High-dimensional imaging of the HPV-induced tumor microenvironment to improve clinical outcomes

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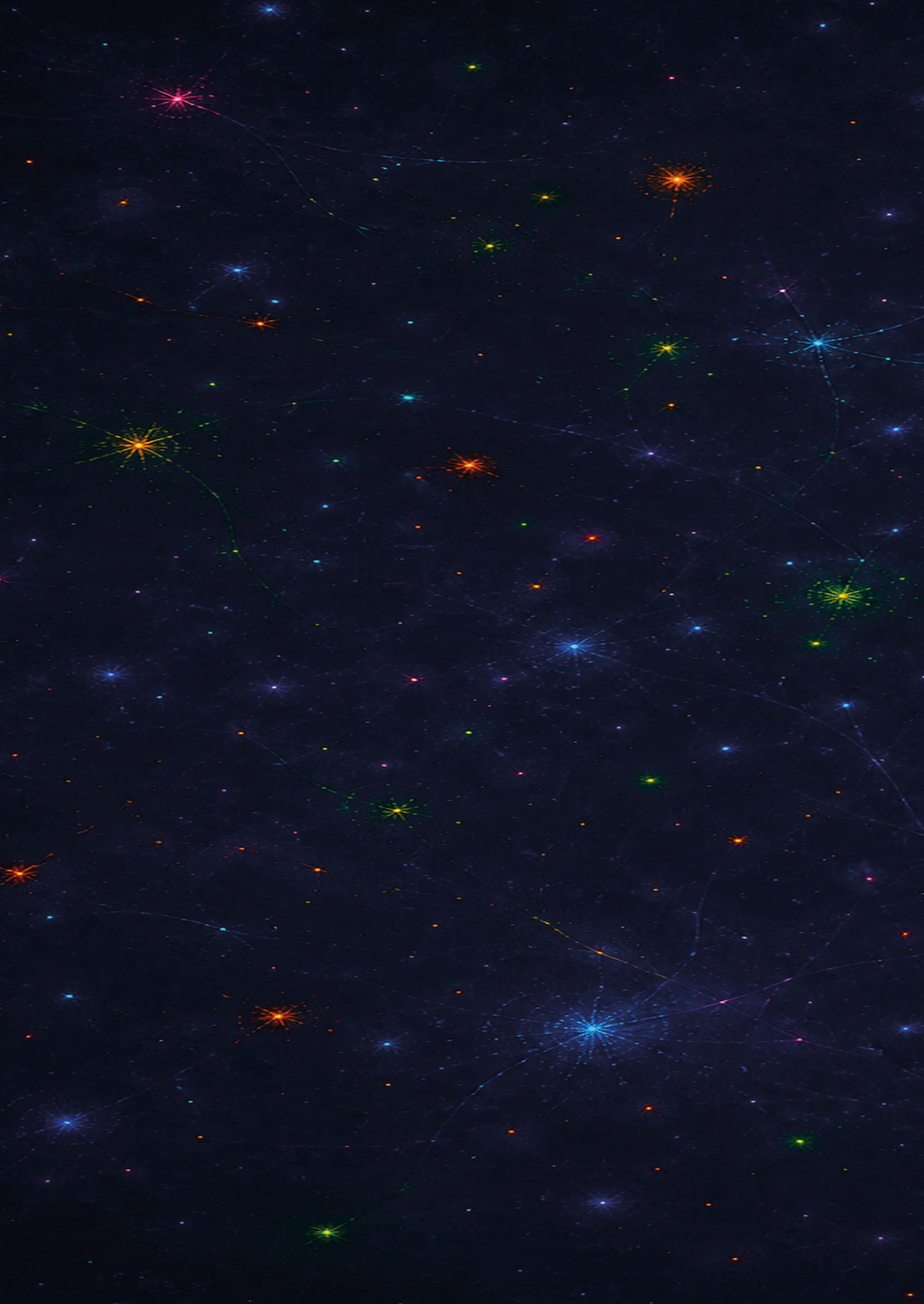

Chapter 1

Introduction

Cancer represents one of the most intricate diseases in medicine, driven not only by the genetic alterations within tumor cells but by a dynamic interplay with the surrounding microenvironment. The tumor microenvironment (TME) is increasingly recognized as a decisive driver of antitumor immunity, determining whether immune cells mediate tumor eradication or contribute to immune escape. Advances in immuno-oncology have revealed that successful therapy depends as much on reprogramming this ecosystem as on targeting the tumor itself. Yet, the precise mechanisms by which spatial organization and cellular composition within the TME shapes therapeutic response remains incompletely understood. This thesis aims to deepen our understanding of these interactions in HPV-induced tumors, leveraging cutting-edge spatial profiling technologies to uncover how the immune landscape governs response to immunotherapy and how it impacts patient survival, ultimately striving to inform the rational design of more effective, personalized treatment strategies.

The tumor microenvironment

Composition of the tumor microenvironment

The TME is a complex ecosystem of cellular and molecular constituents that collectively shape tumor behavior and influence therapeutic outcome. In addition to malignant cells, it comprises diverse immune cell populations, including lymphocytes (e.g. T cells, B cells) and myeloid cells (e.g. macrophages, dendritic cells, neutrophils, mast cells), which together regulate immune surveillance and evasion. Stromal elements, such as fibroblasts and endothelial cells, provide structural integrity while promoting angiogenesis, invasion, and immune modulation. The extracellular matrix (ECM), composed of proteins such as collagen, laminin and fibronectin, acts as a dynamic scaffold that modulates cellular behavior. Soluble factors, including cytokines, chemokines and growth factors, orchestrate communication between tumor and stromal compartments **(Figure 1)**.

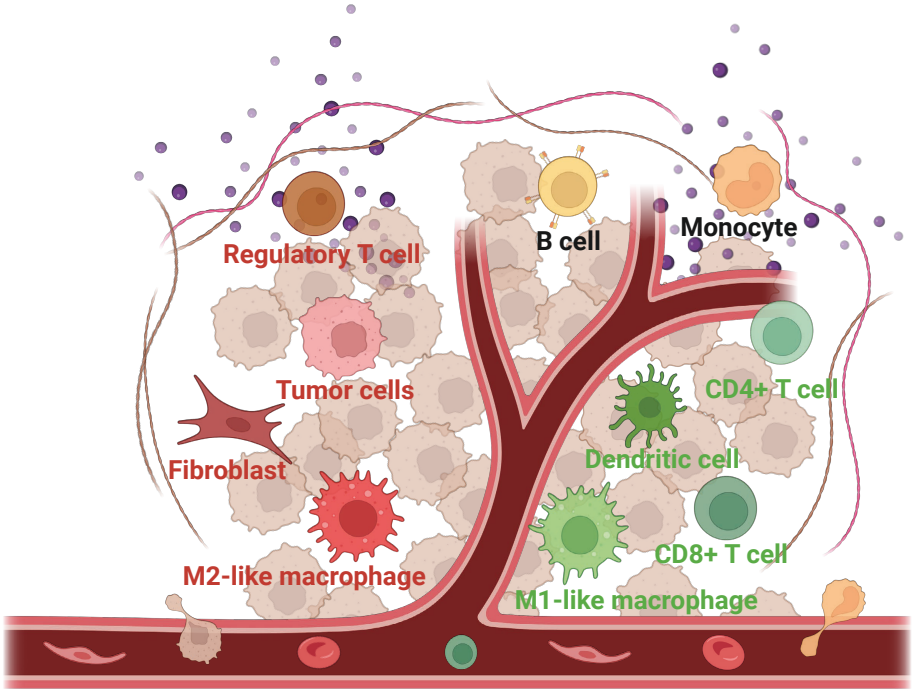

Figure 1. Cellular composition of the tumor microenvironment.

Cell populations associated with effective tumor control are depicted in green, those that promote tumor progression in red and cell types with context-dependent or dual functions are shown in black. Image created with BioRender.

Beyond these individual cellular and molecular components, their reciprocal spatial interactions play a crucial role in tumor dynamics. For example, the proximity of immune cells to tumor cells determines the likelihood of an effective anti-tumor response.[1] Similarly, fibroblast distribution and ECM density can create physical barriers that hinder drug penetration or promote immune exclusion by limiting lymphocyte infiltration.[2] Endothelial cells positioned near tumor nests sustain angiogenesis and facilitate metastatic dissemination.[3] Recent advances in high-resolution imaging have underscored the importance of these spatial relationships. Elucidating these patterns is essential for understanding tumor biology and for designing more effective therapeutic strategies.

The hallmarks of cancer, as described by Hanahan and Weinberg[4], provide a conceptual framework for tumor biology, several of which are tightly linked to the TME. Angiogenesis, for instance, is driven by endothelial cells responding to pro-angiogenic cues, while metastasis depends on ECM remodeling

by cancer-associated fibroblasts (CAFs). Immune evasion is mediated by immunosuppressive populations such as regulatory T cells (Tregs) and tumor-associated myeloid cells (TAMs), often perpetuated by chronic inflammation. Moreover, metabolic reprogramming within the TME allows tumor adaptation to hypoxia and nutrient deprivation. This underscores the TME's centrality in cancer progression and elements therein as promising targets for therapeutic innovation.

Factors shaping the TME

The etiology of a tumor profoundly influences the composition and function of its TME. Tumors arising from carcinogen exposure, such as tobacco-induced lung cancer or ultraviolet light-induced melanoma, typically exhibit a high mutational burden resulting from cumulative DNA damage. This mutational load enhances neoantigen generation and immunogenicity, thereby increasing responsiveness to immunotherapy.[5]

Virus-driven cancers, such as HPV-induced oropharyngeal or genital cancers, EBV-induced nasopharyngeal cancer or Merkel cell polyomavirus-induced skin cancer, often exhibit unique TMEs influenced by viral oncoproteins. Viral antigens enhance immune recognition and provide targets for T cell mediated immunity, supporting immunotherapeutic approaches. Consequently, virus-driven tumors frequently exhibit more inflamed immune profiles, with abundant lymphocytic infiltrates that contribute to improved prognosis and higher responsiveness to immunotherapy.[6-10]

Tumors developing in the context of chronic inflammation, such as colorectal cancer associated with inflammatory bowel disease, arise within an immunologically altered microenvironment. Persistent inflammation recruits innate immune cells and promotes a pro-tumorigenic cytokine milieu characterized by elevated IL-6, IL-10 and TGF-β levels, which suppress adaptive immunity.[11]

The oncogenic pathways activated within tumor cells further shape the immune TME. Aberrant WNT/β-catenin signaling, for instance, suppresses dendritic cell (DC) recruitment and promotes an immune-excluded phenotype. Tumors with active WNT signaling, including colorectal cancer and melanoma, often resist immune checkpoint blockade (ICB) owing to this immune-suppressive milieu.[12 13] Similarly, constitutive activation of the MAPK pathway induces VEGF and other immunosuppressive cytokines, reducing T cell infiltration and

impairing immune recognition.[14] Loss of p53 function can result in unchecked tumor growth and immunosuppression through pathways that enhance TGF-β signaling, which recruits Tregs and MDSCs and creates a tumor-promoting environment.[15] Likewise, *PTEN* loss, frequently observed in prostate and breast cancers, enhances PI3K/Akt signaling, leading to diminished T cell infiltration and poor responsiveness to ICB.[16]

Lastly, the anatomical site of a tumor significantly influences its immune landscape due to the distinct immunological characteristics of each tissue.[17] This aligns with the classical "seed and soil" theory, in which the tumor cells represent the "seed" and the tissue the "soil". Increasing evidence indicates that it is often the "soil" rather than the intrinsic properties of the tumor "seed" that determines outcome: tumors of identical origin can display very different immune profiles and therapeutic outcomes depending on their anatomical location.[18] For example, liver metastases often exhibit a highly immunosuppressive TME, with increased recruitment of Tregs and MDSCs, as well as the expression of immunosuppressive cytokines like IL-10 and TGF-β. This immunosuppressive environment is likely due to the liver's intrinsic tolerogenic properties, which facilitate tolerance to foreign antigens but also hinder effective anti-tumor immunity.[19] Similarly, lung and bone metastases exhibit distinct immune contexts that reflect the primary immune functions of these tissues. Lung metastases may show variable immune infiltration, often influenced by ongoing inflammatory responses in the lung, whereas bone metastases tend to involve immunosuppressive interactions with osteoclasts and other bone-resident cells, limiting effective immune responses.[20] These site-specific differences carry significant clinical implications, as metastases in more immunosuppressive organs tend to respond poorly to immunotherapy, underscoring the need for strategies that either modulate the local TME or enhance systemic immune activation.[17 21]

Impact of the TME on clinical outcome

The TME is a major determinant of clinical outcome in cancer. High infiltration by cytotoxic $CD8^+$ T cells consistently correlates with improved survival across tumor types. These T cells directly recognize and kill tumor cells, and their abundance reflects a pre-existing anti-tumor immune response.[22 23] High intratumoral expression of IFN-γ further enhances anti-tumor activity by upregulating MHC molecules and promoting a pro-inflammatory milieu.[24] In addition, macrophage polarization towards the classically activated M1 phenotype correlates with favorable prognosis; these macrophages secrete

IL-12 and TNF-α, stimulate T cell recruitment and suppress tumor growth, in contrast to immunosuppressive M2-like macrophages.[25] Conversely, increased numbers of immunosuppressive cell populations, such as Tregs and MDSCs, promote an immunosuppressive TME that favors tumor progression and are associated with poor prognosis in many cancer types.[26 27]

The stromal component of the TME also plays a vital role in prognosis. CAFs are key stromal components that secrete ECM molecules such as collagen and laminin, along with cytokines that promote tumor growth and invasion. High CAF density is linked to adverse outcomes, as CAFs drive desmoplasia and generate physical barriers that impede immune infiltration and drug delivery, particularly in pancreatic cancer and breast cancer.[28]

The immune TME is also important in modulating the response to conventional cancer therapies, such as chemotherapy and radiotherapy. Chemotherapy, while primarily targeting rapidly proliferating tumor cells, also exerts profound immunomodulatory effects on the TME, which can either enhance or impede therapeutic efficacy. Anthracyclines, such as doxorubicin, induce immunogenic cell death, characterized by the secretion of danger-associated molecular patterns (DAMPs), which promotes DC recruitment, activation and efficient cross-presentation of tumor antigens. This robust priming of tumor-specific $CD8^+$ and $CD4^+$ T cells fosters durable anti-tumor immunity.[29] In contrast, cyclophosphamide and cisplatin mainly induce conventional apoptosis, releasing limited tumor antigens and DAMPs. Moreover, chemotherapy can deplete critical immune populations through bone marrow suppression, including T cells and dendritic cells, thereby reducing overall immune surveillance.[30]

Similarly, radiotherapy exerts potent immunomodulatory effects that extend beyond its direct cytotoxicity. Ionizing radiation induces DNA damage and immunogenic tumor cell death, enhancing DC recruitment and priming of T cells.[31] Concurrently, radiotherapy reshapes the TME by modulating cytokine secretion and immune cell infiltration, while the tumor vasculature (regulated by stromal and endothelial cells) controls immune cell access to tumor nests, influencing both local immune surveillance and overall therapeutic efficacy.[32]

Immunotherapy

Immunotherapeutic strategies offer the potential for more precise and effective targeting with fewer side effects compared to the above mentioned conventional treatment strategies.

The cancer-immunity cycle and immunotherapy

The cancer-immunity cycle provides a framework for understanding how the immune system interacts with tumors and offers a rational basis for the development of various immunotherapeutic strategies. This cycle describes how tumor antigens are released, captured by dendritic cells and presented to T cells in draining lymph nodes, leading to the priming of tumor-specific effector T cells. These T cells then traffic to and infiltrate the tumor, recognize malignant cells through peptide-MHC complexes and induce their destruction, which in turn releases additional antigens and perpetuates the cycle. However, tumors can often evade immune surveillance by manipulating this cancer-immunity cycle at multiple points.

Immunotherapies differ in their mode of action yet share the common goal of enhancing tumor-specific immune responses. Immune checkpoint blockade (ICB), such as antibodies targeting PD-1 or PD-L1, functions by releasing inhibitory brakes on the immune system, particularly on T cells. This reactivation enables immune recognition and elimination of tumor cells that exploit checkpoint signaling for immune escape. Adoptive cell therapies, including chimeric antigen receptor T cell therapy (CAR-T) and tumor-infiltrating lymphocyte (TIL) transfer, aim to amplify or engineer patients' own T cells for direct tumor targeting. These approaches have shown remarkable efficacy, particularly CAR-T therapy in hematologic malignancies[33] and TIL therapy in melanoma[34]. Cancer vaccines represent another major strategy, designed to activate the immune system against specific tumor antigens. These vaccines can either stimulate a systemic immune response or bolster pre-existing immune activity within the TME, improving tumor recognition and destruction. Oncolytic viruses, which selectively infect and lyse cancer cells while simultaneously inducing immune responses, add another layer of therapeutic intervention by directly disrupting the tumor and activating anti-tumor immunity. This therapy is often used in combination with other approaches to boost immune priming.[35] Collectively, immunotherapeutic interventions act on distinct phases of the cancer-immunity cycle, aiming to overcome immune suppression, amplify tumor-specific responses and achieve durable tumor control.

Impact of the TME on immunotherapy response

The composition of the TME is a key determinant of response to immunotherapy. Tumors with an immunologically "hot" TME, characterized by high infiltration of effector immune populations such as $CD8^+$ T cells, $CD4^+$ helper T cells, dendritic cells, NK cells and M1-like macrophages, along with high IFN-γ signaling and low abundance of immunosuppressive subsets such as Tregs, M2-like macrophages and CAFs, are generally more responsive to immunotherapy. This responsiveness largely reflects the presence of pre-existing, tumor-infiltrating, activated T cells. ICB can then reinvigorate these T cells, allowing them to target tumor cells more effectively.[36] Moreover, hot tumors, such as melanoma and lung cancer, often exhibit high tumor mutational burden (TMB) and abundant neoantigens, promoting immune infiltration and sensitivity to ICB.[5 37]

In contrast, "cold" tumors display immune excluded or immune-desert phenotypes, lacking pre-existing immune activation and thus demonstrating limited responsiveness to ICB. Efforts to convert cold tumors to hot ones through combination therapies, such as the use of oncolytic viruses[38], CXCR4 inhibitors[39], or anti-angiogenic agents[40], are being explored to enhance the efficacy of immunotherapy. A comprehensive understanding of the TME composition and dynamics is essential for optimizing treatment selection, predicting patient outcomes and developing novel therapeutic strategies.

HPV-induced tumors

Etiology of HPV-induced tumors

This thesis focuses on the immunotherapy of HPV-induced tumors. Therefore, we have studied multiple tumor types that are predominantly caused by HPV. These (pre)cancers originate from three different anatomical sites: the vulva, the cervix and the head and neck region **(Figure 2)**. These tumors share overlapping mechanisms of pathogenesis: HPV-driven transformation, mutations in tumor suppressor gene *TP53*, or driven by other oncogenic mutations. Cervical cancer is HPV-driven in approximately 99% of cases, vulvar cancer in 20%[41] and head and neck cancers (HNC), particularly oropharyngeal cancer, in 70-80% of cases[42]. Interestingly, cancers of the vulva and the cervix have a defined premalignant precursor lesion, respectively vulvar and cervical high-grade squamous intraepithelial lesion (HSIL), whereas no equivalent precursor lesion has been clearly identified in HNC. Most HPV infections are transient, with >90% cleared within two years through coordinated innate and adaptive immune responses.[43] Persistent infection, however, may lead to (pre)cancer development.

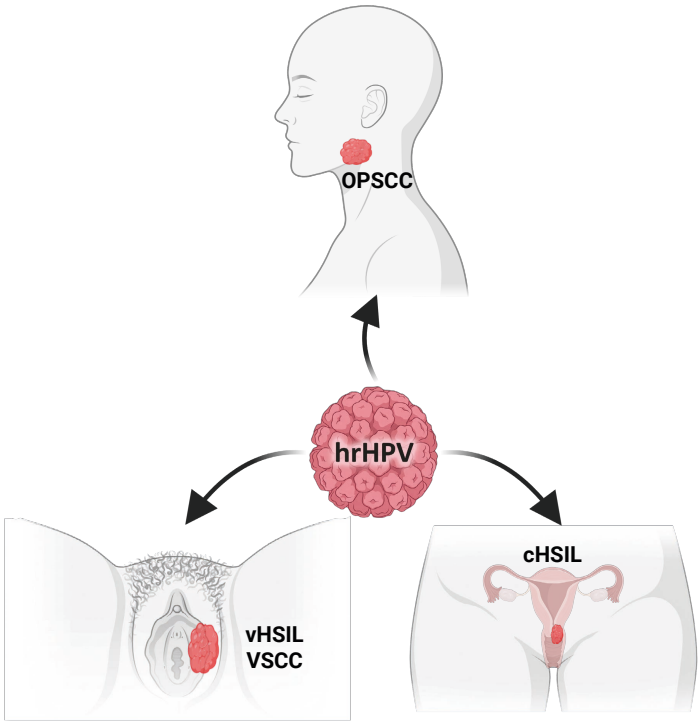

Figure 2. Overview of the HPV-induced tumors studied in this thesis.

High-risk human papillomavirus (hrHPV) is the etiologic driver of cervical high-grade squamous intraepithelial lesion (cHSIL), vulvar high-grade squamous intraepithelial lesion (vHSIL), and the majority of vulvar squamous cell carcinomas (VSCC) and oropharyngeal squamous cell carcinomas (OPSCC). Image created with BioRender.

HPV is a DNA virus that infects basal epithelial cells of stratified squamous epithelium, including the anogenital (cervix, vagina, vulva, penis, anus) and oropharyngeal (tonsils, base of tongue, soft palate, pharynx) regions. It comprises both high- and low-risk genotypes, with HPV16 and HPV18 accounting for over 70% of HPV-associated cancers.[44] Its oncogenicity arises from the expression of the viral oncoproteins E6 and E7. E6 promotes ubiquitin-mediated degradation of p53, thereby disabling p53-dependent apoptosis in DNA-damaged cells.[45] E7 binds and induces degradation of the retinoblastoma protein, promoting unchecked cell-cycle progression.[46] Together, these mechanisms induce genomic instability and malignant transformation.[47] Since E6 and E7 are expressed in all dysplastic cells, they constitute ideal tumor-specific targets for immunotherapy.

HPV-induced (pre)malignancies offer a uniquely powerful model for studying tumor-immune interactions. Because these tumors are driven by a shared and well-defined oncogenic program (primarily the viral oncoproteins E6 and E7) they exhibit far less genomic and etiological heterogeneity than most solid cancers. This molecular homogeneity minimizes confounding variables and creates a "natural experiment" in which inter-patient differences in immune responses are more likely to reflect variation in host immunity or microenvironmental dynamics, rather than underlying oncogenetics. Furthermore, persistent expression of viral antigens provides a controlled framework to examine chronic antigen stimulation and local immune adaptation, processes central to many cancers.[48] Additionally, HPV-driven disease spans a well-defined continuum from high-grade intraepithelial lesions to invasive cancer, enabling a rare opportunity to longitudinally study how the TME evolves during malignant progression. As such, HPV-induced tumors serve as a unique platform to understand generalizable principles of tumor immunology and therapeutic response.

Despite their shared viral origin, HPV-induced tumors differ across anatomical sites[17] due to distinct epithelial contexts, immune environments and clinical behaviors. The next sections summarize the key biological and clinical features of the major HPV-induced (pre)cancers addressed in this thesis.

cHSIL

Cervical high-grade squamous intraepithelial lesion (cHSIL), previously termed cervical intraepithelial neoplasia grades 2/3 (CIN2/3), is a premalignant lesion caused by persistent high-risk HPV (hrHPV) infection. The global incidence of cHSIL is increasing, especially among younger women.[49] Without treatment, up to 50% of cHSIL lesions progress to cervical cancer.[50,51] Standard management of cHSIL involves surgical excision, typically via large loop excision of the transformation zone (LLETZ) or conization, achieving clearance in approximately 90% of cases.[52] Nevertheless, recurrence occurs in ~15% of patients within five years.[53] LLETZ is associated with an increased risk of preterm delivery in subsequent pregnancies, reflecting a 50-70% higher incidence of cervical insufficiency, particularly in women who undergo repeated procedures.[54]

Cervical cancer

Cervical cancer remains the fourth most common cancer type among women worldwide.[55] Given that 99% of cervical cancers are hrHPV-induced, it represents a largely preventable disease, prompting the WHO's Global Cervical

Cancer Elimination Initiative, centered on screening and prophylactic HPV vaccination. For early-stage cervical cancer (FIGO stage I-IIA), radical surgical interventions yield excellent outcomes, with >90% cure rates.[56] Hysterectomy, however, leads to infertility, making fertility-preserving strategies important in younger patients. In locally advanced disease (stage IIB and beyond), cisplatin-based chemoradiation remains the standard of care, achieving five-year survival rates of 66-80%, but at the cost of gastrointestinal and genitourinary toxicities.[56] Recurrent or metastatic disease carries a poor prognosis, with median survival below 12 months despite systemic chemotherapy.[57]

vHSIL

Vulvar high-grade squamous intraepithelial lesion (vHSIL), formerly termed usual vulvar intraepithelial neoplasia (uVIN), is a premalignant lesion predominantly caused by HPV16. This pleiomorphic dermatosis can cause itching or painful burning sensations. If untreated, approximately 3% of vHSIL cases progress to cancer. Current vHSIL management relies on surgical excision or laser ablation, which achieve lesion clearance in ~80% of patients, but have recurrence rates approaching 30%, especially in immunocompromised individuals. Imiquimod is a first-line nonsurgical option, yielding response rates of about 60%.[58] Currently there are no biomarkers to predict treatment success.

Vulvar cancer

Vulvar squamous cell carcinoma (VSCC) is the most common pathological subtype of vulvar cancer. VSCC can arise through three distinct molecular pathways: classical *TP53* mutations (66% of cases, poorest survival), other somatic mutations such as *NOTCH1* or *HRAS* (15% of cases, intermediate survival) and persistent hrHPV infection (19% of cases, best prognosis).[59 60] For early-stage VSCC (FIGO I-II), wide local excision or vulvectomy provides local control in 70-90% of cases depending on disease extent.[61] Advanced (FIGO III-IV) or recurrent VSCC often requires adjuvant chemoradiation, but recurrences remain frequent. In chemoradiation, radiotherapy induces double-strand DNA breaks in rapidly dividing cells (mostly cancer cells). Chemotherapy (cisplatin) acts as a radiosensitizer by forming DNA adducts and crosslinks that distort the DNA helix, which amplifies radiation-induced DNA lesions and slows their repair, thereby increasing the likelihood of cancer cell death.[62] The significant morbidity of these treatments, including chronic genital and urinary complications, underscores the urgency for more effective, less morbid alternatives.

Head and neck cancer

Head and neck cancer (HNC) represents the sixth most common cancer type worldwide, with squamous cell carcinoma comprising 90% of cases.[63] HNC arises from two major etiologic pathways: exposure to carcinogens, such as tobacco and alcohol, leading to oncogenic mutations (e.g. in *TP53*) or persistent hrHPV infection.[64] Currently, 60% of HNC cases are HPV-driven, and this proportion continues to rise.[42] Clinically, patients with HPV-positive HNC exhibit improved survival and reduced recurrence following standard treatments compared with HPV-negative cases.[65] In early-stage HNC, surgery or radiotherapy achieves approximately 75% five-year survival.[66] Locally advanced disease is typically managed with chemoradiation, achieving five-year survival rates of 60-70% but causing significant treatment-related morbidity, including mucositis and dysphagia.[67] In recurrent or metastatic HNC, systemic therapies such as cisplatin or cetuximab (an epidermal growth factor receptor inhibitor) provide limited benefit, with response rates near 15% and median survival below 10 months.[68]

Immunotherapy in HPV-induced tumors

Given the strong immunogenic potential of HPV-induced lesions and the central role of viral antigens in driving tumor biology, numerous immunotherapeutic approaches have been explored to boost antitumor immunity. These include local immune stimulants, vaccines designed to prevent or eliminate HPV-infected cells and checkpoint blockade that reinvigorates T cells.

Imiquimod is a topical immune modulator used in dermatology for its immunostimulatory properties. It is approved for the treatment of basal cell carcinoma, actinic keratosis (precursor of squamous cell carcinoma of the skin), as well as for the treatment of external genital warts (condylomas) caused by low-risk HPV. Imiquimod is a toll-like receptor 7 (TLR7) agonist. TLR7 is expressed on dendritic cells and macrophages, stimulating these cells to release pro-inflammatory cytokines such as IFN-α and IL-12.[69] This cascade triggers local immune activation, recruiting and activating both innate and adaptive immune cells to eradicate dysplastic cells. Clinical studies have reported regression of vHSIL and cHSIL in approximately 60% of patients.[70 71] Nevertheless, inflammatory side effects such as erythema and ulceration limit patient adherence and nearly half of treated patients fail to respond, suggesting that local immune activation alone may be insufficient in these cases.

Prophylactic HPV vaccines (e.g. Gardasil or Cervarix) are based on virus-like particles composed of the HPV L1 capsid protein. These virus-like particles are taken up by antigen-presenting cells as extracellular antigens and therefore processed via the MHC class II pathway, inducing robust $CD4^+$ helper T cell responses that support B cells to produce neutralizing antibodies, which block viral entry into basal epithelial cells. The capsid antigens of prophylactic vaccines are not expressed by infected cells anymore, and therefore these vaccines cannot clear established HPV infections.[72] In contrast, therapeutic HPV vaccines target viral oncoproteins such as E6 and E7, which are expressed within infected or transformed cells. Intracellular expression of these antigens enables processing via the MHC class I pathway, eliciting $CD8^+$ cytotoxic T cell responses, while cross-presentation and professional antigen-presenting cells allow MHC class II presentation to also activate $CD4^+$ helper T cells. This coordinated cellular immunity is critical for recognizing and eliminating HPV-expressing cells.[72] The therapeutic DNA vaccine VGX-3100 targets E6 and E7, and achieved a 50% response rate in cHSIL patients, with complete viral clearance in a subset of responders.[73] Another therapeutic vaccine, ISA101, composed of synthetic long peptides derived from E6 and E7, has induced strong T cell responses but demonstrated limited clinical efficacy in established cancers, with response rates around 30%.[74] This limited efficacy is likely due to the immunosuppressive TME in advanced cancers, where Tregs, MDSCs and cytokines such as TGF-β inhibit cytotoxic T cell activity.[75] Ongoing efforts therefore focus on combination therapies integrating vaccines with ICB or agents that remodel the TME.

Immune checkpoint blockade (ICB) have transformed the treatment landscape across multiple cancer types, but response rates in HPV-associated malignancies remain variable. These agents target inhibitory immune checkpoints, including PD-1 and CTLA-4, expressed on T cells, whose ligands are frequently upregulated on tumor cells and antigen-presenting cells (APCs), thereby suppressing T cell activation. By blocking these pathways, ICB reinvigorates antitumor T cell activity.

Pembrolizumab (a PD-1 inhibitor) has been approved for PD-L1-positive recurrent or metastatic cervical cancer, following trials demonstrating durable responses in approximately 14% of patients.[76] The modest efficacy highlights the need for biomarker-guided patient selection and combinatorial strategies. Anti-angiogenic agents such as bevacizumab, targeting vascular endothelial growth factor (VEGF), have further improved survival in advanced cervical

cancer. When combined with chemotherapy, bevacizumab increases both overall survival and response rates, with reported response rates of 48%, versus 36% with chemotherapy alone.[77] VEGF-targeted therapies may also synergize with immunotherapy by enhancing immune cell infiltration into tumors and mitigating VEGF's immunosuppressive influence in the TME.

For advanced or recurrent VSCC, ICB is under clinical evaluation as an alternative to cytotoxic therapies. Early data show overall response rates around 10%, with median overall survival of six months.[78] Current trials are testing neoadjuvant pembrolizumab to expand treatment options in this setting.[79]

In recurrent or metastatic HNC, nivolumab (a PD-1 inhibitor) demonstrates an overall response rate of 13% in HPV-positive tumors, with durable remissions in a subset of patients.[7] Perioperative (neoadjuvant plus adjuvant) pembrolizumab combined with surgery and radiotherapy significantly improves three-year event-free survival (58% versus 46%) in resectable locally advanced HNC.[80] In addition to ICB, therapeutic HPV vaccines are under evaluation as adjuncts to enhance immune responses in HPV-positive HNC, with early trials showing promising results.[81]

Despite the promise of immunotherapy, a notable subset of patients does not respond to current immunotherapeutic approaches. Importantly, a substantial proportion of ICB-nonresponsive patients do harbor neoantigen-specific T cells, indicating that antitumor immunity is present but not effectively engaged by current therapies.[82] Combination approaches of ICB with radiation or chemotherapy are under investigation to enhance therapeutic efficacy.[83] Previous attempts to identify reliable biomarkers for predicting response to immunotherapy, such as PD-L1 expression, tumor mutational burden and the presence of specific tumor-infiltrating lymphocyte subsets have shown limited clinical usability.[84] Thus, while immunotherapy represents a major step forward in the treatment of cancers, significant challenges remain. The heterogeneity in clinical responses highlights the need for a more comprehensive understanding of the TME and its role in mediating therapeutic efficacy. Identifying predictive biomarkers for patient stratification and developing rational combination strategies are critical steps toward improving response rates and avoiding overtreatment.

Based on prior research linking the TME with survival and immunotherapy response in various cancers, we questioned whether distinct TME features

also contribute to therapeutic outcomes in HPV-induced tumors. Because HPV-induced tumors share the same oncogenic driver, anatomical site and etiological background, they allow precise interrogation of how TME composition shapes immunotherapy response and clinical outcome. Thus, insights gained from studying HPV-induced tumors are broadly informative, revealing principles of TME interactions that may guide treatment strategies across other, more heterogeneous cancer types.

The TME in HPV-induced tumors

The TME of HPV-driven lesions is shaped by a complex interplay of immune and stromal components that influences disease progression and therapeutic response. Immune profiling of cHSIL and cervical cancer has revealed that elevated Treg densities suppress antitumor immunity and correlate with poor prognosis.[85] M2 macrophages contribute to immune suppression by secreting pro-angiogenic and immunoregulatory cytokines, creating a microenvironment favorable for tumor progression.[86,87] MDSCs, recruited through tumor-derived inflammatory cues, inhibit T cell activity via IL-10 and TGF-β secretion and are associated with advanced disease.[88] NK cells in cervical cancer display reduced cytotoxicity and altered receptor expression (e.g. decreased NKG2D), further promoting immune evasion.[89] Moreover, most cervical cancers have an immunosuppressive cytokine environment, with elevated levels of TGF-β, IL-10 and VEGF, promoting Treg and MDSC expansion while inhibiting $CD8^+$ T cell functionality. Concurrently, chronic inflammation driven by cytokines such as IL-6 and TNF-α can support tumor progression by maintaining a pro-tumorigenic environment.[85]

The immune landscape of vHSIL and VSCC has been limitedly explored, as most prior analyses relied on single-marker immunohistochemistry. Available data indicate an increase in $CD14^+$ macrophages during progression from vHSIL to VSCC, with M2-like macrophages exceeding M1-like populations. High densities of stromal $CD14^+$ macrophages correlate positively with intraepithelial Tregs and negatively with activated $CD8^+$ T cells, predictive for reduced recurrence-free survival.[90] Conversely, dense stromal infiltration by activated $CD8^+TIM3^+$ and $CD3^+NKG2A^+$ T cells associates with absence of recurrence and improved outcomes. Interestingly, in VSCC, high T cell infiltration does not consistently predict survival, potentially reflecting functional exhaustion or anergy of TILs.[91]

In HNC, HPV-positive HNC exhibits a more inflamed TME than HPV-negative HNC, characterized by abundant $CD8^+$ and $CD4^+$ T cell infiltration and higher

immune activation, correlating with improved survival.[92 93] The presence of HPV-specific TILs predicts excellent survival.[92] Conversely, HPV-negative HNC, typically associated with tobacco and alcohol use, displays a less inflamed, immunosuppressive TME with reduced lymphocyte infiltration and limited responsiveness to immunotherapy.[94]

Together, these findings highlight that HPV-driven tumors encompass a spectrum of immune microenvironments, ranging from highly inflamed to profoundly immunosuppressive, with significant implications for prognosis and therapy response. Understanding these TME differences is essential for developing more effective immunotherapeutic strategies and thereby improving patient outcomes.

New techniques to high-dimensionally chart the *in situ* TME

Previous in-depth studies of the TME have relied primarily on single-cell analyses of patient-derived materials, such as flow cytometry and single-cell RNA sequencing. These methods have provided key insights into the diversity of cell types and functional cell states within the TME. Nevertheless, a growing body of evidence indicates that the spatial context plays a critical role in shaping clinical outcome.[22 23] At the start of this PhD, the emergence of high-dimensional spatial profiling technologies enabled detailed *in situ* characterization of the cellular and structural organization of the TME. These techniques reveal interactions between tumor cells, immune cells and stromal components that cannot be captured by fluorescence-activated cell sorting (FACS) or bulk RNA sequencing, which lack the spatial dimension **(Figure 3)**. Moreover, they provide a more integrated and comprehensive understanding of the TME than traditional single-marker or low-plex immunohistochemical approaches.

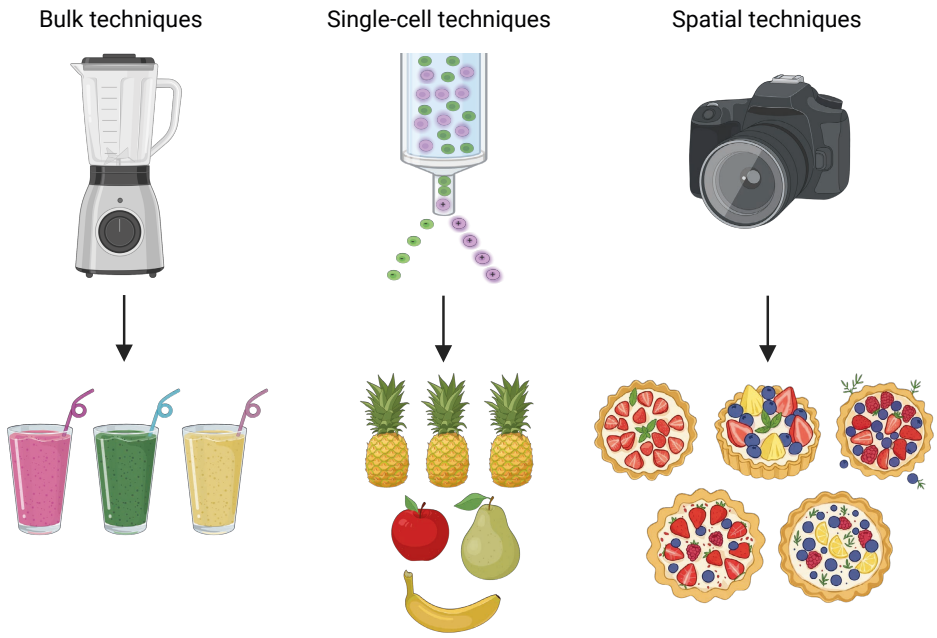

Figure 3. Schematic overview of the techniques to chart the TME.

Bulk techniques generate averaged molecular signatures of the TME. Single-cell technologies disentangle the phenotypic heterogeneity of individual cell types. Spatial techniques localize these cell types within their native tissue architecture, preserving positional context and spatial interactions. Image created with BioRender.

Furthermore, methods such as flow cytometry and RNA sequencing often require fresh tissue, which limits their use for analyzing archival formalin-fixed paraffin-embedded (FFPE) samples. In contrast, technologies including multispectral immunofluorescence (mIF), imaging mass cytometry (IMC) and single-cell spatial transcriptomics enable high-resolution TME analysis directly in FFPE tissue. These approaches facilitate the exploration of archived clinical trial samples, irrespective of size, and allow the discovery of previously unknown cell populations.[95] The spatial information provided by these methods is essential for understanding how the spatial architecture of the TME influences tumor biology, immune evasion and therapeutic response, particularly for immunotherapy, where the proximity and localization of immune cells relative to tumor cells can determine treatment efficacy.[36] Each technique, however, has specific inherent limitations that must be considered.

Multispectral immunofluorescence

At the onset of this PhD, multispectral immunofluorescence (mIF) represented one of the most advanced *in situ* imaging methods available for multiplexed tissue analysis. Today, mIF is a cornerstone technology for studying the spatial organization of immune cells and tumor cells within the TME. Using antibodies conjugated to spectrally distinct fluorophores, mIF enables the simultaneous detection of multiple markers on a single FFPE tissue section. Through spectral unmixing, up to seven fluorescent signals can be quantified, allowing detailed visualization of immune-tumor cell interactions at subcellular resolution (0.5-1µm per pixel).[96] Although highly effective for high-resolution spatial mapping, the limited number of markers that can be analyzed per panel constrains its use in studies requiring broader phenotypic coverage. Furthermore, mIF is a medium-throughput technique, which can restrict its application in large-scale or discovery-driven studies where high multiplexing is advantageous.

Imaging mass cytometry

Imaging mass cytometry (IMC) extends spatial proteomic profiling by allowing simultaneous detection of up to 50 markers. In IMC, antibodies are conjugated to heavy-metal isotopes, and tissue is ablated using a laser, ionizing the tags for quantification by mass spectrometry.[97] This eliminates the spectral overlap inherent to fluorescence-based systems, enabling highly multiplexed spatial analyses. However, IMC's resolution is lower than that of mIF and its throughput is limited by long acquisition times. The integration of spatial data with mass spectrometry-based quantification also requires sophisticated computational workflows for data normalization, visualization and interpretation. Despite these challenges, IMC remains a powerful tool for comprehensive, hypothesis-generating studies of the TME, capable of revealing complex multicellular interactions and signaling networks.[98]

Single-cell spatial transcriptomics

Single-cell spatial transcriptomics represents the next generation of TME profiling by enabling spatially resolved transcriptomic mapping of thousands of genes *in situ*. This approach captures transcriptional heterogeneity within the tissue architecture and links gene expression patterns to specific spatial niches. Platforms such as Visium (10x Genomics), Xenium (10x Genomics) and CosMx (NanoString) preserve spatial integrity while detecting RNA transcripts directly in FFPE tissue sections, generating high-resolution expression maps. While this technology provides an unprecedented view of cellular states, it remains limited by lower throughput and the imperfect correlation between mRNA and

protein expression, which complicates the interpretation of functional immune phenotypes.[99] Moreover, cellular functions influenced by post-translational modifications or protein-protein interactions require complementary proteomic approaches to achieve a more complete understanding of the underlying biology.

Thesis outline

The immune TME plays a critical role in determining patient survival and response to immunotherapy across multiple cancer types, yet it has not been comprehensively investigated in HPV-induced (pre)cancers. The aim of this thesis is to provide an in-depth characterization of the TME in HPV-driven lesions, extending beyond what was previously feasible, and to relate these findings to clinical outcomes. At the onset of this PhD, available immunotherapeutic strategies elicited clinical responses in only a subset of HPV-associated precancer patients, with no reliable predictive biomarkers. In addition, the impact of the TME on survival in HPV-associated HNC and VSCC was largely unexplored. Addressing these gaps is essential to determine whether, and which types of, immunotherapies should be developed for this patient population. These two overarching questions therefore form the foundation of this thesis.

Part I: immunotherapy and TME of HPV-induced precancer

In part I we explored the impact of the TME on response to immunotherapy in HPV-associated precancers. In **chapter 2** we give an overview of what was known about the immune microenvironment of vulvar (pre)cancer at the time this PhD was started. Previously, it was reported that the clinical response to immunotherapeutic vaccination was related to the strength of the systemic T cell response, yet there was too much overlap between responders and non-responders for it to be the sole determinant.[100] Therefore, in **chapter 3** we investigated whether the pre-existing TME of vHSIL was associated with clinical response to immunotherapeutic vaccination. Not only did we show that there were differences in immune infiltration between non-responders and responders, but our study of post-vaccination samples also revealed that therapeutic vaccination did not convert an immunological cold lesion into hot. The outcomes of this study led to two new studies. The first of these explored whether the TME composition is also important for response to imiquimod immunotherapy. Therefore, we studied pre-imiquimod vHSIL samples of

responders and non-responders to imiquimod in **chapter 4**. This revealed that a pre-existing hot TME was required for responses to both imiquimod and therapeutic vaccination, with imiquimod responses being predominantly driven by high infiltration by inflammatory myeloid cells, and vaccination responses being mainly associated with high infiltration by pro-inflammatory T cells. Since these types of immunological analyses were heavily steered to certain known cell types, we applied single-cell spatial transcriptomics to study the TME of therapeutically vaccinated vHSIL patients in **chapter 5**. This more holistic approach to study the TME revealed that responders had a pre-existing type 1 immune contexture and a distinct spatial ecosystem of well-organized immune cells, in contrast to non- and partial-responders. Finally, in **chapter 6** we analyzed whether the findings that were made in vHSIL regarding the predictive capacity of the TME for response to imiquimod could be extrapolated to cHSIL, and further developed this into a predictive biomarker that is currently prospectively tested.

Part II: survival and TME of HPV-induced cancer patients

In part II we investigated the impact of the TME on survival in VSCC and HNC patients. We for the first time explored the composition and impact of the myeloid landscape of VSCC in **chapter 7**. Here, we found that high monocyte infiltration in the TME forms an independent positive prognostic biomarker for survival in these patients. In addition, we applied combined IMC, single-cell and bulk transcriptomics for an in-depth analyses of the TME in HNC in **chapter 8.** Alike our findings in vHSIL, the presence of specific spatial ecosystems were important for survival. In particular, the presence of intratumoral tumor-specific T cells was associated with the presence of intra-epithelial immune microaggregates, comprising $CD4^+$ and $CD8^+$ T cells as well as dendritic cells, and these were associated with excellent survival.

The main findings of this thesis are summarized in **chapter 9** (in English) and **chapter 10** (in Dutch), accompanied by a forward-looking outlook that highlights key clinical implications and identifies promising avenues for future research.

References

1. Li JR, Pan X, Lin Y, et al. Spatial Proximity of Immune Cell Pairs to Cancer Cells in the Tumor Microenvironment as Biomarkers for Patient Stratification. *Cancers (Basel)* 2025;17(14) doi: 10.3390/cancers17142335 [published Online First: 2025/07/29]
2. Grout JA, Sirven P, Leader AM, et al. Spatial Positioning and Matrix Programs of Cancer-Associated Fibroblasts Promote T-cell Exclusion in Human Lung Tumors. *Cancer Discov* 2022;12(11):2606-25. doi: 10.1158/2159-8290.Cd-21-1714 [published Online First: 2022/08/27]
3. Oria VO, Castro JL, de Pina Roque J, et al. Crosstalk between tumor endothelial cells and cancer cells is important for metastasis initiation. *Cell Commun Signal* 2025;23(1):443. doi: 10.1186/s12964-025-02441-3 [published Online First: 2025/10/17]
4. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57-70. doi: 10.1016/s0092-8674(00)81683-9 [published Online First: 2000/01/27]
5. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015;348(6230):124-8. doi: 10.1126/science.aaa1348 [published Online First: 2015/03/15]
6. Piersma SJ, Jordanova ES, van Poelgeest MI, et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* 2007;67(1):354-61. doi: 10.1158/0008-5472.Can-06-3388 [published Online First: 2007/01/11]
7. Ferris RL, Blumenschein Jr G, Fayette J, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. *N Engl J Med* 2016;375:1856-67.
8. Kuo YC, Tai TS, Yang HY, et al. Characterization of the immune cell profile in metastatic nasopharyngeal carcinoma treated with chemotherapy and immune checkpoint inhibitors. *Am J Cancer Res* 2024;14(12):5717-33. doi: 10.62347/sspi9013 [published Online First: 2025/01/13]
9. Huang SCM, Tsao SW, Tsang CM. Interplay of Viral Infection, Host Cell Factors and Tumor Microenvironment in the Pathogenesis of Nasopharyngeal Carcinoma. *Cancers (Basel)* 2018;10(4) doi: 10.3390/cancers10040106 [published Online First: 2018/04/05]
10. Nghiem PT, Bhatia S, Lipson EJ, et al. PD-1 Blockade with Pembrolizumab in Advanced Merkel-Cell Carcinoma. *N Engl J Med* 2016;374(26):2542-52. doi: 10.1056/NEJMoa1603702 [published Online First: 2016/04/20]
11. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010;140(6):883-99. doi: 10.1016/j.cell.2010.01.025 [published Online First: 2010/03/23]
12. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. *Nature* 2015;523(7559):231-5. doi: 10.1038/nature14404 [published Online First: 2015/05/15]
13. Luke JJ, Bao R, Sweis RF, et al. WNT/β-catenin Pathway Activation Correlates with Immune Exclusion across Human Cancers. *Clin Cancer Res* 2019;25(10):3074-83. doi: 10.1158/1078-0432.Ccr-18-1942 [published Online First: 2019/01/13]
14. Sumimoto H, Imabayashi F, Iwata T, et al. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* 2006;203(7):1651-6. doi: 10.1084/jem.20051848 [published Online First: 2006/06/28]
15. Blagih J, Zani F, Chakravarty P, et al. Cancer-Specific Loss of p53 Leads to a Modulation of Myeloid and T Cell Responses. *Cell Rep* 2020;30(2):481-96.e6. doi: 10.1016/j.celrep.2019.12.028 [published Online First: 2020/01/16]
16. Peng W, Chen JQ, Liu C, et al. Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy. *Cancer Discov* 2016;6(2):202-16. doi: 10.1158/2159-8290.Cd-15-0283 [published Online First: 2015/12/10]
17. Santegoets SJ, van Ham VJ, Ehsan I, et al. The Anatomical Location Shapes the Immune Infiltrate in Tumors of Same Etiology and Affects Survival. *Clin Cancer Res* 2019;25(1):240-52. doi: 10.1158/1078-0432.Ccr-18-1749 [published Online First: 2018/09/19]
18. Langley RR, Fidler IJ. The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer* 2011;128(11):2527-35. doi: 10.1002/ijc.26031 [published Online First: 2011/03/03]

19. Liu Y, Yang H, Li T, et al. Immunotherapy in liver cancer: overcoming the tolerogenic liver microenvironment. *Front Immunol* 2024;15:1460282. doi: 10.3389/fimmu.2024.1460282 [published Online First: 2024/09/19]
20. Linde N, Casanova-Acebes M, Sosa MS, et al. Macrophages orchestrate breast cancer early dissemination and metastasis. *Nat Commun* 2018;9(1):21. doi: 10.1038/s41467-017-02481-5 [published Online First: 2018/01/04]
21. Binnewies M, Roberts EW, Kersten K, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med* 2018;24(5):541-50. doi: 10.1038/s41591-018-0014-x [published Online First: 2018/04/25]
22. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313(5795):1960-4. doi: 10.1126/science.1129139 [published Online First: 2006/09/30]
23. Fridman WH, Pagès F, Sautès-Fridman C, et al. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012;12(4):298-306. doi: 10.1038/nrc3245 [published Online First: 2012/03/16]
24. Wei X, Ruan H, Zhang Y, et al. Pan-cancer analysis of IFN-γ with possible immunotherapeutic significance: a verification of single-cell sequencing and bulk omics research. *Front Immunol* 2023;14:1202150. doi: 10.3389/fimmu.2023.1202150 [published Online First: 2023/08/30]
25. Garrido-Martin EM, Mellows TWP, Clarke J, et al. M1(hot) tumor-associated macrophages boost tissue-resident memory T cells infiltration and survival in human lung cancer. *J Immunother Cancer* 2020;8(2) doi: 10.1136/jitc-2020-000778 [published Online First: 2020/07/24]
26. DeNardo DG, Barreto JB, Andreu P, et al. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 2009;16(2):91-102. doi: 10.1016/j.ccr.2009.06.018 [published Online First: 2009/08/04]
27. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10(9):942-9. doi: 10.1038/nm1093 [published Online First: 2004/08/24]
28. Kalluri R. The biology and function of fibroblasts in cancer. *Nat Rev Cancer* 2016;16(9):582-98. doi: 10.1038/nrc.2016.73 [published Online First: 2016/08/24]
29. Casares N, Pequignot MO, Tesniere A, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med* 2005;202(12):1691-701. doi: 10.1084/jem.20050915 [published Online First: 2005/12/21]
30. Bracci L, Schiavoni G, Sistigu A, et al. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ* 2014;21(1):15-25. doi: 10.1038/cdd.2013.67 [published Online First: 2013/06/22]
31. Gupta A, Probst HC, Vuong V, et al. Radiotherapy promotes tumor-specific effector CD8+ T cells via dendritic cell activation. *J Immunol* 2012;189(2):558-66. doi: 10.4049/jimmunol.1200563 [published Online First: 2012/06/12]
32. Forster JC, Harriss-Phillips WM, Douglass MJ, et al. A review of the development of tumor vasculature and its effects on the tumor microenvironment. *Hypoxia (Auckl)* 2017;5:21-32. doi: 10.2147/hp.S133231 [published Online First: 2017/04/27]
33. Haslauer T, Greil R, Zaborsky N, et al. CAR T-Cell Therapy in Hematological Malignancies. *Int J Mol Sci* 2021;22(16) doi: 10.3390/ijms22168996 [published Online First: 2021/08/28]
34. Klobuch S, Seijkens TTP, Schumacher TN, et al. Tumour-infiltrating lymphocyte therapy for patients with advanced-stage melanoma. *Nat Rev Clin Oncol* 2024;21(3):173-84. doi: 10.1038/s41571-023-00848-w [published Online First: 2024/01/09]
35. LaRocca CJ, Warner SG. Oncolytic viruses and checkpoint inhibitors: combination therapy in clinical trials. *Clin Transl Med* 2018;7(1):35. doi: 10.1186/s40169-018-0214-5 [published Online First: 2018/11/15]
36. **36.** Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature* 2017;541(7637):321-30. doi: 10.1038/nature21349 [published Online First: 2017/01/20]
37. Snyder A, Makarov V, Merghoub T, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 2014;371(23):2189-99. doi: 10.1056/NEJMoa1406498 [published Online First: 2014/11/20]

38. Twumasi-Boateng K, Pettigrew JL, Kwok YYE, et al. Oncolytic viruses as engineering platforms for combination immunotherapy. *Nat Rev Cancer* 2018;18(7):419-32. doi: 10.1038/s41568-018-0009-4 [published Online First: 2018/04/27]
39. Chen IX, Chauhan VP, Posada J, et al. Blocking CXCR4 alleviates desmoplasia, increases T-lymphocyte infiltration, and improves immunotherapy in metastatic breast cancer. *Proc Natl Acad Sci U S A* 2019;116(10):4558-66. doi: 10.1073/pnas.1815515116 [published Online First: 2019/02/01]
40. Fukumura D, Kloepper J, Amoozgar Z, et al. Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges. *Nat Rev Clin Oncol* 2018;15(5):325-40. doi: 10.1038/nrclinonc.2018.29 [published Online First: 2018/03/07]
41. Hinten F, Molijn A, Eckhardt L, et al. Vulvar cancer: Two pathways with different localization and prognosis. *Gynecol Oncol* 2018;149(2):310-17. doi: 10.1016/j.ygyno.2018.03.003 [published Online First: 2018/03/21]
42. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* 2011;29(32):4294-301. doi: 10.1200/jco.2011.36.4596 [published Online First: 2011/10/05]
43. Stanley M. Immune responses to human papillomavirus. *Vaccine* 2006;24 Suppl 1:S16-22. doi: 10.1016/j.vaccine.2005.09.002 [published Online First: 2005/10/13]
44. Koutsky L. Epidemiology of genital human papillomavirus infection. *Am J Med* 1997;102(5a):3-8. doi: 10.1016/s0002-9343(97)00177-0 [published Online First: 1997/05/05]
45. Scheffner M, Werness BA, Huibregtse JM, et al. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990;63(6):1129-36. doi: 10.1016/0092-8674(90)90409-8 [published Online First: 1990/12/21]
46. Münger K, Phelps WC, Bubb V, et al. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989;63(10):4417-21. doi: 10.1128/jvi.63.10.4417-4421.1989 [published Online First: 1989/10/01]
47. Doorbar J, Egawa N, Griffin H, et al. Human papillomavirus molecular biology and disease association. *Rev Med Virol* 2015;25 Suppl 1(Suppl Suppl 1):2-23. doi: 10.1002/rmv.1822 [published Online First: 2015/03/11]
48. Gavil NV, Scott MC, Weyu E, et al. Chronic antigen in solid tumors drives a distinct program of T cell residence. *Sci Immunol* 2023;8(84):eadd5976. doi: 10.1126/sciimmunol.add5976 [published Online First: 2023/06/02]
49. Ting J, Rositch AF, Taylor SM, et al. Worldwide incidence of cervical lesions: a systematic review. *Epidemiol Infect* 2015;143(2):225-41. doi: 10.1017/s0950268814001356 [published Online First: 2014/06/01]
50. McCredie MR, Sharples KJ, Paul C, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *Lancet Oncol* 2008;9(5):425-34. doi: 10.1016/s1470-2045(08)70103-7 [published Online First: 2008/04/15]
51. Loopik DL, IntHout J, Ebisch RMF, et al. The risk of cervical cancer after cervical intraepithelial neoplasia grade 3: A population-based cohort study with 80,442 women. *Gynecol Oncol* 2020;157(1):195-201. doi: 10.1016/j.ygyno.2020.01.023 [published Online First: 2020/01/25]
52. Bigrigg A, Haffenden DK, Sheehan AL, et al. Efficacy and safety of large-loop excision of the transformation zone. *Lancet* 1994;343(8888):32-4. doi: 10.1016/s0140-6736(94)90881-8 [published Online First: 1994/01/01]
53. Bogani G, V DID, Sopracordevole F, et al. Recurrence rate after loop electrosurgical excision procedure (LEEP) and laser Conization: A 5-year follow-up study. *Gynecol Oncol* 2020;159(3):636-41. doi: 10.1016/j.ygyno.2020.08.025 [published Online First: 2020/09/08]
54. Kyrgiou M, Athanasiou A, Paraskevaidi M, et al. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: systematic review and meta-analysis. *Bmj* 2016;354:i3633. doi: 10.1136/bmj.i3633 [published Online First: 2016/07/30]
55. Arbyn M, Weiderpass E, Bruni L, et al. Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. *Lancet Glob Health* 2020;8(2):e191-e203. doi: 10.1016/s2214-109x(19)30482-6 [published Online First: 2019/12/10]

56. Cibula D, Pötter R, Planchamp F, et al. The European Society of Gynaecological Oncology/European Society for Radiotherapy and Oncology/European Society of Pathology Guidelines for the Management of Patients With Cervical Cancer. *Int J Gynecol Cancer* 2018;28(4):641-55. doi: 10.1097/igc.0000000000001216 [published Online First: 2018/04/25]
57. Tewari KS, Monk BJ. New strategies in advanced cervical cancer: from angiogenesis blockade to immunotherapy. *Clin Cancer Res* 2014;20(21):5349-58. doi: 10.1158/1078-0432.Ccr-14-1099 [published Online First: 2014/08/12]
58. Wallbillich JJ, Rhodes HE, Milbourne AM, et al. Vulvar intraepithelial neoplasia (VIN 2/3): comparing clinical outcomes and evaluating risk factors for recurrence. *Gynecol Oncol* 2012;127(2):312-5. doi: 10.1016/j.ygyno.2012.07.118 [published Online First: 2012/08/08]
59. Nooij LS, Ter Haar NT, Ruano D, et al. Genomic Characterization of Vulvar (Pre) cancers Identifies Distinct Molecular Subtypes with Prognostic Significance. *Clin Cancer Res* 2017;23(22):6781-89. doi: 10.1158/1078-0432.Ccr-17-1302 [published Online First: 2017/09/14]
60. Williams EA, Werth AJ, Sharaf R, et al. Vulvar Squamous Cell Carcinoma: Comprehensive Genomic Profiling of HPV+ Versus HPV-Forms Reveals Distinct Sets of Potentially Actionable Molecular Targets. *JCO Precis Oncol* 2020;4 doi: 10.1200/po.19.00406 [published Online First: 2020/09/15]
61. Woelber L, Trillsch F, Kock L, et al. Management of patients with vulvar cancer: a perspective review according to tumour stage. *Ther Adv Med Oncol* 2013;5(3):183-92. doi: 10.1177/1758834012471699 [published Online First: 2013/05/02]
62. Boeckman HJ, Trego KS, Turchi JJ. Cisplatin sensitizes cancer cells to ionizing radiation via inhibition of nonhomologous end joining. *Mol Cancer Res* 2005;3(5):277-85. doi: 10.1158/1541-7786.Mcr-04-0032 [published Online First: 2005/05/12]
63. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68(6):394-424. doi: 10.3322/caac.21492 [published Online First: 2018/09/13]
64. Gormley M, Creaney G, Schache A, et al. Reviewing the epidemiology of head and neck cancer: definitions, trends and risk factors. *Br Dent J* 2022;233(9):780-86. doi: 10.1038/s41415-022-5166-x [published Online First: 2022/11/13]
65. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst* 2008;100(4):261-9. doi: 10.1093/jnci/djn011 [published Online First: 2008/02/14]
66. Barsouk A, Aluru JS, Rawla P, et al. Epidemiology, Risk Factors, and Prevention of Head and Neck Squamous Cell Carcinoma. *Med Sci (Basel)* 2023;11(2) doi: 10.3390/medsci11020042 [published Online First: 2023/06/27]
67. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *New England Journal of Medicine* 2010;363(1):24-35.
68. Vermorken JB, Mesia R, Rivera F, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med* 2008;359(11):1116-27. doi: 10.1056/NEJMoa0802656 [published Online First: 2008/09/12]
69. Schön MP, Schön M. Imiquimod: mode of action. *Br J Dermatol* 2007;157 Suppl 2:8-13. doi: 10.1111/j.1365-2133.2007.08265.x [published Online First: 2007/12/11]
70. Tristram A, Hurt CN, Madden T, et al. Activity, safety, and feasibility of cidofovir and imiquimod for treatment of vulval intraepithelial neoplasia (RT^{3}VIN): a multicentre, open-label, randomised, phase 2 trial. *Lancet Oncol* 2014;15(12):1361-8. doi: 10.1016/s1470-2045(14)70456-5 [published Online First: 2014/10/12]
71. van de Sande AJM, Kengsakul M, Koeneman MM, et al. The efficacy of topical imiquimod in high-grade cervical intraepithelial neoplasia: A systematic review and meta-analysis. *Int J Gynaecol Obstet* 2024;164(1):66-74. doi: 10.1002/ijgo.14953 [published Online First: 2023/06/23]
72. Pollard AJ, Bijker EM. A guide to vaccinology: from basic principles to new developments. *Nat Rev Immunol* 2021;21(2):83-100. doi: 10.1038/s41577-020-00479-7 [published Online First: 2020/12/24]
73. Trimble CL, Morrow MP, Kraynyak KA, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet* 2015;386(10008):2078-88. doi: 10.1016/s0140-6736(15)00239-1 [published Online First: 2015/09/21]

74. Sousa LG, Rajapakshe K, Rodriguez Canales J, et al. ISA101 and nivolumab for HPV-16(+) cancer: updated clinical efficacy and immune correlates of response. *J Immunother Cancer* 2022;10(2) doi: 10.1136/jitc-2021-004232 [published Online First: 2022/02/24]
75. Welters MJP, Santegoets SJ, van der Burg SH. The Tumor Microenvironment and Immunotherapy of Oropharyngeal Squamous Cell Carcinoma. *Front Oncol* 2020;10:545385. doi: 10.3389/fonc.2020.545385 [published Online First: 2021/01/12]
76. Frenel JS, Le Tourneau C, O'Neil B, et al. Safety and Efficacy of Pembrolizumab in Advanced, Programmed Death Ligand 1-Positive Cervical Cancer: Results From the Phase Ib KEYNOTE-028 Trial. *J Clin Oncol* 2017;35(36):4035-41. doi: 10.1200/jco.2017.74.5471 [published Online First: 2017/11/03]
77. Tewari KS, Sill MW, Long HJ, 3rd, et al. Improved survival with bevacizumab in advanced cervical cancer. *N Engl J Med* 2014;370(8):734-43. doi: 10.1056/NEJMoa1309748 [published Online First: 2014/02/21]
78. Maiorano MFP, Loizzi V, Cormio G, et al. Immunotherapy and Advanced Vulvar Cancer: A Systematic Review and Meta-Analysis of Survival and Safety Outcomes. *Cancers (Basel)* 2025;17(14) doi: 10.3390/cancers17142392 [published Online First: 2025/07/29]
79. van Poelgeest MIE, Kortekaas KE, van Doorn HC, et al. APOLLO: neo-adjuvant pembrolizumab for primary vulvar squamous cell carcinoma-a multicenter, single-arm, phase II, clinical proof-of-concept study. *Int J Gynecol Cancer* 2024 doi: 10.1136/ijgc-2024-005523 [published Online First: 2024/06/24]
80. Uppaluri R, Haddad RI, Tao Y, et al. Neoadjuvant and Adjuvant Pembrolizumab in Locally Advanced Head and Neck Cancer. *N Engl J Med* 2025 doi: 10.1056/NEJMoa2415434 [published Online First: 2025/06/18]
81. Kumar N, Butler JS, Vashisht J. The Current Landscape for Human Papillomavirus Vaccination in Oropharyngeal Carcinoma. *Cancer J* 2025;31(3) doi: 10.1097/ppo.0000000000000767 [published Online First: 2025/06/03]
82. Puig-Saus C, Sennino B, Peng S, et al. Neoantigen-targeted CD8(+) T cell responses with PD-1 blockade therapy. *Nature* 2023;615(7953):697-704. doi: 10.1038/s41586-023-05787-1 [published Online First: 2023/03/09]
83. Galluzzi L, Buqué A, Kepp O, et al. Immunogenic cell death in cancer and infectious disease. *Nat Rev Immunol* 2017;17(2):97-111. doi: 10.1038/nri.2016.107 [published Online First: 2016/11/01]
84. Goodman AM, Kato S, Bazhenova L, et al. Tumor Mutational Burden as an Independent Predictor of Response to Immunotherapy in Diverse Cancers. *Mol Cancer Ther* 2017;16(11):2598-608. doi: 10.1158/1535-7163.Mct-17-0386 [published Online First: 2017/08/25]
85. Piersma SJ. Immunosuppressive tumor microenvironment in cervical cancer patients. *Cancer Microenviron* 2011;4(3):361-75. doi: 10.1007/s12307-011-0066-7 [published Online First: 2011/06/01]
86. Liu J, Geng X, Hou J, et al. New insights into M1/M2 macrophages: key modulators in cancer progression. *Cancer Cell Int* 2021;21(1):389. doi: 10.1186/s12935-021-02089-2 [published Online First: 2021/07/23]
87. Cortés-Morales VA, Chávez-Sánchez L, Rocha-Zavaleta L, et al. Mesenchymal Stem/Stromal Cells Derived from Cervical Cancer Promote M2 Macrophage Polarization. *Cells* 2023;12(7) doi: 10.3390/cells12071047 [published Online First: 2023/04/14]
88. Kawano M, Mabuchi S, Matsumoto Y, et al. The significance of G-CSF expression and myeloid-derived suppressor cells in the chemoresistance of uterine cervical cancer. *Sci Rep* 2015;5:18217. doi: 10.1038/srep18217 [published Online First: 2015/12/17]
89. Garcia-Iglesias T, Del Toro-Arreola A, Albarran-Somoza B, et al. Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions. *BMC Cancer* 2009;9:186. doi: 10.1186/1471-2407-9-186 [published Online First: 2009/06/18]
90. van Esch EM, van Poelgeest MI, Trimbos JB, et al. Intraepithelial macrophage infiltration is related to a high number of regulatory T cells and promotes a progressive course of HPV-induced vulvar neoplasia. *Int J Cancer* 2015;136(4):E85-94. doi: 10.1002/ijc.29173 [published Online First: 2014/09/16]
91. Sznurkowski JJ, Zawrocki A, Emerich J, et al. Prognostic significance of CD4+ and CD8+ T cell infiltration within cancer cell nests in vulvar squamous cell carcinoma. *Int J Gynecol Cancer* 2011;21(4):717-21. doi: 10.1097/IGC.0b013e3182131f36 [published Online First: 2011/05/06]

92. Welters MJP, Ma W, Santegoets S, et al. Intratumoral HPV16-Specific T Cells Constitute a Type I-Oriented Tumor Microenvironment to Improve Survival in HPV16-Driven Oropharyngeal Cancer. *Clin Cancer Res* 2018;24(3):634-47. doi: 10.1158/1078-0432.Ccr-17-2140 [published Online First: 2017/10/12]
93. Masterson L, Lechner M, Loewenbein S, et al. CD8(+) T cell response to human papillomavirus 16 E7 is able to predict survival outcome in oropharyngeal cancer. *Eur J Cancer* 2016;67:141-51. doi: 10.1016/j.ejca.2016.08.012 [published Online First: 2016/09/27]
94. Cillo AR, Kürten CHL, Tabib T, et al. Immune Landscape of Viral- and Carcinogen-Driven Head and Neck Cancer. *Immunity* 2020;52(1):183-99.e9. doi: 10.1016/j.immuni.2019.11.014 [published Online First: 2020/01/12]
95. Carstens JL, Krishnan SN, Rao A, et al. Spatial Multiplexing and Omics. *Nat Rev Methods Primers* 2024;4(1) doi: 10.1038/s43586-024-00330-6 [published Online First: 2024/01/01]
96. Gerdes MJ, Sevinsky CJ, Sood A, et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proc Natl Acad Sci U S A* 2013;110(29):11982-7. doi: 10.1073/pnas.1300136110 [published Online First: 2013/07/03]
97. Giesen C, Wang HA, Schapiro D, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat Methods* 2014;11(4):417-22. doi: 10.1038/nmeth.2869 [published Online First: 2014/03/04]
98. Jackson HW, Fischer JR, Zanotelli VRT, et al. The single-cell pathology landscape of breast cancer. *Nature* 2020;578(7796):615-20. doi: 10.1038/s41586-019-1876-x [published Online First: 2020/01/22]
99. Marx V. Method of the Year: spatially resolved transcriptomics. *Nat Methods* 2021;18(1):9-14. doi: 10.1038/s41592-020-01033-y [published Online First: 2021/01/08]
100. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. *Proc Natl Acad Sci U S A* 2010;107(26):11895-9. doi: 10.1073/pnas.1006500107 [published Online First: 2010/06/16]