DDX4 granule biology and its clinical significance. This structure clarifies how TIME, histomorphology, AI, and DDX4 findings converge on prognostic and predictive biomarkers.

5.1 Patient and tumor characteristics (Studies I-III)

Clinicopathological characteristics of HNSCC cohorts of studies I-III are presented in Table 5.

Table 5. Patient characteristics of the HNSCC study cohorts.

	Cohort I	**Validation cohort I**	**Validation cohort II**	**Validation cohort III**	**Cohort II**
	RT/CRT Neoadjuvant treated HNSCC patients (n=53)	**Surgically treated HNSCC patients (n=49)**	**RT/CRT treated OPSCC patients (n=51)**	**RT/CRT treated OSCC patients (n=71)**	**HNSCC patients (n=46)**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Sex					
Male	37 (70)	24 (49)	34 (67)	45 (63)	35 (76)
Female	16 (30)	25 (51)	17 (33)	26 (37)	11 (24)
Age at diagnosis					
< 65	31 (58)	9 (18)	40 (78)	40 (56)	NA
> 65	22 (42)	40 (82)	11 (22)	31 (44)	NA
Smoking status					
Current smoker or >30 pack years	26 (49)	12 (25)	18 (35)	32 (45)	20 (43)
Former smoker or 10-30 pack-years	7 (13)	7 (14)	13 (25)	12 (17)	0
Non-smoker or <10 pack years	19 (36)	26 (53)	20 (39)	25 (35)	22 (48)
NA	0	4 (8)	0	2 (3)	4 (9)

	Cohort I	**Validation cohort I**	**Validation cohort II**	**Validation cohort III**	**Cohort II**
	RT/CRT Neoadjuvant treated HNSCC patients (n=53)	**Surgically treated HNSCC patients (n=49)**	**RT/CRT treated OPSCC patients (n=51)**	**RT/CRT treated OSCC patients (n=71)**	**HNSCC patients (n=46)**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Alcohol consumption					
Yes	22 (42)	9 (18)	20 (39)	26 (37)	5 (11)
No	31 (58)	32 (65)	27 (53)	38 (54)	37 (80)
NA	0	8 (16)	4 (8)	7 (10)	4 (9)
Primary tumor site					
Oral cavity	31 (58)	49 (100)	0	71 (100)	22 (48)
Oropharynx	11 (21)	0	51 (100)	0	14 (30)
p16 positive	6		32 (63)		
p16 negative	5		11 (22)		
NA	0		8 (16)		14 (30)
Larynx	5 (10)	0	0	0	0
Other	6 (11)	0	0	0	10 (22)
T Class					
T1-2	21 (40)	49 (100)	39 (76)	32 (45)	25 (54)
T3-4	32 (60)	0	12 (24)	39 (55)	21 (46)
N class					
N0	10 (19)	45 (92)	13 (25)	27 (38)	27 (59)
N+	43 (81)	4 (8)	38 (75)	44 (62)	18 (39)
Stage					
I-II	6 (11)	45 (92)	11 (22)	17 (24)	
III-IV	47 (89)	4 (8)	40 (78)	54 (76)	
Recidive in 5 years					
Yes	25 (47)	17 (35)	9 (18)	38 (54)	24 (52)
No	28 (53)	32 (65)	42 (82)	33 (46)	18 (39)
Living at 5 years					
Yes	16 (30)	25 (52)	37 (72)	23 (32)	17 (37)
No, died of HNSCC	31 (58)	12 (24)	8 (16)	36 (51)	
No, died of other cause	6 (11)	12 (24)	6 (12)	12 (17)	
Treatment					
Surgical treatment					
Local operation	40 (75)	49 (100)	0	0	
Neck dissection	43 (81)	22 (45)	31 (61)	43 (61)	
RT/CRT					
RT only	2 (4)	0	4 (8)	29 (41)	
CRT only	2 (4)	0	47 (92)	42 (59)	
RT + surgery	9 (17)	0	0	0	
CRT + surgery	40 (75)	0	0	0	

5.2 TIME characteristics in HNSCC (Studies I-II)

53 HNSCC patients who underwent neoadjuvant RT/CRT and had matched tumor samples available both before and within six months after treatment was selected for in-depth analysis of the TIME (HNSCC Cohort I, Table 3). Whole sections of FFPE tumor samples were used.

To quantify the immune contexture, IHC panel capturing cytotoxic T cells, macrophage subsets, and PD-L1 was selected for IHC analysis to profile the HNSCC TIME; in total, 489 whole-section stainings were analyzed (HNSCC cohort I). CD8 quantified TILs, CD68/CD206/Clever-1 characterized TAMs, and PD-L1 was assessed as an immune-checkpoint marker. This panel provided a standardized snapshot of key immune compartments for downstream association analyses.

The most characteristic TIME profile in the treatment-naive samples was low CD8$^+$ iTILs (n=36/47, 77 %), high CD8$^+$ sTILs (n=28/46, 61 %) and low CD206$^+$ TAMs (n=37/47, 79 %). Most of the tumors had PD-L1 TPS $\geq$ 10% (n=28/46, 61 %) and CPS $\geq$ 20 (n=27/46, 59 %). These baseline frequencies frame subsequent comparisons and show that PD-L1 positivity and stromal CD8 infiltration were common in this cohort.

To understand the post-therapy immune milieu, marker distributions in the post-RT/CRT resections were studied. The post-treatment TIME was dominated by low stromal CD8$^+$ sTILs (n=44/51, 86 %) and low immunosuppressive TAM markers CD206$^+$ (31/50, 62 %) and Clever-1$^+$ TAMs (36/50, 72%), consistent with substantial treatment-driven remodeling.

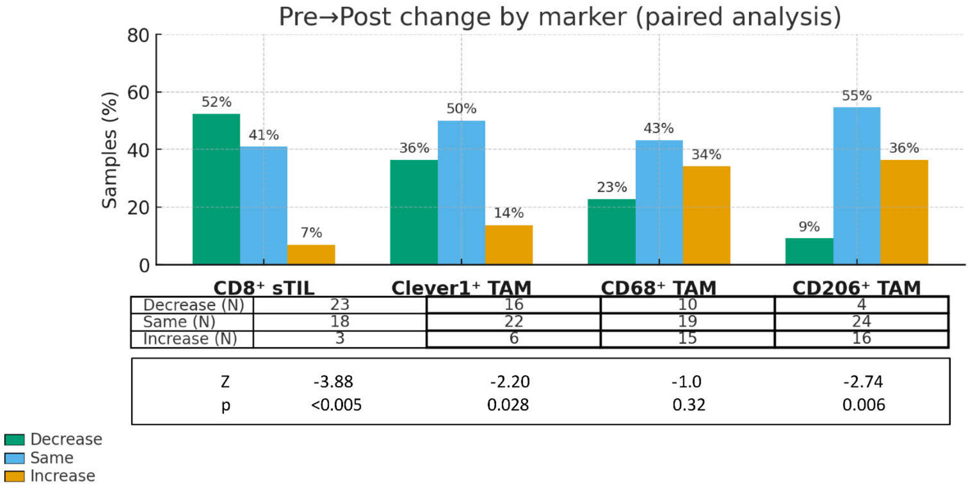
Detailed overall survival analyses with multivariable model revealed that high amount of CD8$^+$ sTILs in baseline HNSCC tumors was significantly associated with improved prognosis in multivariable survival analysis (HR 0.36; 95% CI: 0.16 to 0.79, p=0.011). In addition, high amount of CD206$^+$ TAMs in post-treatment samples (n=19/50, 38 %) were significantly associated with worse overall survival (HR 2.63; 95% CI: 1.22 to 5.65, p=0.013). Interestingly, for patients with residual tumors after RT/CRT, high PD-L1 expression in the tumor cells of post-RT/CRT samples (TPS $\geq$ 10% n=15/25, 60% and TPS $\geq$ 50% n=8/25, 32%) of samples, respectively) was associated with better survival (HR 0.35, 95% CI: 0.12 to 0.97 and HR 0.37, 95% CI 0.14 to 0.94). The results suggest that baseline stromal CD8$^+$ TILs are favorable, whereas post-treatment CD206$^+$ TAM enrichment may signal adverse biology, and PD-L1 in residual tumor may mark improved outcome after RT/CRT.

Because HPV status can influence immune profiles and prognosis in oropharyngeal cancer, we documented p16 status in this subgroup. Among patients with oropharyngeal cancer, 55% (6/11) had p16-positive tumors, indicating HPV-associated disease. However, subgroup level statistical analysis of TIME characteristics was not possible in this work due to the limited number of the subgroup cases.

5.2.1 RT/CRT induced TIME alterations (Study I)

To directly measure therapy-induced immune shifts, paired pre- and post-RT/CRT specimens within the same patients were analyzed (HNSCC Cohort I). Expression of immunological markers in pre-/post-treatment samples from the same patients was compared pairwise and classified as decreased, unchanged, or increased in response to RT/CRT. Pairwise statistical analysis of stromal TILs (sTILs) and macrophage markers (CD68, CD206, Clever-1) was feasible for 46/53 (87%) patients. In contrast, reliable statistical assessment of intratumoral CD8$^+$ TILs and PD-L1 expression was limited, as in a substantial proportion of cases (21/51; 41%) viable tumor cells were markedly reduced or eliminated in post-treatment specimens. Notably, the observed immune changes were largely consistent across primary tumor sites and independent of HPV status (Figure 2 B in Study I).

To pinpoint which immune compartments changed, we evaluated the direction of sTIL and macrophage-marker shifts after RT/CRT. Among the stromal immune cell populations, CD8$^+$ TILs decreased in 52% (n=23/44) of the cases, yielding a statistically significant reduction (p=<0.005). While CD68$^+$ macrophage density remained unchanged, RT/CRT was associated with a significant decrease in Clever-1 expression in 36% (n=16/44) of the cases and a corresponding increase of CD206 expression in 36% (n=16/44) of cases (p=0.006) (Figure 8). Despite these distinct and statistically significant modifications in the TIME following neoadjuvant RT/CRT, none of the observed changes were correlated with overall survival. The absence of a survival association suggests that measurable TIME changes do not translate into prognostic signals in this cohort.



	CD8$^+$ sTIL	Clever1$^+$ TAM	CD68$^+$ TAM	CD206$^+$ TAM
Decrease (N)	23	16	10	4
Same (N)	18	22	19	24
Increase (N)	3	6	15	16

Z	-3.88	-2.20	-1.0	-2.74
p	<0.005	0.028	0.32	0.006

Figure 8. RT/CRT induced changes in TILs and TAMs in TIME.

5.3 Histomorphological features

5.3.1 Histopathological grading (Studies I-III)

Histopathological grading has long been used as a conventional prognostic tool, yet its clinical utility differs markedly between tumor types. In HNSCC, several studies have reported that histological grade is inconsistently associated with outcome and therefore has limited impact on treatment decision-making, whereas in prostate carcinoma grading (Gleason) is a central, treatment-guiding determinant of prognosis. Grading was therefore evaluated in both RT/CRT-treated HNSCC cohorts and in the prostate cancer TMA to (i) benchmark our findings against prior literature and (ii) use grading as a reference standard when evaluating the prognostic performance of novel candidate markers.

In the RT/CRT-treated HNSCC patient cohort (HNSCC cohort I), the impact of the three-tier histological grading system on survival was analyzed. Of the 47 patients, 11 (23%) had well-differentiated grade 1 SCC, 27 (57%) had moderately differentiated grade 2 SCC, and 9 (19%) had poorly differentiated grade 3 SCC. However, the grading did not show a consistent prognostic association: notably, patients with moderately differentiated SCC exhibited better outcomes than those with well differentiated disease (Figure 9) (HR 0.66, 95% CI: 0.31 to 1.41, p=0.28), and a similar pattern was observed in HNSCC cohort II**.** These findings show that conventional grading was of limited prognostic value in HNSCC in this treatment context and had only a limited role in baseline risk stratification.

In contrast, as an internal control in a tumor type where grading is expected to be clinically informative, Gleason patterns (3–5) were recorded for each core in the prostate cancer TMA. Pattern 3 was observed in 74% (n = 122/164) and patterns 4–5 in 26% (n = 42/164) of carcinomas, and grading showed the expected prognostic relationship, with increasing Gleason pattern associated with worse outcome (data not shown). The expected Gleason–outcome relationship supports the validity of our grading workflow and underscores the tumor-type specificity of grading, motivating the search for more reliable tissue-based prognostic and predictive biomarkers in HNSCC.

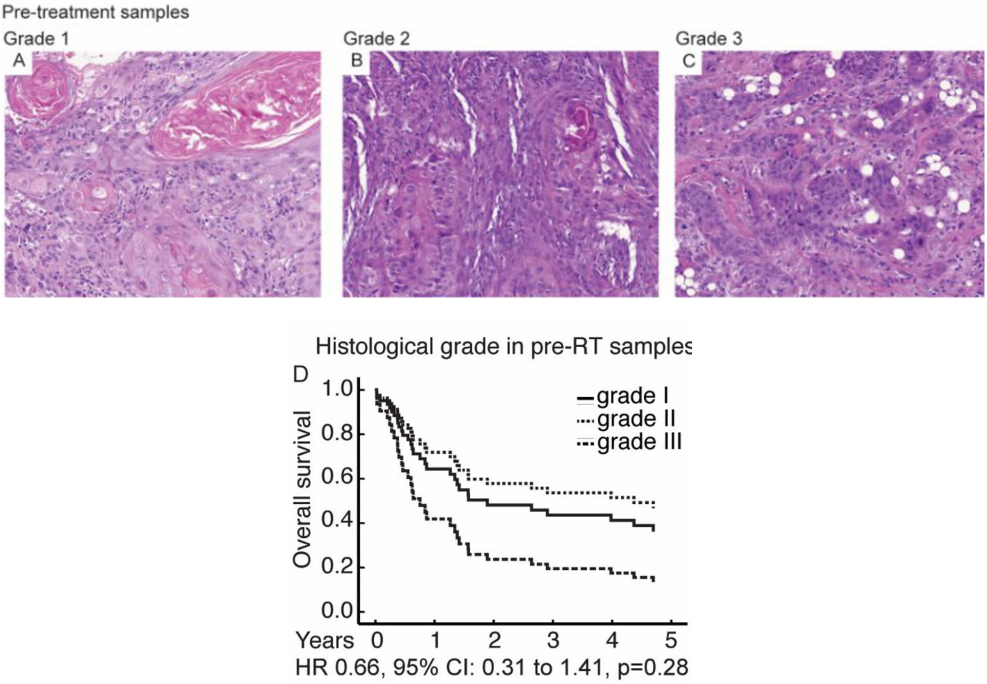


Figure 9. Histopathological tumor grading in head and neck squamous cell carcinoma. **A**: well differentiated grade 1 HNSCC, **B**: moderately differentiated grade 2 HNSCC, **C**: poorly differentiated Grade III HNSCC, **D**: Prognostic significance of histological grading in HNSCC cohort I.

5.3.2 Treatment-naive tumor necrosis as a predictive biomarker in HNSCC (Study I)

To evaluate a simple, clinically accessible morphology marker, baseline tumor necrosis was selected for more detailed analyses. Predictive value of histological tumor necrosis and its potential associations with TILs, TAMs, and PD-L1 expression in treatment-naive tumor samples were studied. Tumor necrosis, defined as present or absent, was identified in 32% (n = 15/47) of pre-neoadjuvant RT/CRT tumors, while 68% (n = 32/47) showed no evidence of necrosis. Morphologically, necrosis was typically observed as small foci within tumor cell islands rather than as extensive confluent areas. The presence of tumor necrosis in pre-neoadjuvant HNSCC samples emerged as a strong predictor of poor outcome, with a 5-year OS of 14.3% in patients with necrosis compared to 48.5% in those without necrosis (HR 2.87; 95% CI: 1.24 to 6.66, p=0.014). Importantly, tumor necrosis was not associated with established clinicopathological prognostic variables, nor with the expression of TIL, TAM, or PD-L1 markers (data not shown). Although tumor necrosis is frequently interpreted as a surrogate for a hypoxic, immunosuppressive tumor microenvironment, this relationship was not evident with the baseline immune markers included in our panel.

To determine whether tumor necrosis is a neoadjuvant–specific marker, the association between necrosis and outcome was assessed in independent HNSCC cohorts outside the neoadjuvant setting. The following primary tumor specimens were analyzed from three validation cohorts comprising oral cavity SCC treated with primary surgery alone (validation cohort I: oral cavity SCC, n = 49) and definitive RT/CRT cohorts (validation cohort II: oropharyngeal SCC, n = 51; validation cohort III: oral cavity SCC, n = 71). In contrast to the neoadjuvant cohort, the presence of tumor necrosis did not correlate with reduced survival in any of these groups (data not shown). The association between necrosis and outcome was observed in the neoadjuvant setting but not consistently across different treatment modalities.

5.3.2.1 AI detection of tumor necrosis

Automated deep learning is a very promising approach to reduce pathologists' manual workload and possibly accelerate necrosis assessment. Therefore, we investigated whether deep learning can automatically detect necrotic areas on H&E-stained sections. The relatively straightforward recognition of histological necrosis in digitized H&E-stained whole-slide images prompted us to explore automated detection using deep learning. Following AI training, testing, and validation, the algorithm demonstrated high accuracy in identifying necrotic regions. The algorithm accurately identified necrotic regions, with area-fraction error rates of 0.02% for tissue outlines and 0.32% for necrosis (false positives), and 0.07% and 0.18% (false negatives), respectively (Study I: Figure 3 D). The low false-positive and false-negative area fractions indicate that AI can quantify necrosis reproducibly, enabling high-throughput studies and potential clinical implementation.

5.3.3 Treatment-induced histomorphological changes in HNSCC (Study II)

Within the scope of the overall project, this part of the study focused on evaluating the effects of neoadjuvant RT/CRT on HNSCC tumor histomorphology and identifying response characteristics that most reliably predict treatment outcome, complementing related analyses of the TIME. In addition, the study aimed to determine how the TIME before and after therapy relates to histopathological treatment response (HTR). Patient selection in HNSCC cohort I appears appropriate in this respect, as the cohort provided representative material for the assessment of both HTR and immune parameters in advanced HNSCC treated with neoadjuvant

RT/CRT. These findings support the suitability of the cohort for integrated HTR–TIME analyses and for deriving robust response metrics.

To identify the most prognostic post-treatment features, we quantified tumor vitality, regression (TRG), and the $CD68^+$ MGC reaction. To assess treatment-induced changes, post-treatment tumor specimens were evaluated for alterations in tumor parenchyma and stroma, relative to treatment-naive samples. Viable tumor was absent in 41% (n=21/51) and present in 59% (n=30/51) of cases. TRG (three-tier) showed at least partial regression (TRG 1–2) in 74% (n=37/50) and, together with tumor vitality, associated with OS in multivariable analysis (Tumor vitality: HR 3.31; 95% CI 1.42 to 7.72, p=0.006 and TRG: HR 0.34; 95% CI: 0.13 to 0.87, p=0.025). A CD68-positive MGC reaction was present in 63% (n=32/51), ranging from small clusters to large aggregates, and independently predicted favorable outcome—outperforming tumor vitality (II: Table 2, Figure 1 h-J). The independent association of MGCs with favorable survival suggests that the host tissue reaction adds prognostic information beyond residual viable tumor alone.

5.3.3.1 Combined histopathological treatment response (cHTR)

To improve interpretability and reproducibility, we condensed heterogeneous treatment response patterns into a two-tier composite response score combined histopathological treatment response (cHTR). While tumors with complete regression (TRG 2) or no regression (TRG 0) showed clear prognostic patterns, the intermediate TRG 1 group—partial regression with residual viable tumor—was heterogeneous and difficult to interpret. Moreover, in practice a two-tier system is easier to apply and more reproducible than a three-tier schema. We therefore developed cHTR parameter that integrates TRG with the presence of MGC. Two-tier grading was defined as follows: good cHTR includes TRG 2 or TRG 1 with MGC; poor cHTR includes TRG 0 or TRG 1 without MGC (Figure 10). Using cHTR, 64% (32/50) were good and 36% (18/50) were poor responders. cHTR provided the strongest prognostic signal (HR 0.21; 95% CI 0.094–0.47; p<0.001), with 5-year overall survival of 59% vs 16%. Among clinical variables, tobacco history showed the strongest—but nonsignificant—association with cHTR. cHTR distinguished good and poor responders, with a marked survival difference observed between the groups after neoadjuvant RT/CRT.

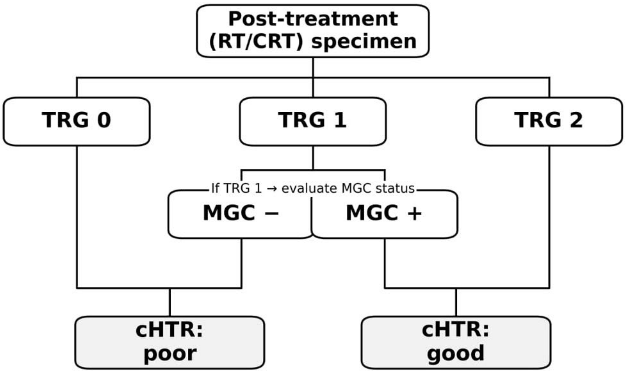


Figure 10. Assessment of combined histopathological therapy response (cHTR). Based on survival analysis TRG 0 and TRG 2 (TRG = tumor regression grade) are significant markers for poor and good therapy response, respectively. TRG 1 group (partial regression with presence of vital tumor cells) was further divided into poor and good therapy response groups by combining the presence of MGC (multinucleated giant cell reaction).

5.3.3.2 cHTR in immunologic context

To connect immune context with histological response, we tested whether pre-/post-treatment TIME markers predict cHTR and MGC formation. To link the TIME with cHTR, we analyzed pre-/post-treatment staining scores for $CD8^+$ TILs, TAM markers, and PD-L1. High intraepithelial $CD8^+$ TIL density (iTILs) and high PD-L1 expression in either tumor or immune cells (TPS ≥50%, CPS ≥20) in pre-treatment samples significantly predicted poor cHTR ($CD8^+$ TILs: HR 7.11; 95% CI: 1.53 to 32.9, p= 0.012 and PD-L1 CPS: HR 4.66; 95% CI: 1.07 to 20.1, p= 0.039). Other immune markers—including stromal $CD8^+$ TILs and TAM markers (CD68, CD206, Clever-1)—were not associated with cHTR in either pre- or post-treatment samples (Table 3 in Study II).

Notably, pre-treatment PD-L1 expression correlated inversely with the presence of post-treatment MGC: cases with PD-L1–positive carcinoma cells rarely exhibited MGC in the corresponding post-RT/CRT samples (Spearman's $\rho = -0.42$; 95% CI -0.64 to -0.13; $p = 0.005$). MGC did not correlate with other immunological markers. Taken together, high intraepithelial $CD8^+$ TIL density and elevated PD-L1 expression at baseline predicted poor cHTR, while the inverse PD-L1–MGC relationship suggests that MGC formation may represent a distinct immune-related response phenotype not fully captured by conventional TIME markers.

5.4 DDX4 in cancer

5.4.1 Characterization of DDX4 granule formation (Study III)

To explore DDX4 as a pan-cancer biomarker, we first mapped where DDX4 granules occur across tumor types and normal tissues (n=4, prostate, breast, colon and lung tissue epithelium and stroma). Normally restricted to germ cells, the germline-specific RNA helicase DDX4 was found to form germ-granule–like cytoplasmic ribonucleoprotein (RNP) granules in a wide range of human malignancies. IHC and immunofluorescence analyses demonstrated these structures in epithelial carcinomas, including HNSCC, prostate, breast, colon, and lung carcinomas, as well as in non-epithelial malignant tumors such as fibrosarcomas and leiomyosarcomas (Figure 1 in Study III). DDX4 granules closely resembled germ granules and were consistently observed across diverse tumor types, while being absent in healthy somatic tissues. DDX4 granules displayed a tumor-restricted, germ granule-like pattern in cancer cells.

To understand formation of DDX4 granules, we examined their appearance across complementary in vitro and in vivo model systems and under defined cellular stress conditions. DDX4 granule formation was not observed by immunofluorescence in standard 2D monolayer cultures of PC3 or UT-SCC-14 cancer cells, although DDX4 protein expression was detectable by immunoblotting and immunofluorescence (Figure 2 B-D in Study III). In contrast, prominent cytoplasmic DDX4 granule formation was consistently induced in both three-dimensional organotypic spheroid cultures and tumor xenografts, indicating that granule assembly depends on microenvironmental conditions that better recapitulate the tumor microenvironment (Figure 2 A-C, E in Study III). To directly test the role of cellular stress, exposure of PC3 cells to the translational inhibitor puromycin was sufficient to induce DDX4 granule formation even in 2D culture (Figure 2 F in Study III). Together, these observations demonstrate that DDX4 granule assembly is a context- and stress-dependent phenomenon, supporting a role for DDX4 granules in tumor adaptation to microenvironmental stress conditions encountered in vivo.

To infer potential functions of DDX4 in cancer, we profiled the protein composition of cancer-associated DDX4 granules by immunoprecipitation and mass spectrometry from PC3 xenograft tumors (Figure 6A, B in Study III). Proteomic analyses revealed that DDX4-associated complexes in vivo are enriched for RNA-binding proteins and splicing regulators, including multiple components characteristic of germ granules, indicating that cancer cells co-opt germline-like RNA regulatory machinery. Enrichment of RNA-binding and splicing regulators suggests DDX4 granules participate in post-transcriptional control programs co-opted by cancer cells.

5.4.2 Functional analysis of DDX4 using knock out and overexpression models (Study III)

To determine whether DDX4 plays a causal role in promoting tumor aggressiveness under conditions that better recapitulate the tumor microenvironment, we investigated its functional requirement in three-dimensional and in vivo tumor models. To test causality, we asked whether DDX4 is required for aggressive behavior in 3D and in vivo tumor models. Functional experiments using CRISPR-Cas9–mediated knockout of DDX4 (DDX4-null cells) in PC3 (prostate) and UT-SCC-14 (HNSCC) models demonstrated that cell proliferation (IncuCyte-based confluence measurements) and apoptosis (Annexin V assay) in 2D cultures were unaffected. In contrast, spheroid growth and invasion (AMIDA-based morphometric analysis), as well as xenograft tumor growth and metastatic burden, were significantly reduced—by approximately 30–60% across assays—in DDX4-null cells compared with wild-type controls.

In vivo, DDX4-null subcutaneous xenografts grew more slowly, were smaller, and exhibited reduced metastatic potential relative to wild-type tumors. Orthotopic inoculation of DDX4-null PC3 cells into the prostate further confirmed a delay in tumor growth and significantly smaller secondary lymph node metastases compared to wild-type tumors. Collectively, the impaired spheroid growth, invasion, xenograft growth, and metastatic phenotypes in DDX4-null cells indicate that DDX4 promotes tumor aggressiveness specifically under TME-like conditions.

To confirm specificity of the knockout phenotype, we performed DDX4 re-expression (rescue) experiments. Overexpression of DDX4 rescued these defects, confirming its functional role in promoting tumor aggressiveness. Rescue experiments support that the observed functional deficits are attributable to loss of DDX4 rather than off-target effects.

Taken together, these findings demonstrate that DDX4 is dispensable for baseline proliferation in conventional 2D culture but is required for aggressive tumor behavior in three-dimensional and in vivo contexts. DDX4 was required for adaptation to microenvironmental conditions, consistent with its tumor-promoting effects observed in vivo.

5.4.3 Transcriptomic and post-transcriptional profiling of DDX4-null cells and tumors (Study III)

To uncover mechanisms, we compared transcriptomes of DDX4-null and wild-type cells and xenografts. Transcriptomic profiling (RNA sequencing) of DDX4-null versus wild-type PC3 prostate cancer cells in vitro, as well as corresponding PC3 xenograft tumors in vivo, revealed extensive transcriptomic changes, with thousands of differentially expressed genes. More than 3,500 genes were dysregulated in 2D culture, and over 140 genes in DDX4-null xenograft tumors, with significant overlap between the two settings. These included upregulation of tumor suppressors (e.g., OPCML, DCC, PROX1) and downregulation of metastasis-promoting genes (e.g., ADAM12, CDH6, CDH7). Transcriptomic changes included also immune checkpoint axis as CEACAM1/5 and ICAM5 were upregulated and CD274 (PD-L1) expression was reduced. Broad dysregulation—including epithelial-mesenchymal transition- (EMT), metastasis-, and immune-checkpoint–related genes—supports DDX4 as an upstream regulator of programs linked to progression and immune modulation.

To validate key molecular signals, selected RNA-seq findings were confirmed at both the messenger RNA (mRNA) and protein levels by quantitative real-time PCR (qRT-PCR) and western blotting**.** This validation demonstrated increased expression of CEACAM1 and reduced expression of SFRP1 and CDH6, as well as increased E-cadherin (CDH1) protein expression in DDX4-null tumors. Together, these molecular changes are consistent with altered epithelial–mesenchymal plasticity and modulation of signaling pathways relevant to tumor invasion.

Because DDX4 is an RNA helicase, we tested whether loss of DDX4 alters alternative splicing patterns. RNA-seq also revealed widespread alterations in alternative splicing: over 1,600 splicing events (in >1,200 genes) were significantly affected in DDX4-null cells, with skipped exons being the most common event. A consistent reduction in intron retention was observed, implicating DDX4 is involved in post-transcriptional regulation of cancer-associated transcripts. Widespread splicing changes and reduced intron retention implicate DDX4 in post-transcriptional transcript shaping in cancer.

To define the molecular context of DDX4 action, we characterized its interaction partners and small RNA signatures in prostate adenocarcinoma cells. Protein interaction analyses revealed that DDX4-containing complexes in cancer cells were enriched for RNA-binding proteins and splicing regulators, including components associated with germ granules and stress granules (e.g., DDX3X, G3BP1).

Given the central role of the PIWI–piRNA pathway in germline DDX4 function, we next examined whether this canonical interaction is preserved in cancer cells. In contrast to its role in the germline, DDX4 did not interact with PIWI proteins, and small RNA sequencing revealed only low levels of piRNA-like reads. Together, these data demonstrate that DDX4 function in cancer cells occurs in the absence of detectable PIWI interactions and without robust activation of the piRNA pathway. The absence of PIWI interactions and the scarcity of piRNA-like reads point to a cancer-specific, piRNA-independent role for DDX4 in stress-associated RNA regulation.

5.4.4 Evaluation of DDX4 expression in patient cohorts

To translate experimental findings, we evaluated the clinical associations of DDX4 expression in HNSCC cohorts II and I. Given experimental evidence that DDX4 drives aggressive tumor phenotypes and may modulate the immune milieu, we investigated its clinical significance across HNSCC and PC. We quantified DDX4 expression and granule formation in HNSCC (cohort II; with complementary analyses in cohort I) and prostate cancer (PC cohort), two epithelial tumor types with divergent natural histories and prognostic frameworks. This enables assessment of whether DDX4 granules track with clinical aggressiveness and patient outcomes.

5.4.4.1 DDX4 in HNSCC and PC (Study III)

To assess prognostic relevance in HNSCC, we quantified DDX4 granules by IHC in cohort II and related them to clinicopathological variables and survival. Cytoplasmic DDX4 granules were observed in 74% (n=34/46) of tumors, with heterogeneous intratumoral distribution. DDX4 expression associated with larger tumor size, higher recurrence rates, and poorer overall survival (Figure 7A and S7A in Study III). These associations support DDX4 as a candidate adverse prognostic biomarker in HNSCC.

To test whether DDX4 associations extend to another epithelial cancer than HNSCC, we analyzed DDX4 granules in a radical prostatectomy cohort by immunofluorescence. In 164 PC patient samples, cytoplasmic DDX4 granules were observed in 74% (n=122/164) of tumors. DDX4 expression in PC epithelial cells was associated with higher Gleason pattern when the 3-tiered primary DDX4 score was used (Figure S7C in Study III). In low-grade disease (Gleason grade group ≤2/5), there was a trend toward worse progression-free survival in patients with DDX4-positive cancer compared to patients with DDX4-negative cancer (Figure 7B in Study III). Links to higher Gleason pattern and worse progression trends suggest DDX4 may mark aggressive biology even within clinically low-grade prostate cancer.

5.4.4.2 DDX4 and TIME

Motivated by indications of immune modulation by DDX4, we explored by IHC whether tumor DDX4 positivity associates with features of the TIME, particularly PD-L1, in treatment-naive HNSCC. Based on the immunogenic and immune-modulating properties of cancer–germline antigens and the transcriptional changes observed in DDX4-null PC3 xenografts—including upregulation of CEACAM1 and downregulation of PD-L1/CD274—we examined potential associations between DDX4 expression and TIME features in HNSCC cohort I. No clear association between DDX4 and TILs, TAMs, or PD-L1 was detected in pre- or post-treatment samples.

To increase power for PD-L1, we analyzed all available treatment-naive HNSCC samples (cohorts I and II combined; n=82). PD-L1 CPS ≥1 was more frequent in DDX4-negative tumors 83.3% (n=30/36) than in DDX4-positive tumors 56.5% (n=26/46) (Figure 11). This exploratory finding should be interpreted cautiously due to potential differences in case mix (site, stage, treatment context) and sampling; TMA cores vs whole sections) between cohorts. While most immune markers showed no relationship, the higher PD-L1 CPS in DDX4-negative tumors is an exploratory signal that warrants confirmation in harmonized cohorts.

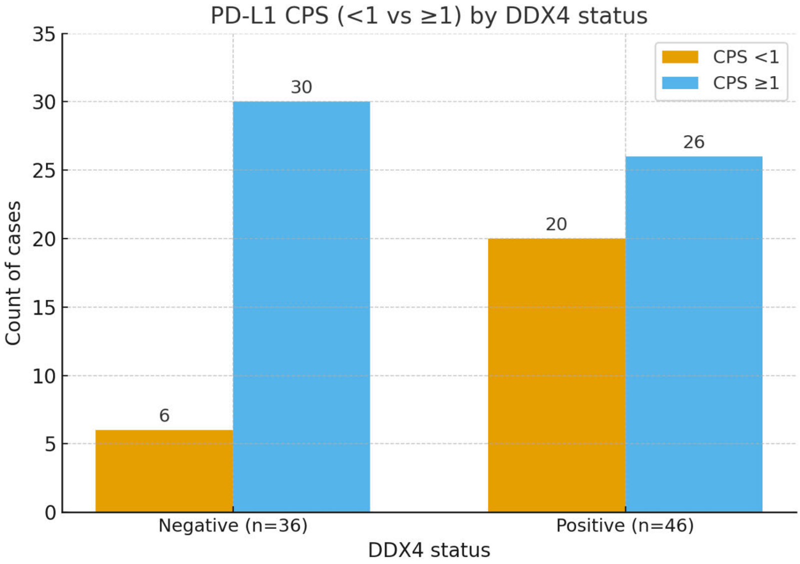


Figure 11. PD-L1 CPS by DDX4-status. Grouped bar chart showing case counts for CPS <1 and CPS ≥1 in DDX4-negative (n=36) and DDX4-positive (n=46) tumors. CPS ≥1 was more frequent in the DDX4-negative group (30/36) than in the DDX4-positive group (26/46); Fisher's exact p = 0.016, OR = 0.26 (positive vs negative), 95% CI 0.09–0.75. Bars display raw counts; labels above bars indicate counts.

5.4.4.3 Predictive significance of DDX4 in RT/CRT

To evaluate predictive (rather than prognostic) utility, we examined whether pre-treatment DDX4 status associates with response to neoadjuvant RT/CRT in HNSCC cohort I. Cytoplasmic DDX4-positive granules were detected by IHC in 38% of treatment-naive tumors (n=17/45). Pre-treatment DDX4 positivity predicted a favorable histopathological response to neoadjuvant RT/CRT (good cHTR; OR 0.15; 95% CI 0.031–0.76; p = 0.022), while not being prognostic within the same cohort.

These findings indicate that DDX4 may serve as a predictive biomarker for histological response to neoadjuvant RT/CRT, rather than a general prognostic marker.

To integrate the DDX4 findings, we summarize how its expression relates to aggressiveness, immune context, and treatment response. Collectively, these findings indicate that DDX4 expression is associated with tumor aggressiveness and adverse outcomes across distinct epithelial malignancies, supporting its potential utility as a broadly relevant cancer biomarker. Importantly, in the neoadjuvant RT/CRT setting, pre-treatment DDX4 positivity was associated with a favorable histopathological treatment response (good cHTR), suggesting predictive value for treatment response. By contrast, in treatment-naive HNSCC samples, PD-L1 CPS ≥1 was more prevalent among DDX4-negative tumors (84% n=37/44) than among DDX4-positive tumors (50% n=19/38); however, this observation was based on a heterogeneous cohort. Overall, DDX4 emerges as a context-dependent biomarker—adverse prognostically across cancers yet potentially predictive for RT/CRT response—highlighting the need for prospective validation.

6 Discussion

The overall aim of this thesis was to identify histopathological and immunological biomarkers that could improve prognostication and treatment stratification in HNSCC, with a particular emphasis on the neoadjuvant RT/CRT setting. By integrating analyses of pretreatment tumor biology, therapy-induced histomorphological changes, the TIME, AI-assisted digital pathology, and functional molecular characterization of the cancer–germline antigen DDX4, this work provides a multidimensional framework for understanding treatment response in HNSCC beyond conventional staging systems.

6.1 Immune landscape and therapy-induced modulation in HNSCC (Study I)

The TIME has emerged as a key determinant of outcome in HNSCC, yet its clinical translation has been limited by marked heterogeneity and a lack of robust, reproducible biomarkers (S. Chen et al., 2023). The availability of paired pre- and post-neoadjuvant RT/CRT tumor samples across the studies included in this thesis enabled a detailed evaluation of therapy-induced immune remodeling. The analyzed cohorts comprised patients with advanced-stage HNSCC treated with curative intent. Consistent with previous reports, high numbers of pretreatment CD8$^+$ TILs, particularly stromal TILs, were associated with improved survival and more favorable response to RT/CRT, underscoring the role of pre-existing antitumor immunity in mediating treatment efficacy (Balermpas et al., 2014).

Importantly, however, RT/CRT did not uniformly induce a favorable immune response. Post-treatment enrichment of protumorigenic CD206$^+$ TAMs was associated with poor outcome, indicating that oncologic therapies may in some cases promote immunosuppressive or tumor-supportive changes within the microenvironment. Together, these findings highlight the heterogeneity of therapy-induced immune modulation in HNSCC and emphasize that immune parameters should be interpreted in conjunction with morphologic and molecular features rather than as isolated biomarkers (Binnewies et al., 2018). Collectively, these in-depth analyses provide deeper insight into how RT/CRT remodels the TIME in HNSCC and provide a biological rationale for future validation of immune-related response biomarkers.

6.2 Pretreatment tumor necrosis as a dominant predictive marker (Study I)

A main finding of this thesis was the strong prognostic impact of pretreatment histological tumor necrosis in the neoadjuvant RT/CRT setting. In the neoadjuvant cohort analyzed, baseline necrosis emerged as the single most powerful predictor of poor OS, outperforming individual immune markers and remaining independent of TNM stage. Notably, this adverse prognostic effect was specific to the neoadjuvant context and was not observed in surgically treated or definitive RT/CRT cohorts.

This context dependency suggests that pretreatment necrosis may identify biologically aggressive tumors that are intrinsically resistant to neoadjuvant oncologic therapies. Rather than representing a uniform biological entity, histological necrosis likely reflects a convergence of adverse tumor features, including hypoxia, impaired perfusion, and rapid proliferative growth (Yee & Li, 2021). These features may limit both direct cytotoxic effects of RT/CRT and the induction of effective antitumor immune responses (Beckers et al., 2024; Lee et al., 2010).

At the same time, treatment-induced tumor cell death—including necrotic cell death—can, under some conditions, promote immunogenic signals and antitumor immunity (Krysko et al., 2012). Thus, while therapy-induced cell death may be immunostimulatory, extensive pretreatment necrosis in this neoadjuvant cohort identified HNSCCs with an adverse biology and poor outcome. The biological impact of histological necrosis therefore appears to be context dependent, with potential for both immunostimulatory and immunosuppressive consequences (Krysko et al., 2012; Zapletal et al., 2023).

In the neoadjuvant RT/CRT setting studied here, tumors exhibiting extensive baseline necrosis may derive limited benefit from neoadjuvant oncologic therapies and could warrant consideration of alternative primary treatment strategies, as well as intensified investigation with respect to targeted therapeutic approaches.

Despite its well-established prognostic relevance in several other solid malignancies, histological tumor necrosis has remained under-recognized in HNSCC and is currently not incorporated into major pathology reporting guidelines (Brierley et al., 2025; WHO Classification of Tumours Editorial Board, 2023). The findings of this thesis, together with emerging independent evidence, support renewed investigation of necrosis as a clinically accessible and biologically meaningful biomarker in HNSCC, particularly as a biomarker that may help identify patients less likely to benefit from neoadjuvant RT/CRT (Z. Chen et al., 2023).

6.3 AI-assisted digital pathology as an enabler of reproducible morphologic biomarkers (Study I)

The application of AI-based deep learning for automated necrosis detection represents an important translational component of this work. AI-assisted necrosis quantification demonstrated strong prognostic performance while addressing key limitations of conventional histopathological assessment, including interobserver variability. Importantly, AI-based analysis does not merely automate visual assessment but fundamentally standardizes it, enabling reproducible quantification across whole tumor sections and cohorts (Takamatsu, 2025).

These findings illustrate how AI-assisted digital pathology can facilitate the clinical adoption of morphologic biomarkers that have historically been underutilized due to challenges in reproducibility. In the context of neoadjuvant therapy, where pretreatment decision-making may rely on limited biopsy material, such standardized approaches may be particularly valuable.

6.4 A novel histopathological therapy response assessment method incorporating MGC (cHTR) (Study II)

As interest in neoadjuvant therapies in HNSCC grows, the need for reliable and practical HTR assessment has become increasingly evident (Bhatia & Burtness, 2023; International Collaboration on Cancer Reporting (ICCR), 2025b; The Royal College of Pathologists of Australasia, 2025). Traditional TRG systems in colorectal carcinomas rely on estimating residual viable tumor in relation to therapy-induced fibrosis, a process prone to subjectivity and limited reproducibility (Mandard et al., 1994; Becker et al., 2003; Janssen et al., 2022). In this thesis, post-treatment histomorphological features—including residual tumor vitality, TRG, and histiocytic MGC reaction—were systematically evaluated. To enable a clinically practical two-tier response classification, we developed a combined histopathological therapy response parameter (cHTR) that integrates conventional TRG with the presence of MGCs, classifying tumors into good versus poor response. Among these features, the presence of MGCs emerged as a particularly strong prognostic marker, outperforming TRG and residual tumor alone. MGCs were associated with favorable overall survival and appeared to reflect an active, therapy-induced immunologic process rather than passive clearance of necrotic debris. This interpretation is supported by the inverse association between MGC response and pretreatment PD-L1 expression, suggesting that MGCs preferentially occur in a distinct immune context characterized by lower checkpoint activity. In line with this interpretation, Uppaluri et al. reported MGCs in association with favorable response following neoadjuvant pembrolizumab in HNSCC, supporting the concept that

MGCs may reflect therapy-associated immune activation (Uppaluri et al., 2020). Mechanistically, the absence of an MGC reaction after RT/CRT may reflect impaired immune-mediated tumor clearance, potentially contributing to residual disease and recurrence. Clinically, this may identify a subgroup with insufficient therapy-associated immune activation in whom strategies aimed at reinvigorating antitumor immunity warrant further evaluation.

Importantly, pretreatment immune features also showed predictive associations with histopathological response: higher pretreatment PD-L1 expression (in HNSCC cells and/or immune cells) and increased $CD8^+$ intraepithelial TILs (iTILs) were associated with poorer cHTR, indicating a reduced likelihood of a favorable combined response after neoadjuvant RT/CRT. These findings also emphasize that the prognostic and predictive relevance of $CD8^+$ TILs may depend on their spatial compartmentalization (Binnewies et al., 2018). In this thesis, stromal $CD8^+$ TILs were prognostic and associated with improved overall survival, whereas increased intraepithelial $CD8^+$ TILs were associated with poorer cHTR. This dissociation suggests that stromal $CD8^+$ infiltration may reflect broader antitumor immune competence, while intraepithelial $CD8^+$ T cells—particularly when accompanied by PD-L1 expression—may represent a tumor–immune interface characterized by adaptive immune resistance and functionally restrained cytotoxic activity (Borsetto et al., 2021; Teng et al., 2015). In this scenario, RT/CRT-induced HNSCC cell death may be insufficient to overcome checkpoint-mediated immune suppression, resulting in less frequent MGC-associated immune remodeling and a poorer histopathological response.

Clinically, these observations suggest that pretreatment immune cell infiltration and checkpoint expression do not necessarily translate into a favorable histopathological response to RT/CRT. Accordingly, patients with higher pretreatment PD-L1 expression and increased $CD8^+$ iTILs—particularly in the absence of a post-treatment MGC reaction—may represent a subgroup in whom strategies aimed at augmenting antitumor immunity, including immunomodulatory combinations, merit further study. More broadly, quantifying $CD8^+$ TILs without accounting for their localization may obscure clinically relevant biology and may partly explain inconsistencies across studies (Binnewies et al., 2018).

Building on these observations, the cHTR demonstrated strong and independent prognostic value in multivariable analyses, even when pretreatment tumor necrosis was included in the model. The cHTR thus seems to provide a biologically informed, reproducible, and clinically practical framework for response assessment that aligns with the evolving role of neoadjuvant therapies in HNSCC. An important direction for future research is to determine whether cHTR is generalizable to other malignancies and to neoadjuvant immunotherapy settings.

6.5 DDX4 as a context-dependent cancer–germline biomarker linking tumor aggressiveness and treatment response (Study III)

A central molecular component of this thesis was the cancer–germline antigen DDX4. More broadly, the recurrent activation of germline programs in somatic malignancies highlights how cancer cells can repurpose evolutionarily conserved, germline-restricted mechanisms to support malignant fitness (Bruggeman et al., 2023). In this framework, DDX4 is a particularly illustrative example: rather than functioning through its canonical PIWI–piRNA axis, DDX4 appears to represent a cancer-adapted, PIWI-independent RNA regulatory module that can be mobilized to reshape post-transcriptional control under conditions relevant to tumor growth.

Conceptually, these findings support the idea that DDX4 contributes less to baseline cell-autonomous proliferation and more to context-dependent tumor competence—that is, the ability of cancer cells to cope with microenvironmental constraints such as metabolic stress, hypoxia, fluctuating nutrient availability, and other stressors encountered in vivo (Nakahara et al., 2023). The stress-associated, granule-forming behavior of DDX4 in tumor-like conditions is consistent with a model in which DDX4 helps buffer transcriptome complexity and maintain adaptive RNA processing and translation programs when cells face microenvironmental pressure. This interpretation also aligns with prior literature linking higher expression of RNP granule–associated proteins with adverse outcomes in cancer, supporting the relevance of stress granule–like biology in aggressive disease (Lavalée et al., 2021).

Clinically, IHC detected cytoplasmic DDX4 granules were associated with aggressive disease and adverse outcomes in two different epithelial malignancies, HNSCC and prostate cancer, supporting DDX4 as a broadly relevant marker of tumor aggressiveness. Importantly, however, in the neoadjuvant RT/CRT setting, pretreatment DDX4 expression was not prognostic but instead predicted favorable histopathological treatment response, as defined by good cHTR. This apparent paradox underscores the importance of distinguishing prognostic from predictive biomarkers and suggests that biologically aggressive HNSCC may nevertheless be particularly susceptible to therapy-induced damage under specific treatment contexts (Oldenhuis et al., 2008).

Motivated by the immunogenic nature of cancer–germline antigens and transcriptional changes observed in DDX4-null xenografts, the relationship between DDX4 expression and the tumor immune microenvironment was explored in treatment-naive HNSCC (Gjerstorff et al., 2015). Overall, no consistent associations were observed between DDX4 and TILs, TAMs, or PD-L1 when pre- and post-treatment samples were analyzed separately. Taken together, these analyses suggest that any immunologic effects of DDX4 in HNSCC are unlikely to manifest as uniform differences in bulk TIL/TAM density or PD-L1 status, and may instead depend on spatial organization, functional immune states, or treatment context.

From a translational perspective, these observations strengthen the rationale for considering DDX4 as both a biomarker and a potential therapeutic target. Its germline-restricted expression in normal epithelial tissues provides a conceptual basis for tumor selectivity, while its ATP-dependent helicase activity raises the possibility of pharmacologic targeting, albeit with fertility-related considerations. Finally, the apparent linkage between DDX4-dependent RNA programs and pathways relevant to invasion, epithelial–mesenchymal plasticity, and immune-related signaling suggests that DDX4 may sit upstream of multiple clinically consequential phenotypes. In addition, because several cancer–germline antigens are immunogenic, activation of germline-like programs could plausibly influence how tumors are perceived by the immune system in the setting of therapy-induced cell death, although this remains speculative. This broader conceptual link is discussed further below in the context of immunogenic cell death (ICD).

A notable implication of these findings is that DDX4 may have remained underappreciated in cancer research because it is unlikely to be captured by mutation- or rearrangement-focused discovery approaches. As a germline-restricted RNA helicase, DDX4 may be activated through epigenetic derepression or context-dependent transcriptional programs rather than recurrent genomic alterations and thus may not emerge as a prominent candidate in whole-genome or exome sequencing studies. Consistent with this concept, ectopic DDX4 expression in cancer cells is likely influenced by epigenetic regulation, as is typical for germline-restricted genes reactivated in somatic malignancies (De Smet & Loriot, 2013). In addition, RNA-level profiling may underestimate DDX4 if expression is heterogeneous, confined to specific tumor niches, or regulated post-transcriptionally; transient or rapidly turned-over transcripts could further reduce detectability in bulk mRNA datasets (Bruggeman et al., 2018).

Together, these considerations highlight the continued importance of protein-level and spatially resolved analyses—including IHC and single-cell and spatial approaches—for identifying clinically relevant, context-dependent cancer biology that may be missed by genomics alone (Makin, 2020; Lopez Janeiro et al., 2024). These points motivate future work in harmonized clinical cohorts and with spatial or single-cell approaches to clarify where and when DDX4 is engaged within tumors and how this engagement relates to treatment response and immune remodeling.

6.6 Immunogenic cell death (ICD) as a conceptual framework linking necrosis, DDX4, and treatment response

Taken together, the findings of this thesis raise the question of whether a shared biological framework could link pretreatment tumor necrosis, DDX4 expression, immune remodeling, and differential response to neoadjuvant RT/CRT. One potential conceptual framework is ICD, in which RT and CRT induce distinct forms of tumor cell death that differ in their capacity to stimulate antitumor immunity (Zitvogel et al., 2008).

In this context, extensive baseline necrosis may mark biologically aggressive tumors with hypoxic and poorly perfused microenvironments, conditions that can blunt both the direct efficacy of RT/CRT and the development of effective antitumor immune activation. Conversely, DDX4-dependent stress-coping mechanisms could influence how tumor cells handle RT/CRT-induced damage and thereby shape downstream immune consequences of therapy-induced cell death. Given that several cancer–germline antigens can be immunogenic, germline-like programs could plausibly modulate post-therapy immune recognition, although whether DDX4 itself contributes to such immunogenicity remains speculative (Bruggeman et al., 2018). Although ICD was not directly assessed in this thesis, this framework provides a plausible biological context for integrating the observed associations between necrosis, DDX4 status, immune remodeling, and histopathological treatment response.

6.7 Strengths, limitations, and future perspectives

The major strengths of this thesis include the use of paired pre- and post-neoadjuvant tumor samples, whole-section histopathological analyses, integration of AI-assisted digital pathology, and the combination of clinical, morphologic, immunologic, and functional molecular data. Together, these approaches provide a more comprehensive view of tumor biology and therapy-induced changes than any single methodology alone. The paired design enables direct assessment of treatment-related alterations within the same tumor context, while whole-section analyses improve representativeness by better capturing spatial heterogeneity than limited-area sampling. The integration of AI-assisted digital pathology further supports objectivity, reproducibility, and future scalability of morphologic assessment. Importantly, this multimodal and translational framework enhances the clinical relevance of the study by connecting mechanistic observations with histopathological features that may have future utility in biomarker development and treatment guidance.

Limitations include modest cohort sizes, heterogeneity of tumor subsites and sampling methods, and restriction of functional DDX4 studies to selected cancer models. In practice, these limitations may reduce statistical power, constrain subgroup analyses, and limit the generalizability of the findings across different HNSCC subsites and clinical settings. Variation in sampling methods may also affect tissue representativeness and the assessment of spatially heterogeneous histopathological and immune features. In addition, because functional DDX4 experiments were performed only in selected cancer models, the mechanistic findings should be interpreted with caution and require validation in broader and more disease-relevant systems.

These limitations emphasize the need for prospective, multicenter validation and single-cell-level analyses to better resolve intratumoral and immune cell heterogeneity and to clarify the cell-type-specific and context-dependent functions of DDX4. Future studies integrating spatial and single-cell approaches with standardized digital pathology may further refine biomarker-driven treatment stratification in HNSCC. Further subgroup-specific analyses may also be informative. In laryngeal HNSCC, pretreatment necrosis could be explored as a potential predictive marker for identifying patients who may be sufficiently treated with chemoradiotherapy alone versus those who may require additional surgery. In oropharyngeal HNSCC, future studies could likewise evaluate whether DDX4 expression and its prognostic or predictive significance differ according to HPV status.

7 General Conclusions

This thesis addresses the unmet need for reproducible, biologically grounded, and clinically interpretable biomarkers to improve prognostication and treatment guidance in HNSCC beyond conventional staging. By integrating analyses of pretreatment tumor biology, therapy-induced histomorphological changes, and the TIME, and by leveraging AI-assisted digital pathology and mechanistic studies of the cancer–germline antigen DDX4, this work advances a context-aware pathology framework for neoadjuvant RT/CRT-treated HNSCC.

Across paired pre- and post-neoadjuvant samples, therapy-induced immune remodeling was characterized in advanced-stage HNSCC with curative treatment intention. Pretreatment $CD8^+$ TILs—particularly stromal TILs—were associated with improved outcome, whereas post-treatment enrichment of protumorigenic $CD206^+$ TAMs indicated that RT/CRT can also shape the TIME in unfavorable ways. These findings support the view that immune markers are most informative when interpreted together with morphologic and molecular cancer features.

A key morphologic result was the identification of pretreatment histological tumor necrosis as a dominant and context-specific predictor of poor outcome in the neoadjuvant RT/CRT setting, independent of TNM stage. Given its accessibility on routine H&E sections and its current omission from major HNSCC reporting guidelines, these data provide a strong rationale for renewed evaluation of necrosis as a clinically useful biomarker for identifying patients less likely to benefit from neoadjuvant RT/CRT. Importantly, AI-assisted necrosis detection demonstrated that such underutilized morphologic features can be standardized and quantified reproducibly, supporting future implementation in diagnostic workflows.

To improve assessment of post-treatment response, this thesis established a practical two-tier combined histopathological therapy response metric cHTR, which integrates conventional TRG with the MGC reaction. MGCs were associated with favorable survival and, together with cHTR, captured biologically relevant therapy-associated immune activity. Moreover, pretreatment PD-L1 expression and increased $CD8^+$ intraepithelial TILs were associated with poorer cHTR. These findings suggest that an “inflamed” pretreatment phenotype does not necessarily indicate effective antitumor immunity, but may instead coexist with checkpoint-mediated restraint and reduced likelihood of favorable histopathological response. This may have implications for future immunomodulatory combination strategies.

Finally, this thesis delineated the biological and clinical relevance of DDX4 as a cancer–germline biomarker. Functional analyses supported a context-dependent role for DDX4 in promoting aggressive behavior of carcinoma through stress-associated RNA regulatory mechanisms, while clinical data linked cytoplasmic DDX4 granules with adverse outcomes across two epithelial malignancies. Notably, in neoadjuvant RT/CRT-treated HNSCC, pretreatment DDX4 expression predicted favorable histopathological response, underscoring the importance of distinguishing prognostic from predictive biomarkers and motivating prospective validation in harmonized cohorts.

In conclusion, the findings of this thesis support a move toward integrated, biology-driven pathology in HNSCC—combining conventional morphology, immune context, AI-assisted quantification, and molecular insights—to enable more precise risk stratification and ultimately more personalized treatment strategies aimed at improving survival and quality of life for patients with HNSCC.

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