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## Immune cell phenotypes of head and neck lesions associated with viral infections

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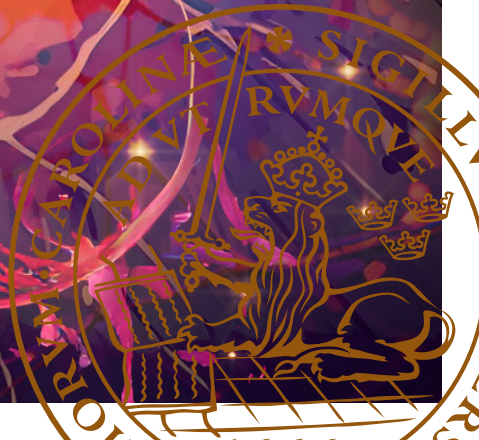
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# Immune cell phenotypes of head and neck lesions associated with viral infections

AASTHA SOBTI

DEPT OF IMMUNOTECHNOLOGY | FACULTY OF ENGINEERING | LUND UNIVERSITY





# Immune cell phenotypes of head and neck lesions associated with viral infections

Aastha Sobti



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## DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on 21<sup>st</sup> of April at 09.00 in Hörsalen, Medicon Village, Schleeletorget 1, Lund

*Faculty opponent*  
Professor emeritus Tina Dalianis  
Department of Oncology-Pathology  
Karolinska Institutet  
Stockholm, Sweden

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**Title and subtitle:** Immune cell phenotypes of head and neck lesions associated with viral infections

**Abstract:**

Tonsillar cancer (TC) and nasopharyngeal cancer (NPC) are associated with high-risk human papillomavirus (HPV) and Epstein-Barr virus (EBV), respectively. Similarly, benign lesions, known as laryngeal papilloma (LP), are associated with low-risk HPV. Recent advances have included immunotherapy as a part of the treatment regimen for recurrent and metastatic HNC and LP, although with limited response rates.

An in-depth understanding of the genetics of the lesions and molecular underpinnings has identified molecular changes that may guide patient care. In papers I-III, key immune players, including CD8<sup>+</sup> T cells and antigen-presenting cells (APCs), were studied in NPC and TC. Using quantitative density of CD8<sup>+</sup> T cells in NPC, three phenotypes were defined: "inflamed", "immune-excluded", and "desert". Based on CD8<sup>+</sup> T cells infiltration, the inflamed phenotype was associated with higher survival rates than the "immune-excluded".

In paper II, digital spatial technology was used to further investigate the defined phenotypes. Higher median expression of protein markers such as CD11c and IDO1 and lower median expression of fibronectin in NPC stromal regions were associated with improved survival. In paper III, we demonstrated a correlation between the levels of APCs and CD8<sup>+</sup> T cells in HPV<sup>+</sup> TC, and showed that patients with high levels of CD8 transcripts had improved survival

In papers IV-V, we investigated LP using gene expression and flow cytometry and observed an inverse relationship between CD8<sup>+</sup> T cells and neutrophils. In addition, a streptococcus subspecies was associated with severe clinical symptoms and high neutrophil counts in chronic LP (paper V: a case report). These findings provide a basis for further investigations of neutrophil and bacterial associations in HPV-associated lesions.

In conclusion, the cell types and biomarkers investigated in this thesis highlight the immune heterogeneity of NPC, TC, and LP. Validations in larger patient cohorts are needed to develop clinically applicable biomarkers for prognostics and patient stratification.

**Key words:** human papillomavirus, Epstein-Barr virus, tonsillar cancer, nasopharyngeal cancer, laryngeal papilloma, tumour microenvironment, immune cells, flow cytometry, spatial proteomics

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*'Nothing in life is to be feared; it is only to be understood. Now is the time to understand more, so that we may fear less.'*

– Marie Curie



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# Original research articles

This thesis is based on the following articles, referred to by their Roman numerals (I-V):

- I. Nilsson JS, Sobti A, Swoboda S, Erjefält JS, Forslund O, Lindstedt M, Greiff L. *Immune phenotypes of nasopharyngeal cancer*. *Cancers* 2020; 12: 3428.
- II. Sobti A, Sakellariou C, Nilsson JS, Askmyr D, Greiff L, Lindstedt M. *Exploring spatial heterogeneity of immune cells in nasopharyngeal cancers*. Manuscript.
- III. Jimenez DG\*, Sobti A\*, Askmyr D, Sakellariou C, Santos SC, Swoboda S, Forslund O, Greiff L, Lindstedt M. *Tonsillar cancer with high CD8<sup>+</sup> T-cell infiltration features increased levels of dendritic cells and transcriptional regulation associated with an inflamed tumor microenvironment*. *Cancers* 2021; 13: 5341. \*Shared first authorship.
- IV. Sobti A, Sakellariou C, Nilsson M, Schwartz S, Olofsson K, Rydell R, Lindstedt M and Forslund O. *Immune delineation of laryngeal papilloma reveals enhanced neutrophil associated gene profile*. *Eur J Immunol* 2021; 51: 2535-2539.
- V. Sobti A, Lindstedt M, Andersson F, Rydell R, Forslund O. *Case Report: Co-Infection of Streptococcus dysgalactiae subspecies equisimilis and HPV11 in laryngeal papilloma*. *Ann Case Report* 2022; 7: 993.

## **My contributions to the articles**

- I. Analysed the RNA sequencing data, produced figures related to the transcriptional analysis, and participated in manuscript writing and revision. Did not perform the immunohistochemistry stainings or analysis.
- II. Participated in the conceptualization and design of the study. Performed the spatial biomarker analysis as well as survival data analysis and visualization. Wrote the first draft of the paper.
- III. Participated in experimental design. Performed sample processing, flow cytometric analysis, RNA-seq, and qPCR data analysis with co-authors. Prepared figures and participated in manuscript writing and revision. Did not perform the HPV DNA and RNA quantification.
- IV. Performed the transcriptional data analysis and produced all figures. Was involved in discussions about data presentation, wrote the first draft, and revised the manuscript with the co-authors.
- V. Prepared the fresh and paraffin-embedded biopsies, performed the flow cytometric analysis and multiplex stainings, and wrote the first draft of the paper. Did not perform the microbial analysis.

## Additional papers

- I. Jimenez DG, Altunbulakli C, Swoboda S, Sobti A, Askmyr D, Ali A, Greiff L, Lindstedt M. *Single-cell analysis of myeloid cells in HPV<sup>+</sup> tonsillar cancer*. *Front Immunol* 2023; 13: 1087843.

# Abbreviations

ACT	Adoptive T cell transfer
AJCC	American joint committee on cancer guidelines
APCs	Antigen-presenting cells
CAFs	Cancer-associated-fibroblasts
CD	Cluster of differentiation
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DCs	Dendritic cells
DFS	Disease-free survival
DSP	Digital spatial profiler
EBV	Epstein Barr virus
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
FFPE	Formalin-fixed paraffin-embedded
FOXP3	Forkhead box protein 3
FN1	Fibronectin
GNLY	Granulysin
GZM	Granzyme
HAVCR2	Hepatitis A virus cellular receptor 2
HL	Healthy-looking laryngeal tissue
HLA	Human leukocyte antigen
HNC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HT	Healthy tonsil
IC	Immune checkpoint
ICOS	Inducible T cell co-stimulator
IDO1	Indoleamine 2,3-dioxygenase 1
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
LAG3	Lymphocyte activation gene
LCs	Langerhans cells

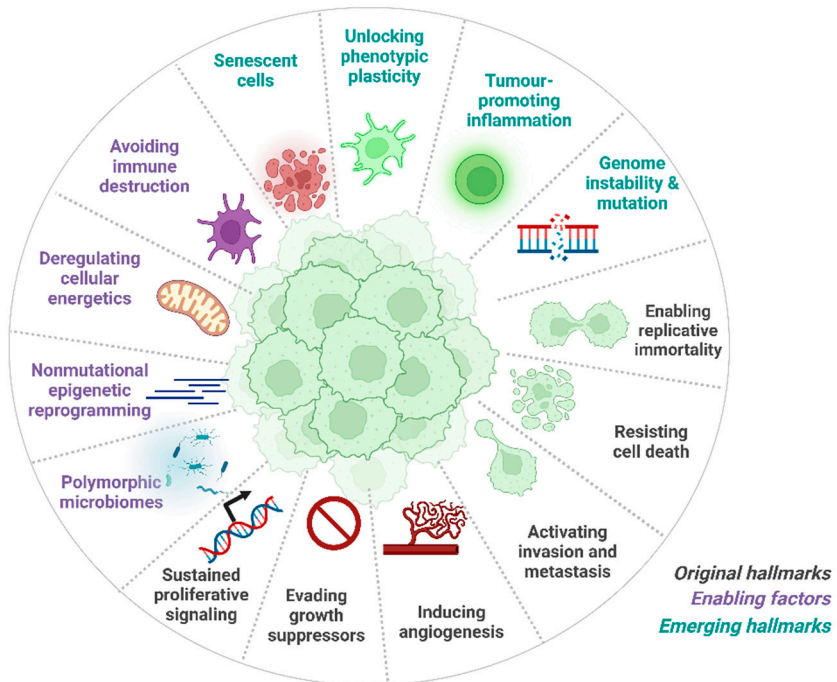
LP	Laryngeal papilloma
MDSCs	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
NK cells	Natural killer cells
NLRP3	Nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing proteins (NLR) family member
NKG7	Natural Killer Cell Granule Protein 7
NPC	Nasopharyngeal cancer
OPC	Oropharyngeal cancers
OX40	Tumour necrosis factor receptor superfamily, member 4
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
RCT	Randomised controlled study
RRP	Recurrent respiratory papillomatosis
scRNA-seq	Single-cell RNA sequencing
STAT3	Signal transducer and activator of transcription 3
TAECs	Tumour-associated vascular endothelial cells
TAMs	Tumour-associated macrophages
TCGA	The cancer genome atlas
TGF- $\beta$	Transforming growth factor
T <sub>h</sub>	T helper
TIGIT	T cell immunoglobulin and ITIM domain
TILs	Tumour infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin-domain containing 3
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TNM	Tumour site, lymph node, and metastasis
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
T <sub>regs</sub>	Regulatory T cells
TC	Tonsillar cancer
VHI	Voice handicap index
VISTA	V-domain Ig suppressor of T cell activation
WHO	World health organization

# Chapter 1: Introduction

According to the World Health Organization (WHO), cancer is the second leading cause of death before the age of 70 in 112 of 183 countries (1, 2). Cancer is a complex disease caused by abnormal growth of cells that gain an advantage over the immune system. Cancer immunotherapies use our immune system by activating cells or blocking inhibitory mechanisms to stimulate anti-tumour responses. Hanahan and Weinberg first summarised the six hallmarks of cancer 22 years ago, including "self-sufficiency in growth signals", "limitless replicative potential", "evasion of apoptosis", "insensitivity to anti-growth signals", "sustained angiogenesis", and "invasion/metastasis" (3). However, the significance of cancer cells "interacting with and evading immune cells" has increased over the last decade and has been added as an additional hallmark (4). More recently, "unlocking phenotypic plasticity", "non-mutational epigenetic reprogramming", "polymorphic microbiomes", and "senescent cells" were proposed as possible new hallmarks and enabling traits (**Figure 1**) (5).

Interestingly, the concept of immune cells eliminating cancer cells dates back to as early as 1909, when Paul Ehrlich, a physician and biochemist, proposed that the immune system can detect and eradicate early cancers (6). The term "cancer immunoediting" was coined to describe the dynamic process by which immune cells play a dual role: protecting the host from cancer formation or promoting cancer growth (7). It evolves through three states, known as "elimination", "equilibrium", and "escape", which can operate independently or sequentially (**Figure 2**) (7).

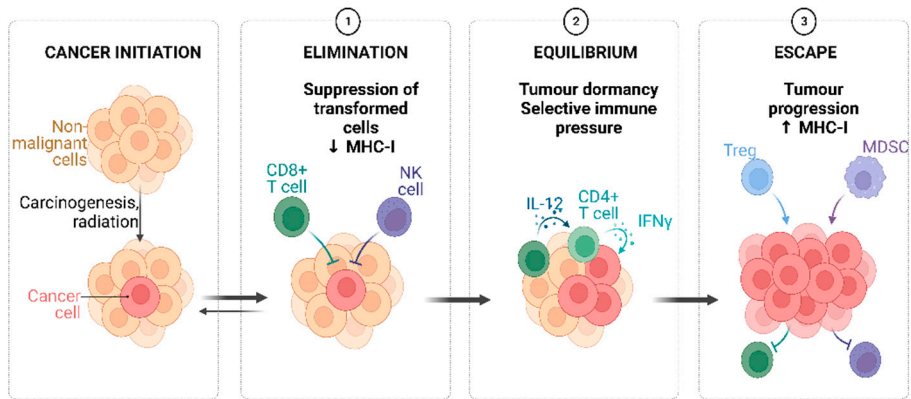




**Figure 1.** Hallmarks of cancer with the original six and added proposed mechanisms. Adapted from Hanahan and Weinberg (5). Figure created in Biorender.

Tumour-associated immune cells are classified into two types: 1) tumour-antagonising immune cells, such as T lymphocytes (including the cytotoxic CD8<sup>+</sup> T cells and effector CD4<sup>+</sup> T cells), natural killer (NK) cells, dendritic cells (DCs), M1-polarized macrophages, and N1-polarized neutrophils, and 2) tumour-promoting immune cells, which primarily consist of regulatory T cells (T<sub>regs</sub>) and myeloid-derived suppressor cells (MDSCs) (8, 9, 10, 11). Both groups of cells play diverse roles in various stages of cancer growth and form a part of tumour-infiltrating leukocytes (8, 12, 13). In addition, during the first phase of "elimination", the tumour-antagonising immune cells carry out immune surveillance, a process by which the immune system detects and eliminates malignant cells (14, 15, 16). This process is proposed to be more active in immunologically "hot" or "inflamed" tumours with significant infiltration of leukocytes, as opposed to "cold" or "desert" tumours that lack infiltrating leukocytes (14, 15).

## Cancer Immunoediting



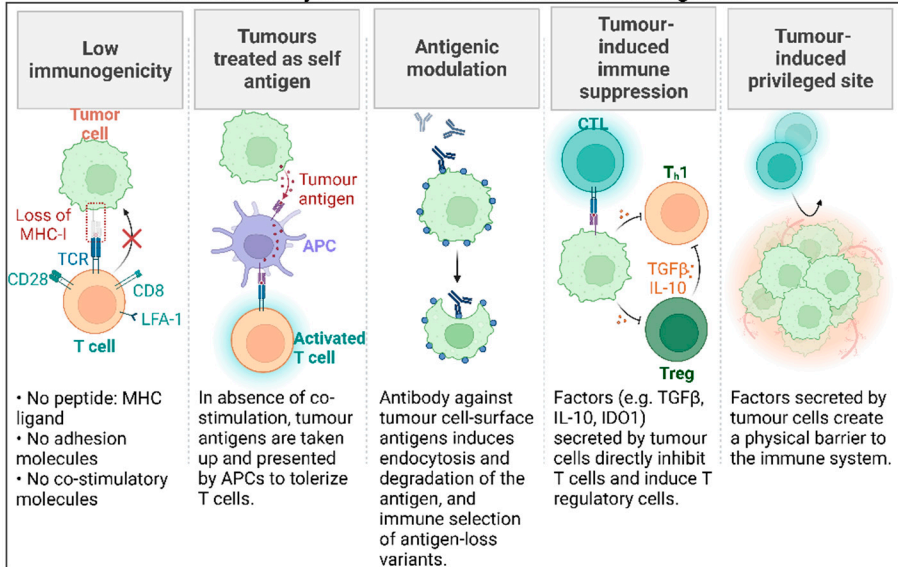
**Figure 2.** Cancer immunoediting. Three-step process following cancer initiation. Adapted from Dunn *et al.* (7). Figure created in Biorender.

During the second phase, our immune system and malignant cells that have survived elimination enter an equilibrium (16). This process is a dynamic state in which lymphocytes and interferons (IFN) and interleukins (IL) exert significant and constant selection pressure on cancer cells, confining but not completely extinguishing the tumour, which consists of numerous genetically unstable and mutating tumour cells (17, 18, 19). A study by Koebel *et al.*, revealed that inhibiting adaptive immune cells, such as CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with monoclonal antibodies or blocking cytokines promoting adaptive immunity, such as IFN- $\gamma$  and IL-12, induced dormant murine cancer cells to grow (19). Furthermore, monoclonal antibodies that depleted NK cells (anti-NK1.1), inhibited NK cell recognition (anti-NKG2D), or reduced NK cell effector activity (anti-tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), did not result in establishment of progressively growing tumours (19). These findings support the hypothesis that adaptive immunity, rather than innate immunity, is essential for maintaining the equilibrium phase (19). Besides that, other seminal studies demonstrated that IL-23, produced by macrophages in mouse tumour models, plays a vital role in counteracting anti-tumour activities by IL-12, allowing cancer cells to remain in an immune-mediated dormancy and preventing cancer cell elimination (20, 21). Simultaneously, cytokines known to regulate tumour immunity, such as IL-4, IL-17, tumour necrosis factor (TNF), and IFN $\alpha/\beta$ , did not appear to play an essential role in controlling the equilibrium phase (21). An additional study in a mouse sarcoma model compared the cellular environment of tumours in

equilibrium to tumours that "escaped" (22). The authors discovered that high proportions of cytotoxic CD8<sup>+</sup> T cells and NK cells as well as low proportions of NKT cells, forkhead box protein 3 (FOXP3<sup>+</sup>) T<sub>regs</sub> cells, and MDSCs in the tumour microenvironment (TMA) were associated with keeping cancer in an immune-mediated equilibrium state (22).

Dormancy can be disrupted, and tumours with low immunogenicity and immunosuppressive TMEs can begin to grow and finally become clinically discernible (7). Cancer cells evade immune cells during the escape phase, the third phase, through a variety of mechanisms (**Figure 3**), including downregulation or loss of tumour antigens, release of immunosuppressive exosomes and proteins such as IL-10 (23) and transforming growth factor (TGF- $\beta$ ) (24), loss of soluble major histocompatibility complex (MHC)-I (25), expression of indoleamine 2,3-dioxygenase (IDO) (26), and resistance to anti-apoptotic molecules such as Fas ligand (27) and TRAIL (28, 29). The immunosuppressive activity of the TME also depends on recruitment of stromal and immune cells, particularly myeloid cells. Tumour stroma encompasses vasculature, extracellular matrix (ECM) components, and extracellular vesicles, including exosomes and non-immune cells such as cancer-associated fibroblasts (CAFs) and tumour-associated vascular endothelial cells (TAECs). CAFs promote myeloid infiltration, production of tolerogenic DCs (tDCs), activation of immunosuppressive M2-phenotypic macrophages via TGF- $\beta$ /IL-10 secretion, and removal of antigen-presenting cells (APCs) via signal inhibition (30, 31). TAECs regulate immune cell migration as well as tumour cell intravasation and extravasation through angiogenesis (32, 33). Furthermore, IDO, a tryptophan-metabolising enzyme in the kynurenine metabolic pathway, promotes immunosuppression under normal conditions to moderate inflammatory responses (34). However, metabolic changes in the kynurenine pathway may also be involved in cancer immune evasion because IDO1 is produced by tumour cells, tumour-associated immune cells, DCs, and macrophages (34, 35). Fibronectin 1 (FN1), a member of the extracellular matrix glycoprotein family, is broadly expressed by a range of cell types and has been explored as a therapeutic target for numerous malignancies (36, 37). Recently, it was discovered that overexpression of FN1 accelerated head and neck cancer (HNC) growth and increased macrophage M2 polarization in an *in vitro* model of HNC (38).

### Mechanisms by which Tumours Avoid Immune Recognition



**Figure 3.** Mechanisms by which cancers avoid immune recognition. Figure created in Biorender.

The immune system is instrumental in developing, establishing, and progressing lesions and malignancies of the head and neck. Therefore, a better knowledge of immune system dysregulation and evasion in the formation and progression of such lesions may lay the groundwork for better therapeutics and improved patient outcomes. Immune checkpoint (IC) inhibitors, costimulatory agonists, antigenic vaccines, oncolytic virus therapy, and adoptive T cell transfer (ACT) are examples of immunotherapeutic strategies being developed for HNC (39). One such IC inhibitor, the anti-receptor programmed death-1 (PD-1), is currently used in clinical settings to treat residual or recurrent HNC when traditional salvage measures have failed, albeit with a response rate limited to only 20-30% of patients (40, 41, 42). The limited response rate might be attributed to immunological escape by the tumour caused by a variety of mechanisms, including physical blockade, cytokine dysregulation, changes to other IC ligands, recruitment of inhibitory cell populations, adverse effects of exosomes, and competitive metabolism of cancer cells, all of which prevent the immune system from detecting and eliminating cancer cells (39, 43, 44). Additional research is required to identify novel biomarkers that can lead to improved patient stratification and innovative therapeutic measures for HNC.

Oropharyngeal cancer (OPC) and nasopharyngeal cancer (NPC) are subsets of HNC. The former is often associated with high-risk human papillomavirus (HPV) (45, 46), even if traditional risk factors such as tobacco smoking and alcohol abuse remain essential. The latter (i.e., NPC) is frequently related to Epstein-Barr virus (EBV) (47, 48, 49). The TME of virus-associated HNCs may differ from that of cancers caused by other carcinogens and may be regarded as distinct types of cancer. Immune cells such as tumour-infiltrating lymphocytes (TILs) and other myeloid cells frequently infiltrate virus-driven cancers. Also, the presence of T<sub>regs</sub>, tumour-associated macrophages (TAMs), and MDSCs, as well as expression of IC ligands and downregulation of MHC expression by tumour cells, indicate an immunosuppressive state within the TME of virus-associated OPC and NPC (44, 50, 51, 52, 53).

In addition to high-risk HPV-associated cancers, non-malignant lesions associated with low-risk HPV types 6 and 11, such as laryngeal papilloma (LP), potentially related to the failure of local innate and/or adaptive immune responses, have also been observed and investigated (54). Enrichment of immunosuppressive CD4<sup>+</sup> FOXP3<sup>+</sup> T<sub>regs</sub> and lack of maturation factor CD83 in Langerhans cells (LCs) have been confirmed in LP (55, 56). The majority of studies have focused on T cells and mainly T<sub>regs</sub> within the LP, with limited depth in other immune cell populations (54, 57, 58). Thus, further investigations are required to delineate the pathophysiological roles of immune cells within LP.

Knowledge of stromal diversity and stromal-tumour interactions in the TME of HNC and LP is limited. Improved insight into the heterogeneous TMEs of these conditions is vital for designing individualised, targeted therapies to activate anti-tumour immunity and develop predictive biomarkers. Therefore, the main objective of this thesis was to explore the immune landscape of NPC, tonsillar cancer (TC), (a subset of OPCs) and LP.

In **papers I-III**, we described the immunophenotypes/profiles of two HNC subsets: NPC and TC. In **paper I**, we used immunohistochemistry to quantify CD8<sup>+</sup> T cells and CD207<sup>+</sup> DCs in paraffin-embedded formalin-fixed (FFPE) NPC samples. Data related to the presence and distribution of CD8<sup>+</sup> T cells were used to define three distinct NPC phenotypes; "inflamed", "immune-excluded", and "desert". The inflamed phenotype had better disease-free survival (DFS) than the immune-excluded. CD207<sup>+</sup> DCs were abundant only in cancer cell areas and not in the surrounding stroma. A transcriptomic analysis revealed a higher expression of pro-IFN in samples expressing high levels of CD8A and B genes compared with (*cf.*) low levels. To further explore the biomarker profiles of immune cells within the TME, we utilised digital

spatial profiling (DSP) of CD45<sup>+</sup> immune cells within cancer cell islets and tumour stroma, respectively, of the same NPC samples as in **paper I (paper II)**. Morphological assessments using PanCK, CD8, and CD45 staining guided spatial profiling of 49 protein biomarkers. Similar to paper I, paper II highlighted different cancer phenotypes and revealed that the "inflamed" phenotype had higher levels of markers associated with B cells, NK cells, macrophages, and myeloid cells, while the "immune-excluded" phenotype had higher levels of markers associated with suppressive populations of myeloid cells and T cells, and the "desert" profile had higher levels of granulocyte markers and immune-regulatory markers.

In **paper III**, we aimed to assess the frequencies and activity of relevant immune cells in HPV<sup>+</sup> TC with varying CD8<sup>+</sup> T cell infiltration levels compared to HPV<sup>-</sup> TC and healthy control tissue. For this purpose, fresh biopsies of tumours and paired healthy tonsils (HT) from TC patients were obtained at the time of diagnosis. The biopsies were analysed in terms of T cell abundance, APC populations, HPV status (p16), HPV type, quantity/load of HPV16, HPV integration status, and viral transcriptional activity. A strong correlation between the levels of CD8<sup>+</sup> T cells and DCs was observed in fresh TC biopsies using flow cytometry. In addition, transcriptional analysis was conducted utilizing a publicly available TC RNA data set available through the Cancer Genome Atlas (TCGA). Tumour samples with high scores of CD8 transcripts in HPV<sup>+</sup> TC (*cf.* CD8<sup>low</sup> TC) displayed an inflammatory profile associated with increased effector T cell transcripts and gene expression linked to active antigen uptake, processing and presentation, co-stimulation, and myeloid cell types.

In **paper IV**, we investigated the immunological aspects of LPs. We used RT-PCR and nCounter pan-cancer immune profiling for multiplex gene expression analysis to assess transcriptional characteristics of LP compared to healthy-looking laryngeal tissue (HL). Using cell typing via deconvolution, an increase in neutrophils was observed within the LP. An independent cohort of fresh tissue samples was used for further validation. The increased number of neutrophils in one chronic LP patient corresponded to the clinical presentation of severe symptoms (**paper V**). This case report used microbial cultures from topical swabs to supplement the study of immune cells in fresh LP biopsies. Certain streptococci species were identified, and the intervals between hospital visits were extended after antibiotics were administered. The relationship between immune cells and microbial culture is understudied in LP, and our case report is one of the few observations on the association between bacteria, HPV, and immune cells.

The current literature, combined with our findings, is covered in the following chapters. The next chapter contains a general reflection on the management and treatment of HNC and LP. Chapter 3 describes the viral association and its influence on the TME of TC/NPC and LP lesions. Chapter 4 focuses on cutting-edge technologies, which may complement traditional diagnostics and treatments. Finally, in Chapter 5, the key results from the presented studies are given together with a future outlook.

# **Chapter 2: Epidemiology and clinical management of head and neck cancer and laryngeal papilloma**

HNC is a globally prevalent cancer with an annual death toll of approximately 375,000 (59, 60). Cancers of the lips, oral cavity, oropharynx (tonsil, base of tongue, lateral pharyngeal wall, soft palate), nasopharynx, hypopharynx, and larynx are all examples of HNC (39, 59). When HNC is diagnosed at a high stage, it is linked to a high rate of primary-site recurrence and metastatic spread (61). Common side effects of HNC and its treatment are loss of sense of taste, dry mouth, and dysphagia/dysphonia (62). Smoking and alcohol consumption are well-known risk factors for HNC. Genetic mutations and viral infections, such as EBV and high-risk HPV, have also been identified as causative factors for some HNCs, namely NPC and OPC/TC, respectively (63, 64). The aetiology, anatomy and varying levels of immune infiltration highlight that these HNCs ought to be regarded as separate entities. Knowledge about their immune characteristics and heterogeneities is needed to improve patient stratification and define immunotherapeutic measures.

## **2.1 Oropharyngeal and nasopharyngeal cancer**

The incidence rates of TC and NPC in Sweden per 100,000 individuals are 2.6 and 0.22, respectively (60). The highest incidence of NPC, 3.0 per 100,000, is found in East Asian countries compared to 0.7 per 100,000 in the rest of the world (65, 66). Presence of EBV has been recorded in almost all undifferentiated, non-keratinized lesions (47, 67). Apart from EBV, host genetics and environmental factors have been suggested as contributors in the



development of NPC. Genetic susceptibility has been linked to NPC, and HLA genes located in the MHC region on chromosome 6p21 have been identified as essential risk loci in populations in Southeast Asia and China (65, 68).

Like many HNCs, tobacco smoking and alcohol abuse has been the most common risk factors of TC. However, more than 70% of TC, e.g., in Sweden, are now associated with HPV, primarily affecting younger patients (45, 69, 70). It has been observed that HPV-associated TC patients have a better prognosis than patients with HPV-negative disease (46, 71).

### **2.1.1 Surgical resection, chemotherapy, and radiotherapy**

The classical staging is carried out using the TNM guidelines of the American Joint Committee on Cancer guidelines (AJCC) (72), where the T-category is based on tumour site and size, the N-status describes the involvement of regional lymph nodes, and the M-status relates to further metastatic spread. Furthermore, additional markers have been added for the TNM classification for subgroups of HNC, such as the surrogate marker for HPV, i.e., p16<sup>INK4A</sup>, included for OPC in the 8<sup>th</sup> edition of the TNM-classification system (72, 73).

Radiotherapy is usually the first-line treatment for TC as well as NPC. Concurrent radio- and chemotherapy (e.g., cisplatin) may be offered for locally advanced disease (74, 75, 76). Surgery, when applied, is determined based on tumour size, extent of invasion, stage, and relationship to surrounding structures. The goals of surgery include free margins and restoration of local anatomy and function. In some cases, transoral robotic surgery has proven to be helpful in TC treatment (77, 78). In recurrent disease, open salvage surgery may be considered for NPC as well as TC.

### **2.1.2 Immunotherapy**

Immunotherapy works primarily by mobilizing immune cells within or outside the TME to target and attack cancer cells (8, 79). IC inhibitors, such as anti-PD-1 antibodies, are one such treatment that aims to block receptors that inhibit T cells from attacking cancer cells. Indeed, the PD-1/PD-L1 axis has, to date, been the most extensively researched therapeutic target for reversing T cell exhaustion and restoring anti-tumour activity. PD-1 inhibitors prevent the interaction between PD-L1 on the surface of cancer cells or APCs and PD-1 on the surface of CD8<sup>+</sup> T cells. As a result, the tumour-killing potential of CD8<sup>+</sup> T cells is restored, resulting in anti-cancer actions (80, 81).

In 2016, the FDA approved pembrolizumab (KEYTRUDA<sup>®</sup>), an immunotherapeutic agent consisting of an antibody blocking PD-1, for the treatment of recurrent or metastatic HNC (82). Thus far, the response rate has been approximately 20%, but somewhat higher for HPV<sup>+</sup> than HPV<sup>-</sup> disease: 24% and 16%, respectively (42). CAPTAIN and JUPITER-02 trials included anti-PD-1/PD-L1, toripalimab, and camrelizumab with human-specific binding capacity or humanised as treatment for recurrent or metastatic NPC. Compared with placebo, the overall response rates in CAPTAIN and JUPITER-02 were 34% and 77%, respectively (40, 41).

In addition to PD-1/PD-L1, a combination of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), intensity-modulated radiotherapy, and anti-PD-1 therapy has been tested (NCT01860430 and NCT01935921). CTLA-4 suppression lowers T<sub>reg</sub> accumulation and activity in the TME in some conditions, such as melanoma, and is currently being clinically evaluated in HNC (83, 84, 85). Other immunotherapeutic agents being explored include anti-IDO1 (KEYNOTE-037) (86), CD276 (NCT02381314), and OX40 (NCT02274155). The main idea is to stimulate anti-tumour T cell function while inhibiting tumour-specific T cell suppression and anergy as well as T<sub>reg</sub> activation (14, 35, 87).

In combination with approved immunotherapeutic drugs, a few clinical trials have explored other types of treatments, such as T cell-targeting therapies, HPV vaccines, cytokines, and oncolytic viruses. In clinical trials, such as NCT01585428 and NCT01585428II, the effects of using and modulating tumour-infiltrating leukocytes have been studied in HPV<sup>+</sup> HNC. Similarly, HPV-related E7 DNA (NCT02163057) and a vaccine combined with nivolumab (NCT02426892) have been tested in HPV<sup>+</sup> HNC to enhance the anti-tumour effect of T cells (88). In NPC, to stimulate CD4 and CD8 T cell responses, EBV-associated proteins like EBNA1 and LMP2 have been the prime choice of antigen for vaccine development (89). HLA-matched EBV vaccines targeting EBNA1 and LMP2 proteins are currently undergoing clinical trials to evaluate their efficacy and safety (NCT01094405).

## **2.2 Laryngeal papilloma and recurrent respiratory papilloma**

### **2.2.1 Disease characteristics**

The prognosis and treatment frequency for patients with recurrent respiratory papillomatosis (RRP) vary depending on the severity and recurrence rate. Therefore, a cumulative staging plus scoring system, the Derkay staging system, was developed by combining the disease's severity, treatment response, and clinical presentation (90). Scoring in adults is primarily based on the anatomical context of the tissues involved, whereas in paediatric patients, a higher score reflects a shorter interval between surgical treatments (91). Owing to the anatomical presentation of the lesions, breathing difficulties and voice dysfunction often indicate surgical resection. The Voice Handicap Index (VHI-10) (92), a patient-based questionnaire, has a positive correlation with the Derkay score and is instrumental in designing treatment plans for patients with RRP (93).

### **2.2.2 Current treatment options**

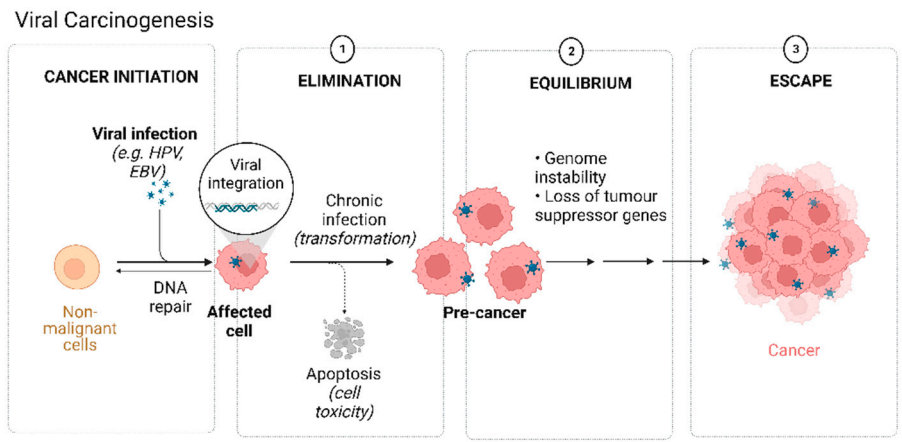
The current standard treatment for LP is surgery to remove the lesions at a flare-up (94). The surgical methods include carbon dioxide (CO<sub>2</sub>) laser, sharp dissection, and microdebridement (95). Although each modality has its merits and disadvantages, most patients continue to experience recurrent illnesses. Therefore, other therapies may potentially be an option, such as prophylactic and therapeutic vaccinations (96), anti-viral medication (97), and, most recently, immunotherapeutic drugs (98). Because RRP has a relapsing-remitting pattern, therapeutics that reduce surgery frequency and lengthen disease-free intervals are warranted. A randomised controlled study of adjuvant IFN indicated that a considerably greater number of patients achieved remission and that the rate of papilloma growth decreased in the first six months following therapy compared to controls, but this advantage was not sustained in the long-term (97). In addition, cidofovir, an antiviral drug, has been recognised as a possible off-label intralesional treatment for RRP (99, 100).

Quadrivalent HPV vaccination (Gardasil®) has been shown to minimise childhood HPV infections, which otherwise often lead to RRP. It can also be used as a therapeutic vaccine to treat active RRP (101, 102). Accordingly, a

few experimental studies have demonstrated a lower recurrence rate of RRP after HPV vaccination (102, 103). Moreover, immune-modulating treatments have recently been investigated for the treatment of RRP, and IC-blockade by specific antibodies targeting the PD-L1/PD-1 axis is considered to have therapeutic potential (104). In a clinical trial, patients with aggressive RRP (n=12) were treated with an FDA-approved antibody that blocks the PD-L1/PD-1 interaction, and the results indicated some beneficial outcomes with limited side effects (98). Two additional phase II clinical trials, i.e., NCT 02632344 and NCT 02859454, have been conducted to examine the efficacy of pembrolizumab and avelumab for the treatment of RRP (95, 101). However, it is crucial to note that many of the treatments discussed above are still in the early stages of clinical trials.

# Chapter 3: Viral and immune associations in the HNC microenvironment

NPC and TC can be carcinogen- and/or virus-driven. The viruses, i.e., HPV and EBV, have been shown to cause cell-cycle changes in the normal epithelium leading to cancer (**Figure 4**) (48, 49, 105, 106). Understanding the immune landscapes of these two unique malignancies may benefit in therapy options. In NPC and TC, TILs constitute approximately 40-50% of the TME (107, 108, 109, 110). Infiltrating leukocytes are vital components of the host antitumour immune response, associated with increased survival in various cancers, including HNCs (107, 108, 109, 110). However, as reviewed by Perri *et al.*, a more profound phenotyping of immune infiltration is critical because TILs may be functionally active or inactive owing to secondary exhaustion or anergy (111).



**Figure 4.** Viral carcinogenesis as seen in terms of cancer immunoeediting. Figure created in Biorender.

A number of studies based on TILs have focussed on cytotoxic T lymphocytes (CTL), and high levels of CTLs within a TME have been linked to improved overall survival (112, 113). Furthermore, Karpathiou *et al.*, in patients with OPC subjected to induction chemotherapy, demonstrated that high densities of T cells (CD3<sup>+</sup>, CD8<sup>+</sup>) and NK cells (CD57<sup>+</sup>), and a low density of macrophages (CD163<sup>+</sup>), were associated with better treatment response (114). Furthermore, they observed an unfavourable relationship between survival and CD4<sup>+</sup> cells, mainly T helper 2 (T<sub>h</sub>2) cells. Another CD4<sup>+</sup> T cell subtype, FOXP3<sup>+</sup> T<sub>regs</sub>, which play a role in maintaining immunological tolerance to host antigens, are regarded as suppressor of the anti-tumour immune response (115). Interestingly, in some HNC subtypes, including HPV<sup>+</sup> OPC, increased T<sub>reg</sub> numbers appear to be related to a better prognosis (116, 117). Previously, in HPV<sup>+</sup> and EBV<sup>+</sup> HNC, compared to other cancer types, studies have demonstrated a higher exhaustion profile of CD8<sup>+</sup> and CD4<sup>+</sup> T cells expressing exhaustion markers like PD-1, lymphocyte activation gene (LAG3), T cell immunoglobulin and ITIM domain (TIGIT), hepatitis A virus cellular receptor 2 (HAVCR2), CTLA4, and thymocyte selection-associated high mobility group box factor (TOX) as well as co-activation markers like granzyme (GZM)B and K, IFN- $\gamma$ , natural Killer Cell Granule Protein 7 (NKG7), granulysin (GNLY), and IL2 (8, 87, 118, 119, 120, 121, 122, 123). These findings verify that immune infiltration composition is key to a cancer's sensitivity to immunotherapy.

Cancer cells in EBV<sup>+</sup> NPC and HPV<sup>+</sup> TC interact with TILs in the TME and those tumours with high infiltration of CTLs are usually considered "inflamed" (110, 123, 124). A few studies have applied predictive immunoscore to correlate infiltrating immune cells in NPC and TC with overall survival and DFS, using their density and gene signatures (125, 126, 127, 128, 129). One such example is the quantitative assessment or transcriptional expression of CD3<sup>+</sup> T cells, specifically CD8<sup>+</sup>/FOXP3<sup>+</sup> and CD8<sup>+</sup>/CD4<sup>+</sup> ratios within the TME of NPC and TC, respectively, where a high ratio of the aforementioned cell populations associated with improved survival (125, 126, 127). Apart from TILs, immune cells such as CD1a<sup>+</sup> LCs, a subtype of DCs, and CD68<sup>+</sup> cells have also been linked to tumour progression and treatment response in HPV<sup>+</sup> tumours (128, 129). The studies mentioned above are examples of the intricacies of the TME and highlight a need for research that focuses on all immune cells within the TME rather than just one immune cell population.

### 3.1 EBV infection, NPC, and immune implications

EBV is associated with several cancers, including NPC (47). NPC represents 0.7% of cancers worldwide, with a 5-year survival rate ranging from 58% in patients with progressive disease to up to 90% in those showing complete response after treatment (59, 65). Nilsson *et al.*, observed that the presence of EBV DNA in NPC biopsies was associated with improved DFS (130). Moreover, Lee *et al.*, highlighted pre-treatment EBV plasma levels as a prognostic marker for overall survival and a potential risk-stratification tool (131). Therefore, estimating EBV DNA levels in the lesion and plasma may assist in the diagnostic phase. The accumulation of genomic instability over time, combined with persistent EBV infection, has been observed to drive the development of NPC (132). EBV oncogenic proteins, i.e., latent membrane proteins (LMP1 and LMP2A), have been linked to an increase in the population of stem-cell-like cancer cells (48, 49).

The immunogenicity of EBV<sup>+</sup> cancer cells is mediated by the downregulation of MHC I (133, 134). Moreover, immune subsets, such as immunosuppressive FOXP3<sup>+</sup> T<sub>regs</sub> and TAMs, have been identified in the NPC TME (135). In addition, using immunohistochemistry, the density and distribution of TILs have been identified as independent favourable prognostic factors (112). Further utilising flow cytometry, a diverse expression of DCs subsets has been observed in NPC TME (136). These findings suggested that DCs populations like CD1c<sup>+</sup> monocytic DCs might help to facilitate cross-presentation of antigens and aid cell-mediated antitumour effects (136). The immunosuppressive role of LMP1 has been associated with enhanced PD-L1 expression in cancer cells and with MDSC expansion (137, 138). Fang *et al.*, reported that LMP1 enhanced PD-L1 expression in NPC cells via the mitogen-activated protein kinases (MAPK)/activator protein-1 (AP-1), Janus kinase 3 (JAK3)/signal transducer and activator of transcription 3 (STAT3), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathways (138). Also, they reported that LMP1<sup>+</sup> NPC cells expressed higher levels of PD-L1 after IFN- $\gamma$  intervention, indicating a stimulatory effect of IFN- $\gamma$  in PD-L1 regulation in NPC cells (138). These observations suggest that NPC is a good candidate for immunotherapy based on PD-1/PD-L1 therapies.

Metabolic reprogramming of NPC cells via LMP1 was shown to increase IL-10, IL-6, and GM-CSF secretion, encouraging CD33<sup>+</sup> MDSC proliferation and immune suppression, and a presence of MDSCs were linked to a poor prognosis (137, 139). The MDSCs expansion and EBV-LMP1 associated metabolic shift and cytokine expression have been associated with the

nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing proteins (NLR) family member (NLRP3) inflammasome, cyclooxygenase-2 (COX-2), and protein p65, which subsequently stimulate the STAT-3 signalling pathway (137, 140). Wang *et al.*, employed computational pathology to analyse distinct signatures for IC receptors, such as PD-L1, IDO1, B7-H3, and B7-H4 on cancer cells and PD-L1, B7-H3, B7-H4, IDO-1, V-domain Ig suppressor of T cell activation (VISTA), inducible T cell costimulatory (ICOS), T cell immunoglobulin, mucin-domain containing-3 (TIM-3), LAG3, and OX40 on intralesional immune cells, and correlate them with plasma EBV-DNA burden (141). Regardless of location, patients with increased PD-L1 and IDO1 markers had better overall survival. Higher expression of LAG3, VISTA, and ICOS, and lower expression of B7-H3 in tumour-associated immune cells, on the other hand, was related to improved overall survival, particularly in patients with high pre-treatment EBV-DNA levels in plasma (141). Using scRNA-seq, Chen *et al.*, identified cell signatures for macrophages, pDCs, CLEC9A<sup>+</sup> DCs, and NK cells associated with improved survival (118). By combining single-cell technology and validation with bulk mRNA, Gong *et al.*, predicted an immunosuppressive role of MDSCs (signature: S100A9 and S1000A8) and TAMs (signature: FCGR3A, CD14, CD163, and MS4A4A) in NPC (119). An in-depth analysis of myeloid cells within NPC revealed better survival rates with the presence of mast cell populations, a finding unique to NPC compared to other cancer types (142). The same study also reported an interaction between mast cells and IL-1B<sup>+</sup> macrophages (142). Thereby, as mentioned above, EBV genes and their products are oncogenic because of the several molecular events of genomic instability that occur during NPC progression, ending in a dysregulated malignant proliferative neoplastic tumour. However, information regarding the heterogeneity of the immune compartment of NPC and its association with molecular changes and clinical characteristics, and survival is still limited.

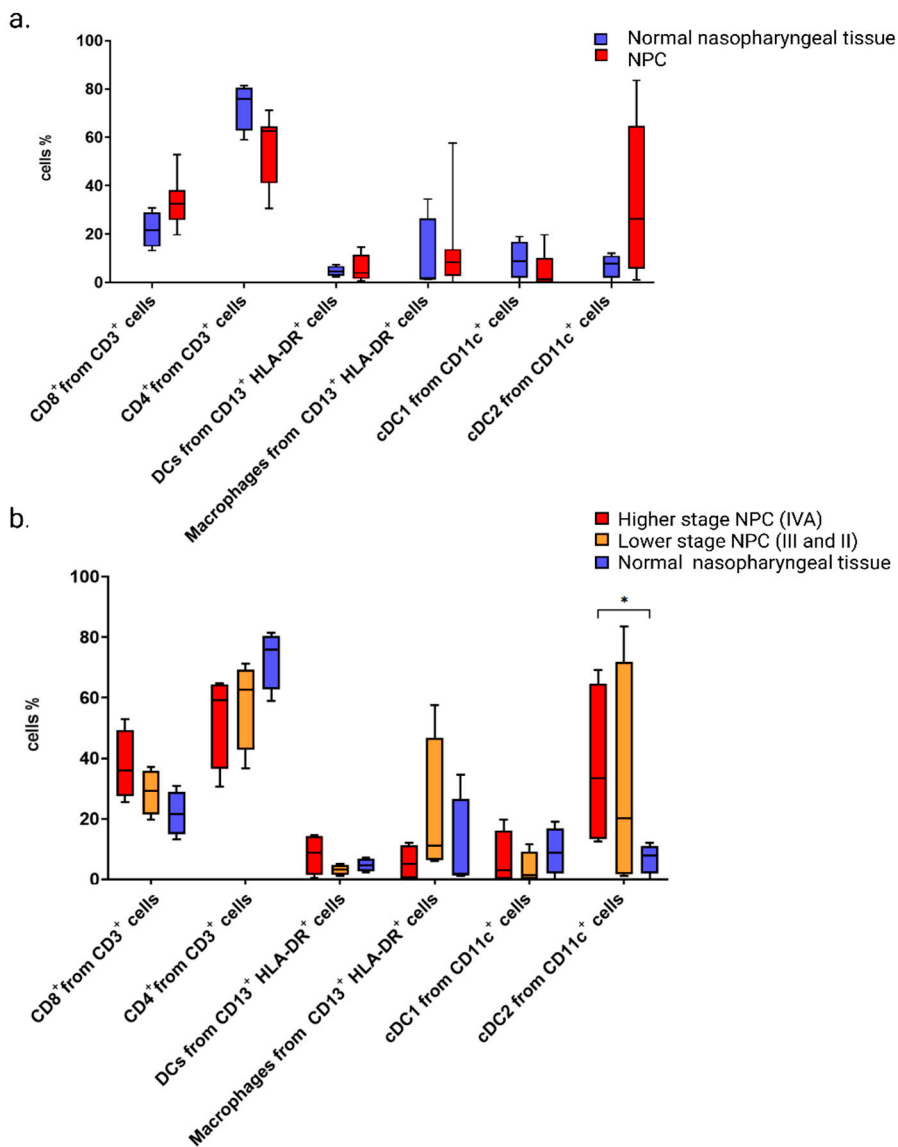
In **paper I**, we assessed aspects of immune infiltration in NPC, focusing on CD8<sup>+</sup> T cells and CD207<sup>+</sup> DCs. Immunohistochemistry was used to quantitatively evaluate CD8<sup>+</sup> T cells and CD207<sup>+</sup> DCs in treatment-naïve NPC biopsies. We conducted an immune phenotype sub-division of the samples based on the presence and distribution of CD8<sup>+</sup> T cells, resulting in "inflamed", "immune-excluded", and "desert" phenotypes. CD8<sup>+</sup> T cells were observed in areas of cancer cells and in the surrounding stroma, whereas CD207<sup>+</sup> DCs were observed only in areas with cancer cells. A higher frequency of CD8<sup>+</sup> T cells was observed in EBV<sup>+</sup> *cf.* EBV<sup>-</sup> NPC. In contrast, no difference in CD207 expression was observed between the groups. Survival analysis was conducted on immune phenotypes. A better DFS for the "inflamed" than for the "immune



excluded" phenotype was observed. Our findings were consistent with the results of an independent meta-analysis in which Liu *et al.*, stressed that high levels of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cell subsets predicted a favourable prognosis for NPC patients (143). Moreover, they suggested that a combination of the AJCC staging system and TIL analysis may provide a more reliable way of identifying patients with high-risk NPC, which may aid in therapeutic decision-making and result in increased survival rates (143).

Chao *et al.*, have reported a dampening effect on DCs via annexin A2-recognizing DC-SIGN in NPC, resulting in immunosuppressive cytokine production by DCs through DC-SIGN signalling (144). Additionally, the same study observed IL-12 production as well as an increase in immunosuppressive IL-10 by NPC cells (144). Moreover, LAMP3<sup>+</sup> DCs have been detected in the TME of NPC at single-cell resolution. These highly mature DC cells express suppressor proteins, release several cytokines to recruit T<sub>regs</sub>, and limit effector T cell functions (145). However, information regarding the functional role of DC populations in NPC remains limited. Moreover, the presence of CD207<sup>+</sup> DCs near cancer cells in **paper I** need functional evaluation.

We wanted to extend our group's prior findings to learn more about the immune composition of NPC. Therefore, we conducted an in-house immune cell profiling by flow cytometry of eight NPCs and four unpaired control nasopharyngeal tissue (**Figure 5a**) and between NPC and peripheral blood (results not shown). There was a trend towards conventional DCs subtype, i.e., cDC2, being abundant in NPC *cf.* nasopharyngeal tissue without malignancy. The increase in cDC2 in NPC coincided with enhanced Epstein-Barr virus-encoded small RNAs (EBER) copy numbers (>1000 copies/ml) and clinical nodal involvement. Furthermore, the CD207<sup>+</sup> cDC2 sub-set made up 26% to 85% of cDC2s within the EBER<sup>+</sup> NPC in comparison with EBER<sup>-</sup> NPC, where the subset comprised 2-6% of the total cell population (not shown). These findings align with our CD207<sup>+</sup> histological staining on FFPE samples (**paper I**), where EBER<sup>+</sup> lesions contained more infiltrating CD207<sup>+</sup> *cf.* EBER<sup>-</sup> NPC. No differences in immune cell populations were seen when comparing clinical stages (**Figure 5b**). To conclude, an enhanced understanding of myeloid cell variability, subset-specific traits, roles within the TME, and their potential impact on NPC patient survival is required to identify new therapeutic options.



**Figure 5.** Immune cell frequencies within NPC and normal nasopharyngeal tissue. Immune cells (CD45<sup>+</sup>) were gated as CD3<sup>+</sup> Lineage<sup>-</sup> T cells (Lin; CD19, CD20, and CD56) and subdivided into CD8<sup>+</sup> and CD4<sup>+</sup> T cells. Antigen-presenting cells (CD3<sup>-</sup> Lin<sup>-</sup> HLA-DR<sup>+</sup>) were divided into CD14<sup>-</sup> DCs and CD14<sup>+</sup> macrophages. cDCs were classified as CD11c<sup>+</sup> CD13<sup>+</sup> and further classified as cDC1 (CD141<sup>+</sup>) and cDC2 (CD1c<sup>+</sup>). **(a)** NPC samples compared to normal nasopharyngeal tissue. **(b)** Immune cells in high-stage NPC, low-stage NPC, and normal

nasopharyngeal tissue, where these tissues were obtained as diagnostic specimens and proven to be without any malignancy by pathologists. Figure created in Biorender.

## 3.2 Influence of HPV on the TME in TC

OPC accounts for 0.5% of newly diagnosed patients as well as 0.5% of cancer-related deaths worldwide and is most prevalent in the tonsils (59, 146). The number of OPC cases associated with traditional risk factors such as tobacco smoking and alcohol abuse has decreased, whereas those driven by high-risk HPV have increased, accounting for 61-91% of the cases in Denmark and approximately 70% in Southern Sweden (147, 148). Patients with HPV<sup>+</sup> OPC often require less treatment than HPV<sup>-</sup> patients and have a better prognosis (71, 149).

HPV, with approximately 200 genotypes, can be divided into low-risk (e.g., 6 and 11) and high-risk (e.g., 16, 18, and 33) types, the latter associated with several malignancies, including cervical, uterine, penile, anal cancer, and OPC (150). HPV infects the basal layer of the epithelium, and cell cycle-associated changes occur with the expression of early oncogenic proteins, mainly E6 and E7 (105, 106). These changes also initiate upregulation of p16, a surrogate marker for HPV-associated disease (151). Higher immune infiltration of HPV<sup>+</sup> cancer has been correlated with increased intensity of oncogenic proteins (E6 and E7) (152, 153). The presence of CD8<sup>+</sup> T cells in the TME of HPV<sup>+</sup> OPC has been associated with better survival than HPV<sup>-</sup> disease (153). HPV can integrate into the host genome, a typical cancer development process. It has been demonstrated that HPV<sup>+</sup> patients without detectable integration have a better survival than integration-positive and HPV<sup>-</sup> patients. (149). Further, patient samples with non-integrated HPV<sup>+</sup> OPC display intensely heightened signatures for immune cells compared to integration-positive disease. In contrast, integration-positive cancers are enriched for events involved in keratinisation and RNA metabolism (149).

Numerous studies have implied an immune escape mechanism in HPV<sup>+</sup> cancer by highlighting a decrease in antigen processing, enhancement of T<sub>regs</sub>, and IDO1 expression (154, 155). Based on gene expression, a recent study subdivided treatment naïve HPV<sup>+</sup> OPC samples into subgroups, i.e., "immune-rich", "mesenchymal", and "xenobiotic" (156). Further, based on IHC, they demonstrated that high numbers of CD8<sup>+</sup> T cells in the TME and PD-1 expression in tumour cells were associated with the "immune-rich" subgroup and an improved response to anti-PD-1 treatment. This study suggests that

stratifications of HPV<sup>+</sup> OPC into immune phenotypes can be used to predict treatment responses and guide immunotherapy treatment decisions.

Most studies have focused on OPC (from the tonsil, the base of the tongue, the lateral oropharyngeal wall, and the soft palate) as one group. However, TC and base of tongue cancer, arising in lymphoid structures with a high natural presence of immune cells, are expected to have unique compositions of immune infiltration and, therefore, a distinct immune response to HPV infection. Thus, we focused exclusively on TCs to ask questions related to HPV load and immunological features. In **paper III**, we assessed the frequency and activity of relevant immune cells in HPV<sup>+</sup> TC with varying CD8<sup>+</sup> T cell infiltration compared to HPV<sup>-</sup> TC and healthy control tissue. For this purpose, we collected paired biopsies, i.e., tumours and paired HT, from TC patients at the time of diagnosis. T cell and APC population abundance, HPV status (p16), HPV type, quantity/load of HPV16, integration status, and viral transcriptional activity were all investigated. In addition, transcriptional analysis was performed using publicly available TCGA data from 38 samples. The HPV status of the fresh biopsies was determined using clinical p16 staining and HPV16 E7 DNA and mRNA quantification. HPV<sup>+</sup> samples were p16<sup>+</sup>, had levels of E7 DNA greater than one copy per cell, and detectable levels of HPV16 E7 mRNA, whereas HPV<sup>-</sup> samples had E7 DNA titres less than one copy per cell and undetectable oncogene expression, regardless of p16 status. In agreement, it has been demonstrated that a complex mechanism involving epigenetic changes, such as the methylation of E2 binding sites (157, 158) and chromatin re-modelling (159), can affect the expression of oncogenic HPV16 E6 and E7 proteins. Therefore, these results suggest that the mere existence of HPV or only p16 positivity in the tissue is insufficient for terming the cancer as HPV<sup>+</sup> and that a multiplex analysis ought to be conducted even in the clinics to determine the HPV status.

Additionally, the flow cytometric analysis of fresh TC biopsies revealed higher CD8<sup>+</sup> T cell, DC, and macrophage abundance in HPV<sup>+</sup> TC *cf.* HPV<sup>-</sup> TC and HPV<sup>+/-</sup> HT (**paper III**). In our study, DC and macrophage levels were higher in HPV<sup>+</sup> TC than in HT, but they were not significantly different from those observed in HPV<sup>-</sup> TC. Overall, DCs levels, but not macrophage or HPV16 E7 DNA and mRNA levels, correlated with CD8<sup>+</sup> T cell infiltration. We concluded that myeloid cells, such as DCs, require further investigation to understand their possible function in HPV<sup>+</sup> TC. In a subsequent investigation, we used scRNA-seq to investigate differences between four HPV<sup>+</sup> TC and HT with regard to CD13<sup>+</sup> HLA-DR<sup>+</sup> myeloid cell populations (160). We identified unique polarization states of DCs and macrophage lineages, demonstrating the

influence of a type-I/II IFN-rich TME on myeloid cells. This was consistent with the hypothesis that HPV<sup>+</sup> TC is enriched in type-1 immune responses. Moreover, we recognized cDC1 as an appealing therapeutic target across all populations due to its favourable association with precursor populations, increased abundance, and capacity to mature in the tumour lesion. It significantly influences TC and HNC patient survival. These populations hold potential for targeted therapy and patient stratification, pending functional evaluation (160).

### **3.3 Benign lesions associated with low-risk HPV**

Low-risk HPV types have been linked to benign, slow-growing lesions in regions such as the oral mucosa, larynx, and anogenital areas (161). Two low-risk types, HPV6 and HPV11, have been associated with LP (162). HPV infection leading to LP can either be vertically transmitted from the mother to the child or in adults via oro-genital contact (163). The mechanisms by which infection leads to lesion development are unknown. Out of the low-risk virus types causing LP, some studies have documented an aggressive behaviour of HPV6 in LP with young age onset (164, 165). However, HPV11 has been clinically linked as being the more aggressive of the two (166, 167). Patients with LP typically exhibit frequent recurrences in the supraglottic, glottic, and subglottic parts of the larynx (168). Not only does this lead to a reduced quality of life due to the effect on the voice, but it can also lead to airway obstruction (169, 170). The associated healthcare-related costs are also a burden for society (171). Papilloma appears in two stages of life: juvenile (till 12 years) and adult (onset often from 20-40 years). The juvenile LP can resolve spontaneously or cause consequences such as dyspnoea and dysphonia (94). RRP rarely transforms into laryngeal carcinoma (172).

Despite complete debridement of the lesions, recurrences are often observed (170, 173). It has been postulated that neighbouring tissues may act as the latent virus reserve, waiting for trigger signals. These, e.g., a surgical procedure, may stimulate reactivation and multiplication of latent viruses (174, 175). Thus, further research on the interplay between immune cells in LP, and how HPV infection influences the progression of disease is warranted.

A few studies have compared the immune compartment in peripheral blood from LP patients and healthy control individuals and found increased monocyte subpopulations in LP (56) and a deficit in NK cytolytic activity in

RRP (54, 176). Bonagura *et al.*, also demonstrated a specific subset of peripheral blood CD4<sup>+</sup> T cells expressing T<sub>h</sub>2-like cytokines in LP patients (54). Compared to LP patients' peripheral blood, biopsies obtained from patients with severe LP featured higher numbers of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> FOXP3<sup>+</sup> and CD8<sup>+</sup> CD28<sup>-</sup> T cells, both expressing the T<sub>h</sub>2-like cytokines IL-10 and TGF-β (55, 57). In addition, the role of immune inhibitory receptors in HPV resistance and RRP development was recently studied. Transcriptomic expression of PD-L1, located on APCs, and TIGIT on T<sub>regs</sub>, were observed to be upregulated in LP (58, 101, 104), suggesting an attenuation of immune activity, which may enable persistent HPV infection and the development of RRP.

In **paper IV**, we analysed the frequency of immune populations in ten LP samples and eight adjacent healthy laryngeal (HL) fresh tissue samples using flow cytometry and validated our findings using transcriptomic data. Leukocytes (CD45<sup>+</sup>) in the LP and HL samples were assessed for T cell, B cell, and neutrophil frequencies. A higher frequency of neutrophils and lower frequencies of CD8<sup>+</sup> T cells and B cells was detected in LP *cf.* HL. In addition, we studied a specific patient with chronic LP (a case report). Fresh samples from this chronic HPV11<sup>+</sup> LP patient were collected over a period of two years and analysed by flow cytometry (**paper V**). An inverse relation between neutrophils and CD8<sup>+</sup> T cells was consistently observed when the patient was not feeling well. Notably, when swab samples were obtained from LP, the increase in neutrophils correlated with the presence of *Streptococcus dysgalactiae* subspecies *equisimilis* (SDSE, EMM type stG480). After prescribing antibiotics, the patient's surgeries decreased to 2 per year *cf.* 4.5 per year. Further studies of the correlation between bacterial flora and immune contribution in LP are warranted.

# Chapter 4: Dissection of the tumour microenvironment using high-dimensional omics technologies

Precision medicine is primarily used to tailor therapy based on a tumour's molecular characteristics. The TME surrounds the cancer cells and includes components such as immune cells, stromal cells, blood vessels, extracellular vesicles, and nerves. The crosstalk between cancer cells and their surrounding may vary depending on the cause, site, histology, immunological makeup, and other factors. (177, 178). The uniqueness of each tumour's phenotypic interactions leads to distinct changes in the molecular profile of cancer cells, giving these cells and subclones a selective advantage to spread or to resist cancer therapies (179).

Recent technological advancements have enabled scientists to gain unprecedented insights into the cellular and molecular components of the TME, and how these are affected by treatment. These include next-generation sequencing (NGS), single-cell and spatial transcriptomics, proteomics, multiplex imaging, and flow cytometry. Furthermore, these advancements allow for identification of the variability and heterogeneity of tumour architecture, different types of immune cells that infiltrate the tumour, and essential biomarkers that can be used for patient stratification or discovery of new treatment targets, making precision medicine a reality (178, 180). Using multi-omics techniques, combined technological improvements have aided in transitioning pre-clinical knowledge into in-depth precision medicine. For example, a current HNC clinical trial (NCT05296135) is identifying onco-immune phenotypic molecular characteristics in FFPE tumour tissue by utilising NGS-mRNA PD-L1 values to generate a predictive score and classifier for patients considered for immunotherapy. Another study (NCT00230308) applies sequencing techniques to identify numerous

biomarkers to better understand the development and progression of HNC by 2030. They intend to use the discovered biomarkers to develop novel techniques and therapeutics for identifying and treating HNC, bridging a translational gap.

Predicting a phenotype from the transcriptome is not straightforward due to multiple tissue levels and sample manipulation. Spatial resolution would be necessary to improve this. Obtaining a spatial context of transcriptomic signatures was difficult until recently, but high-dimensional imaging has enabled the combination of multiple approaches (181, 182). In this thesis, numerous cutting-edge workflows and technologies were used to identify biomarkers that can potentially identify novel treatment targets and be translated into improved patient stratification for treatment.

## 4.1 Transcriptomic analysis and cell characterization using deconvolution techniques

ScRNA-seq provides transcriptomic information on single cells, such as immune cells and cancer cells, and has been utilized in numerous oncological disorders to decipher intra- and intertumoral composition and heterogeneity (183, 184). In addition, this technique has made significant advances in investigating cancer signalling pathways, cell crosstalk, and TME in various cancers, including HNC (184, 185).

The TCGA, and other public repositories, such as GEO and XENA, provide transparent dataset accessibility. Moreover, the development of statistical techniques such as CIBERSORTX (186), xCell (187), and Ecotyper (188) have made it possible to correlate gene expression with phenotypes. These workflows are powerful for *in silico* characterisation of cell subsets and currently encompass 69 diverse cell signatures. Furthermore, they can be applied using biologically relevant or newfound signatures (e.g., from single-cell transcriptomics) to identify distributions of cell types of interest in, e.g., bulk RNA samples.

Bioinformatic pipelines, such as those mentioned above, are also advantageous for mapping differences between cohorts as well as at individual levels. In **papers I, II, and III**, we conducted cell characterisation in addition to differential expression analysis. CIBERSORTX (186) was used on publicly available datasets in the first two studies. We used the method by Danaher *et*



*al.*, (189) in the third study, which is specially designed for NanoString® GEN2 nCounter® data.

#### **4.1.1 Gene expression evaluation in NPC and transcriptomics-based association with immune phenotypes**

In **paper I**, gene expression analysis, focusing on cell signatures, was performed using publicly available NPC and healthy control data (134, 190). The CD8<sup>+</sup> T cell signature from Puram *et al.*, and Newman *et al.*, via CIBERSORTX (185, 186, 191) assisted in dividing the NPC into CD8<sup>HIGH</sup> and CD8<sup>LOW</sup> samples. Differences in immune cell signatures between the NPC subgroups (CD8<sup>HIGH</sup> and CD8<sup>LOW</sup>) and healthy controls were observed. There was a considerable increase in "Macrophages" for CD8<sup>HIGH</sup> NPC compared to controls. Increased expression of signatures linked to macrophages, pDCs, NK cells, and CLEC9A<sup>+</sup> DCs have been shown to be associated with improved survival, suggesting an anti-tumoural role of these cells in the NPC TME (118). In addition, Pearson's correlation analysis was carried out between CD8A transcript levels and DE genes from multigroup analysis from CD8<sup>HIGH/LOW</sup> NPC and healthy controls. A significant ( $r > 0.7$ ) correlation was observed between the CD8A gene and the immune checkpoint marker LAG3. Chen *et al.*, also established an association between LAG3 with tumour infiltrating CD8<sup>+</sup> T cells in NPC (118). Higher expression of LAG3 along with PD-1 in NPC TILs has been associated with poor DFS (192). Pre-clinical mouse models-based studies had earlier shown that LAG-3 inhibition enhanced T cell effector capacities and was complementary to anti-PD-1/PD-L1 (193, 194). Therefore, combining anti-LAG3 and anti-PD-1 therapy for NPC treatment may be favourable for immune activation and response in NPC.

Apart from TILs, other studies using scRNA-seq have demonstrated that tumour-infiltrating myeloid cells are a heterogeneous mix of cell types, including mature mast cells, monocytes, macrophages, DCs, and MDSCs (119, 142). Interestingly, macrophage maturation and polarization do not follow the conventional M1/M2 model in the NPC TME. Instead, the invading macrophages express both M1 and M2-polarized markers such as FCGR2A, FCGR3A, TREM2, and APOC1 (119). Although the role of MDSCs enriched in cancer remains unclear in NPC, a high presence of circulating HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs and high MDSC mRNA expression in NPC correlate with poor DFS (119, 195). Further functional studies at the cellular level are required to assess MDSCs' modulatory potential in NPC.

### 4.1.2 Correlating HPV status and TME profile in TC based on gene expression data

In **paper III**, we examined the transcriptional activity of HPV<sup>+</sup>/HPV<sup>-</sup> TC using TCGA RNA sequencing data. Similar to **paper I**, various cell signatures (185, 186, 191) aided in understanding the TME profile. Pathway analysis revealed higher leukocyte infiltration, antigen presentation, and T cell priming in HPV<sup>+</sup> TC *cf.* HPV<sup>-</sup> TC. Considering our observations from fresh tissue analysis, a subdivision of HPV<sup>+</sup> TC samples from TCGA data was performed based on CD8<sup>+</sup> T cell signatures. Previously, increased CD8<sup>+</sup> T cells associated with HPV have been noted in HPV-driven OPC (TC and base of the tongue cancer) (196). An increase of CD8<sup>+</sup> T cells populations has also been demonstrated in HPV<sup>+</sup> HNC, as compared to HPV<sup>-</sup> HNC, by scRNA-seq analysis (127). The distinction between HPV<sup>+</sup> and HPV<sup>-</sup> OPC has been attributed to the presence of viral antigens, which may lead to early innate immune responses and an increase in the T cells' adaptive immunological response, resulting in different immune profiles in the TME (196, 197). Previous studies had included all anatomic subsites in HNC and OPC; however, we used TCGA data in **paper III** to study the TC-specific immune profiles. CD8 T cell signatures from both bulk and single-cell mRNA sequencing data were utilised to identify and categorise HPV<sup>+</sup> TC samples from the TCGA into CD8<sup>HIGH</sup>/CD8<sup>LOW</sup> (185, 191). Interestingly, the CD8<sup>HIGH</sup> HPV<sup>+</sup> samples, but not HPV<sup>-</sup> ones, correlated significantly with increased overall survival and higher inflammatory scores, as defined by Thorsson *et al.* (198).

When looking into CD8A correlated genes, we observed that IC markers like PDCD1 (PD-1), LAG3, and TIGIT were expressed at a higher level in the CD8<sup>HIGH</sup> HPV<sup>+</sup> group. Other studies have also reported the expression of PD-1 in some fractions of CD8<sup>+</sup> T cells in HPV<sup>+</sup> HNC (127, 199, 200). Consistent with our findings, Wu *et al.*, using scRNA-seq analysis, also demonstrated increased expression levels of CTLA4, PD-1, and LAG3 in CD8<sup>+</sup> T cells from HPV<sup>+</sup> HNC (127). Badoaul *et al.*, reported that patients with high levels of PD-1-expressing T cells in the TME had a higher five-year overall survival (93.9%) than those with low levels (63.6%) (200). Currently, therapeutic antibodies targeting PD-1 and LAG3 (NCT01968109) are under investigation to treat advanced solid tumours, including TC. However, there is still a low response rate for these therapies and a lack of clear understanding of the spatial relationship of IC marker expression and immune cells within the TME. The expression of other IC markers, such as LAG3 and TIGIT, and their correlation to various immune cell populations within HPV<sup>+</sup>/TC may shed light on the different subgroups for future clinical purposes.

### 4.1.3 Laryngeal papilloma and transcriptional data

Several studies have revealed a suppressive TME in LP with a shifting balance between  $T_{h1}$  and  $T_{h2}$  responses (58, 201). The expression of IL-4 by  $CD4^+$  T cells, upregulation of CCL20 mRNA, and downregulation of CCL19 and CCL21 mRNA in LP *cf.* control tissue supports a  $T_{h2}$ -like profile (201, 202). Moreover, when comparing blood samples from LP/RRP-patients with healthy subjects, increased monocyte subpopulations were observed in LP (56), with a defect in the NK cell cytolytic activity for RRP patients (54).

To evaluate the ongoing immune responses in the TME of LP, we compared the transcriptional levels of immune- and tumour-related genes in LP and paired unaffected neighboring healthy laryngeal tissue (HL) (**paper IV**). Transcriptional analysis was performed using the NanoString<sup>®</sup> GEN2 nCounter<sup>®</sup> Analysis System with the PanCancer Immune Profiling Panel. This resulted in 113 transcripts, out of 494 included in the panel, being significantly DE after background threshold and variance filtering were applied. A comparative analysis showed that 37 genes were expressed at higher levels in LP, whereas 76 genes were expressed at lower levels ( $p < 0.05$ ,  $FC \geq 2$ ). The Danaher *et al.*, method (189) for *in silico* immunoscore aided in assessing the distribution of various immune cells associated with significantly expressed genes in LP *cf.* HL, resulting in immune signature scores for 14 immune cell profiles. Due to a lack of probe availability in the refined data set (i.e., for "NK  $CD56^{dim}$  cells" and " $T_{reg}$  cells"), twelve signatures out of 14 expected cell profiles were established. Cell profile scoring revealed a significant increase in expression profiles related to "neutrophils" in LP *cf.* HL ( $p$ -value  $< 0.05$ ), and a significant decrease in "cytotoxic cells", " $CD8$  T cells", "B cells", and "T cells". A recent RNA-seq study examined the transcriptome characteristics of juvenile RRP and adjacent normal tissues (203). Pathway enrichment analysis in RRP revealed that HPV6 RRP was associated with transcripts revealing downregulation of pathways such as " $T_{h1}$  and  $T_{h2}$  cell differentiation", " $T_{h17}$  cell differentiation", "T cell activation", "B cell activation", and "humoral immune response", whereas HPV11 RRP was associated with transcripts revealing negative enrichment of "T cell activation", "neutrophil activation", "neutrophil degranulation", and "dendritic cell migration" (203). Also, TGF- $\beta$ 1-mediated reduction of NK cell cytotoxicity from HPV6/HPV11<sup>+</sup> RRP with downregulated NK cell-activating receptors in RRP peripheral blood has been reported (176). Previously, lower serum IgG levels and a poor prognosis in early-onset RRP were linked to a reduced frequency of plasma B and memory B cells ( $CD19^+$ ) and impaired IL-21 release by T-follicular helper cells in peripheral blood *cf.* healthy controls

(204). Interestingly, to our knowledge, none of the previous studies have reported an increased abundance of neutrophils related to LP, as seen in **paper IV**, and this finding needs to be further evaluated.

## 4.2 Spatial proteomics and transcriptomics

The evolution of genomics and transcriptomics from bulk to single-cell analysis has provided profound insights into the cellular makeup of tumours. On the one hand, single-cell resolution has successfully dissected subpopulations within the TME, but spatial resolution remains unclear (205). On the other hand, single-cell resolution may result in a loss of information on dissociation-sensitive or lowly-expressed protein markers (205). However, advances in spatial resolution techniques, such as GeoMx DSP, Phenocycler, PhenoImager, COSMx, and 10x Visium, have made it possible to correlate the dynamic architecture of TMEs with 100-1000's of proteins/transcripts. Combinatorial analysis merging single-cell data with spatial structures has the potential to shed light on changes in biomarkers in various settings relevant to cancer.

In DSP, using three tissue-specific morphological markers (and one nuclear stain), one can visualise and obtain information on selected regions of interest (ROIs) for specific proteins or transcripts (206). With this technique, the information is limited to a minimum of a certain number of cells: for example, for protein analysis, a minimum of 20 cells is required (207). Interestingly, NanoString® technologies recently launched COSMx, which generates data from single-cell to intracellular levels integrating spatial information with transcriptomics and proteomics (208). Until recently, in techniques such as Phenocycler (Akoya Biosciences®), approximately 100 protein targets have been tagged and visualised at the single-cell level. However, transcriptomics-based information is lacking (209). The advantages of including spatial omics can range from biomarker findings to validations and verifications of treatment responses. Using multiplex staining, PhenoImager, and Phenocycler, researchers have visualised and calculated fold-increases within specific cell populations in dose-response studies for anti-PD-1 or anti-PD-L1 therapy in human FFPE samples (210). Additionally, DSP has been applied to understand proteomic changes within the distinctive tumour morphologies (181, 182). One of the first HNC spatial proteomic studies using the DSP platform was conducted by Kulasinghe *et al.* (211). This study examined tissue morphology using 44 protein markers in 7 metastatic HNC patients treated with nivolumab

and pembrolizumab. Progressive disease was linked to expression of protein markers CD4, CD45RO, CD68, IDO-1, P-ERK, Ki67, PD-L1 PD-1, GZMB, CD45, OX40, STAT3, P-STAT3, CD44, STING, CD66b, P-AKT, and PTEN (211).

Other studies based on multiplex staining, such as PhenoImager, De Sousa *et al.*, reported the effect of the HPV-peptide vaccine in combination with nivolumab in patients with advanced HPV16<sup>+</sup> HNC where only responders showed an increase in activated CTLs (PD-1<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup>), T cells (PD-1<sup>+</sup>CD3<sup>+</sup>), and total macrophages (PD-L1<sup>+</sup>CD68<sup>+</sup> and PD-L1<sup>-</sup>CD68<sup>+</sup>) (212). Furthermore, only C3aR expression in macrophages was associated with progression-free survival (212). Korpela *et al.*, visualised the effect of epidermal growth factor receptor (EGFR) treatment using the PhenoImager pre- and post-treatment in advanced-stage patients. Patients with an excellent response to the drug showed a 4-fold increase in CD8<sup>+</sup> T cells compared to those with a poor response (213).

#### 4.2.1 Delineating spatial heterogeneity in NPC

The NPC TME has an intricate architecture rich in infiltrating leukocytes, potentially making NPC an attractive target for immunotherapy. T cells, B cells, mast cells, macrophages, and neutrophils are among the immune cells forming the TME. Prior research has suggested that an increase in specific subtypes of macrophages (CD68<sup>+</sup>), mast cells, and neutrophil infiltration in NPC might lead to an anti-tumour response (112, 119, 136, 214, 215). Keeping this in mind, we obtained information on 42 protein targets in 30 NPC FFPE samples (**paper II**). Variations between the tumour regions were explored, where immune cells interacting with the cancer cells ("immune-rich cancer cell islet") had higher expression of markers such as GZMB, CD56, CD20, FOXP3, PD-1, and CD68. In contrast, "surrounding stromal leukocytes" regions showed higher expression of B7-H3, CD163, FN1, and CD44, as well as of IC markers such as PD-L1, TIM-3, VISTA, and LAG3. Our findings align with those of Wang *et al.*, who employed digital pathology to show that ICs like PD-L1, B7-H3, B7-H4, and IDO-1 were expressed by both tumour cells and associated immune cells in NPC. In their study, IC markers LAG3, VISTA, TIM3, ICOS, and OX40, on the other hand, were only found to be expressed by immune cells in tumour stroma (141).

Transcriptomic investigations have previously revealed that CD4<sup>+</sup> and CD8<sup>+</sup> T cell clusters in NPC are highly activated and exhausted, as they co-express exhaustion markers LAG3, TIGIT, PD-1, HAVCR2, CTLA4, and TOX and

effector molecules GZMB, GZMK, IFNG, NKG7, and GNLY (118, 119, 141, 145). By multiplex staining of a cohort of 304 NPC patients, Liu *et al.*, identified a combination of biomarkers, i.e., PD-L1, CD163, CXCR5, and CD117, which suggestively could be used to identify low-risk patients that may benefit from IC-therapy (216). In our study, a few CAF-associated markers, such as FN1, were highly expressed in "surrounding stromal leukocytes" regions. FN1 has been found to inhibit cell death in NPC cells by activating the NF- $\kappa$ B pathway, which increases the levels of BCL2 and p65. Additionally, FN1 promotes the migration and invasion of NPC cells by increasing the levels of matrix metalloproteinase 9 (MMP9) and MMP2: enzymes, which break down the extracellular matrix (ECM), and are primarily produced by CAFs (217, 218). Similarly, markers such as B7-H3, which are equally elevated in the surrounding stroma, have been associated with tumour growth in various cancers (219, 220). Furthermore, a significant decrease in cancer cell adhesion to FN1, and a substantial reduction in migration and invasion in B7-H3 depleted cells, have been demonstrated *in vitro* (221). Prior research suggests that B7-H3 is overexpressed in numerous tumour tissue types, which limits the activity of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and may be used as a potential immunotherapy target (222, 223, 224). In addition, combining B7-H3 and FN1 expression in NPC and its stroma may reflect disease progression and be employed as a predictive diagnostic marker. Overall, we were able to identify protein markers associated with immune cells in the TME and correlate them with three phenotypes (**paper II**) and overall survival. Functional studies of these markers might explore their potential therapeutic use in NPC.

# Chapter 5: Concluding remarks and future outlook

HNCs, such as NPC and TC, are heterogeneous lesions characterised by substantial immune cell infiltration and a tolerogenic milieu that promotes tumour progression. Advanced molecular methods, including gene expression analysis and proteome profiling, are increasingly used for biomarker development, with emphasis on the TME, to enable HNC patient stratification and the identification of new therapeutic targets (e.g., **papers I-III**). However, due to the apparent biological complexities involved, it is improbable that a single marker can predict a specific outcome, whereas immune profiles may do so.

Microarray and single-cell sequencing technology enable gene profiling at DNA and RNA levels for nearly all expressed genes as well as for protein profiling, allowing for investigations of the prognostic and therapeutic importance of biomarkers related to tumour characteristics such as cause, site, and stage. In **papers I-III**, fresh biopsies and biobanked FFPE materials of NPC and TC were used to outline biomarkers and profiles focusing on APCs and CD8<sup>+</sup> T cells.

**Paper I** was a retrospective assessment in which immunological characteristics of NPC were investigated and correlated to EBV status, clinical stage, and survival. Based on the number and location of CD8<sup>+</sup> T cells, three immunological phenotypes were identified: "inflamed", "immune-excluded", and "desert" NPC. Additionally, the presence and distribution of CD207<sup>+</sup> cells, presumably a subset of antigen-presenting DCs, which may be used in immune cell targeting, was described. Immune profiles differed between NPC and control tissue as well as between NPC subgroups based on CD8 expression, as evidenced by gene expression (CD8<sup>HIGH</sup> vs. CD8<sup>LOW</sup>). More research into in-depth protein markers is needed to identify different immune cells and comprehend the interaction of all APCs to T cells. Nevertheless, our findings highlight the significance of categorizing pre-treatment NPC.

Limiting aspects of biomarker utility includes tumour heterogeneity, discrepancies between different techniques, and variability in scoring. We used spatial profiling combined with morphological staining in **paper II** to assess the distribution of proteins between various sites, such as cancer cell islets and surrounding stroma in NPC. We further characterized the differences between the phenotypes defined in **paper I** and correlated survival to various markers on the protein level and to independent transcriptomic data. However, the sample size in this study was limited. In the future, larger sample sizes may provide insight into NPC biomarkers' biological impact.

In **paper III**, we compared the immunological profiles of fresh TC samples and contralateral HT and included DNA quantification and RNA expression analyses to characterize HPV status. We showed a link between CD8<sup>+</sup> T cell numbers and APC abundance and revealed differentially expressed biomarkers and signalling pathways in CD8<sup>HIGH</sup> vs. CD8<sup>LOW</sup> HPV<sup>+</sup> TC. In summary, our study reports distinct immunological phenotypes and immune compartment heterogeneity in HPV<sup>+</sup> TC. More research is needed to determine whether this information can aid in patient prognosis and treatment decisions. Furthermore, the diversity of immune cells needs exploration to identify the effector cells and activated cells essential for an anti-tumour response.

This thesis also includes extensive molecular characterisation of LP (**paper IV** and **V**). In **paper IV**, we identified cellular and transcriptional features of LP *cf.* HL. One interesting finding from the case report (**paper V**) sheds light on the microbial interaction within the RRP microenvironment. Our results suggest the possibility of immune cell populations being involved in the progression of LP and thus may aid in developing future therapeutics aimed at reversing the immunosuppressive environment in LP by restoring effective anti-HPV responses.

Understanding the genetics and molecular underpinnings of cancers have made it possible to identify molecular changes that may guide disease management. However, the absence or minimal overlap of biomarkers with high prognostic value identified by similar studies, and the diversity in cancer-specific biological and physiological characteristics, are key factors raised as criticisms of biomarker discovery studies. To overcome such limitations, studies with larger sample sizes, validation of trends or variances in gene expression profiling, and consistent tumour specimen features are necessary. Biomarkers paired with imaging modalities can aid in the management of patients. Pending further clinical studies, biomarkers associated with HPV and EBV, like those mentioned in this thesis, may assist in clinically examining HNC and LP.



In the end, several pre-clinical and clinical studies are being conducted globally, with an aim to identify and treat cancers as early and effectively as possible. Moreover, thereby constructively visualising our work with future motivation to continue working in the field, I would like to conclude with a quote I firmly believe in "We do not need magic to change the world; we carry all the power we need inside ourselves already: we have the power to imagine better"- J.K. Rowling.

# Popular science summary

Cancer is the world's second-most common cause of death. Tumours can arise in any part of the body when normal cells (the building blocks of our bodies) change their behaviour as a result of stimuli within or outside the body. Head and neck cancers (HNC) include tumours of the lips, sinonasal cavities, oral cavity, pharynx (including the naso-, oro-, and hypopharynx), larynx, and salivary glands. Nasopharyngeal cancer (NPC) occurs in the nasopharynx, whereas tonsillar cancer (TC) is an oropharyngeal cancer subtype. NPC and TC are most common in people between the ages of 30 and 60. In addition to deadliness, both are associated with disease-related side effects and adverse effects of existing treatments.

An emerging goal is to treat patients individually to maximise treatment efficacy and minimise side effects. It has been established that NPC linked with Epstein-Barr virus (EBV) and TC linked with human papillomavirus (HPV) respond to treatment better than virus-negative disease. Therefore, in these conditions, we conducted studies to identify disease-specific immune profiles, which are biological qualities of the immune system that can be assessed and associated with a specific disease and may be used to stratify patients in order to guide treatment decisions.

Cancer cells interact with the body's disease-fighting mechanisms, such as immune cells (i.e., white blood cells), in diverse ways. Immune cells can damage and destroy cancer cells. However, cancer cells can modify and stop this response, turn it in their own favour, and continue to grow. It is essential to realise that not all cancers have the same makeup; they behave differently depending on what causes them, where they are located, and what surrounding factors, such as immune cells, are present. In a series of studies, we used cutting-edge technologies and procedures to study differences at molecular and cellular levels to provide an improved understanding of the immune compartment in the tumour microenvironment.

We also investigated laryngeal papilloma (LP), also known as recurrent respiratory papillomatosis (RRP). It is a non-invasive laryngeal condition caused by low-risk HPV, which can cause hoarseness and breathing

difficulties. LP can appear at any age, from childhood to maturity, and reappear anytime. Through our investigations into the lesions, their potential links to bacteria, and their interactions with immune cells, we gained a more comprehensive knowledge of LP and set a course for future biomarker-based studies.

Overall, different high-end technologies and detailed analyses helped us to identify characteristics and differences in the architecture and behaviour of invading immune cells in TC, NPC, and LP. Furthermore, we associated these data with clinically essential parameters, including survival. Finally, we used our findings to make recommendations on specific targets and profiles that may aid in categorising patients for treatment and serve as new prospective treatments.

# लोकप्रिय विज्ञान सारांश

कैंसर दुनिया का दूसरा सबसे महत्वपूर्ण कारण है। ट्यूमर शरीर के किसी भी हिस्से में उत्पन्न हो सकते हैं जब सामान्य कोशिकाएं (हमारे शरीर के निर्माण खंड) शरीर के भीतर या बाहर उत्तेजनाओं के परिणामस्वरूप अपने व्यवहार को बदलते हैं। सिर और गर्दन के कैंसर (एचएनसी) में होंठों के ट्यूमर, साइनोनेसल गुहा, मौखिक गुहा, ग्रसनी (नासो-, ओरो-और हाइपोफैरिक्स सहित), स्वरयंत्र और लार ग्रंथियां शामिल हैं। नासोफैरेनजल कैंसर (एनपीसी) नासोफैरिक्स में होता है, जबकि टॉन्सिलर कैंसर (टीसी) एक ऑरोफरीन्जियल कैंसर उपप्रकार है।

एनपीसी और टीसी 30 और 60 वर्ष की आयु के बीच के लोगों में सबसे आम हैं। समय सीमा के अलावा, दोनों रोग से संबंधित दुष्प्रभावों और मौजूदा उपचारों के प्रतिकूल प्रभावों से जुड़े हैं। इसके अलावा, एक गैर-इनवेसिव लारेंजियल स्थिति जिसे लारेंजियल पैपिलोमा (एलपी) के रूप में जाना जाता है, जिसे आवर्तक श्वसन पैपिलोमैटोसिस (आरआरपी) के रूप में भी जाना जाता है, को स्वरयंत्र में चिकित्सकीय रूप से पहचाना जाता है। एलपी किसी भी उम्र में, बचपन से परिपक्वता तक दिखाई दे सकता है, और किसी भी समय फिर से प्रकट हो सकता है।

एक उभरता हुआ लक्ष्य उपचार प्रभावकारिता को अधिकतम करने और दुष्प्रभावों को कम करने के लिए रोगियों का व्यक्तिगत रूप से इलाज करना है। यह स्थापित किया गया है कि एपस्टीन-बार वायरस (ईबीवी) और मानव पेपिलोमावायरस (एचपीवी) से जुड़े टीसी से जुड़े एनपीसी वायरस-नकारात्मक बीमारी की तुलना में बेहतर उपचार का जवाब देते हैं। नतीजतन, हमने रोग-विशिष्ट प्रतिरक्षा प्रोफाइल की पहचान करने के लिए अध्ययन किया, जो प्रतिरक्षा प्रणाली के जैविक गुण हैं जिनका मूल्यांकन किया जा सकता है और एक विशिष्ट बीमारी से जोड़ा जा सकता है और उपचार के निर्णयों का मार्गदर्शन करने के लिए रोगियों को स्थिर करने के लिए उपयोग किया जा सकता है।

कैंसर कोशिकाएं शरीर के रोग से लड़ने वाले तंत्र, जैसे प्रतिरक्षा कोशिकाओं (यानी, सफेद रक्त कोशिकाओं) के साथ विभिन्न तरीकों से बातचीत करती हैं। प्रतिरक्षा कोशिकाएं कैंसर कोशिकाओं को नुकसान पहुंचा सकती हैं और नष्ट कर सकती हैं। हालांकि, कैंसर कोशिकाएं इस प्रतिक्रिया को संशोधित और रोक सकती हैं, इसे अपने पक्ष में मोड़ सकती हैं, और बढ़ती रहती हैं। यह महसूस करना आवश्यक है कि सभी कैंसर में एक ही मेकअप नहीं होता है; वे इस बात पर निर्भर करते हैं कि उनके कारण क्या हैं, वे कहां स्थित हैं, और आसपास के कारक, जैसे प्रतिरक्षा कोशिकाएं, मौजूद हैं। अध्ययनों की एक श्रृंखला में, हमने ट्यूमर माइक्रोएन्वायरमेंट में प्रतिरक्षा डिब्बे की बेहतर समझ प्रदान करने के लिए आणविक और सेलुलर स्तरों पर अंतर का अध्ययन करने के लिए अत्याधुनिक तकनीकों और प्रक्रियाओं का उपयोग किया।

हमने एलपी घावों पर भी शोध किया - कम जोखिम वाले एचपीवी के कारण होने वाली सौम्य लारेंजियल बीमारी का एक प्रकार, जो बोलने और सांस लेने में कठिनाई का कारण बन सकता है। घावों में हमारी जांच के माध्यम से, बैक्टीरिया के लिए उनके संभावित लिंक, और प्रतिरक्षा कोशिकाओं के साथ उनकी बातचीत, हमने एलपी का अधिक व्यापक ज्ञान प्राप्त किया और भविष्य के बायोमार्कर-आधारित अध्ययनों के लिए एक कोर्स निर्धारित किया।

कुल मिलाकर, विभिन्न उच्च अंत प्रौद्योगिकियों और विस्तृत विश्लेषणों ने हमें टीसी, एनपीसी और एलपी में हमलावर प्रतिरक्षा कोशिकाओं की वास्तुकला और व्यवहार में विशेषताओं और अंतरों की पहचान करने में मदद की। इसके अलावा, हमने इन

आंकड़ों को जीवित रहने सहित नैदानिक रूप से आवश्यक मापदंडों के साथ जोड़ा। अंत में, हमने उनका उपयोग विशिष्ट लक्ष्यों और प्रोफाइल पर सिफारिशें करने के लिए किया जो हो सकते हैं।

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- A.P.J. Abdul Kalam

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As of February 15th, 2023, information on ongoing clinical studies was acquired from the NIH database ClinicalTrials.gov (<http://clinicaltrials.gov/>).

# Paper I-V









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