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Immune Responses and Tumour Cell Detection in Lymph Nodes of Head and Neck Cancer

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Immune Response and Tumour Cell Detection in Lymph Nodes of Head and Neck Cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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The dissertation:
At 9.00 am Friday the 9th of November 2018
In J3:12 Nanna Svartz at Nya Karolinska Sjukhuset (NKS)

”Allting är mycket osäkert och det är just det som lugnar mig”
Too-Ticki / Tove Jansson

ABSTRACT

Many patients with head and neck squamous cell carcinoma present with regional spread to the cervical lymph nodes. Lymph nodes metastases are today the most important factor influencing both the treatment and outcome for this type of cancer. The developments of new cancer treatments and especially immune oncology with the use of new antibodies have changed the field of cancer medicine. In order to fully benefit from this development we have to improve the select patients for the various treatments that can be offered. The overall goal of this thesis is to study the immune responses in tumour tissue, lymph nodes and blood in patients with head and neck cancer with focus on leukocytes, flow cytometry detection of tumour cells and T cell activity.

Paper I delineates the leukocyte ratios in blood from patients with oropharyngeal cancer in an attempt to correlate this with relevant clinical findings. Patients with oropharyngeal cancer displayed signs of increased systemic inflammation. Generally, large tumours seemed to be associated with a high neutrophil to monocyte ratio whereas patients with metastatic node spread showed a low corresponding value.

Paper II characterizes different neutrophil subsets in head and neck cancer and investigates their role in the disease. A specific neutrophil subset, $CD16^{high}CD62L^{dim}$ appeared to have anti-tumour properties, with the ability to inhibit cancer cell migration and proliferation and to induce apoptosis. Further, the elastase in the activated neutrophils created neutrophil extracellular traps and a high rate of $CD16^{high}CD62L^{dim}$ neutrophils corresponded to a better survival.

Paper III is a proof-of-concept study that appraises flow cytometry as a method for detecting lymph node metastases in oral cancer. The results could be presented within 6 hours of the time of biopsy and the data correlated precisely with the clinical histopathologic investigation performed in parallel. In addition, the obtained data indicated that flow cytometry can be a very sensitive tool also for finding micro metastases.

Paper IV was made in order to establish a reliable and clinically useful protocol for sentinel lymph node biopsies in elective neck dissections in patients with oral cancer. Various techniques for identification of sentinel nodes in oral cancer were evaluated in a clinical setting. A combination of techniques was found to constitute a reliable, clinical adaptable work concept. An injection of radioactive technetium Tc99m carried on tilmanocept started the process. The lymph nodes were visualized with SPECT-CT before surgery and with indocyanine green fluorescence dye in combination with a hand-held gamma probe during surgery.

Paper V evaluates the immune response in lymph nodes of oral cancer patients with focus on T lymphocyte activation and linkage to PD-1/PD-L1 expression Lymph node metastses in oral cancer exhibited a higher level of activated T cells than cancer free lymph nodes. CD69, a marker of T cell activity was generally higher in sentinel lymph nodes than in regular nodes. PD-L1 on tumour cells did not correlate to the expression of activation markers on T lymphocytes in the tumour. $CD8^+$ T lymphocytes with high $CD71^+$PD-1 expression were more abundant in the tumours than in the sentinel nodes. Altogether this indicates that immunologic activity of the sentinel node might be used to predict selection for immunological treatment.

LIST OF SCIENTIFIC PAPERS

I. Kågedal Å, Rydberg-Millrud C, Häyry V,Kumlien-Georén S, Lidegran M, Munck-Wikland E, Cardell LO. *Oropharyngeal squamous cell carcinoma induces an innate systemic inflammation, affected by the size of the tumor and the lymph node spread.* Clin Otolaryngol. (2018) Apr 21. doi: 10.1111/coa.13122.

II. Rydberg Millrud, Å Kågedal C, Kumlien Georén S, Uddman R, Razavi R, Munck-Wikland E, Cardell LO. *NET-producing CD16high CD62L-dim neutrophils migrate to tumour sites and predict improved survival in patients with HNSCC.* Int J Cancer. 2017 Jun 1; 140(11):2557-2567.

III. Häyry V, Kågedal Å, Hjalmarsson E, Farrajota Neves da Silva P, Drakskog C, Margolin G, Munck-Wikland E, Winqvist O, Cardell LO. *Nodal staging of neck dissection specimens with flow-cytometry.* British Journal of Cancer (2018) 118,421-427

IV. Kågedal Å, Margolin G, Häyry V, Farrajota Neves da Silva P, Munck-Wikland E, Cardell LO. *A novel sentinel lymph node biopsy approach in oral squamous cell carcinoma.* Manuscript

V. Kågedal Å, Hjalmarsson E, Häyry V, Farrajota Neves da Silva P, Kumlien Georen S, Margolin G, Munck-Wikland E, Winqvist O, Cardell LO. *Immunology staging using sentinel nodes – a new predictive concept for oral cancer?* Manuscript

LIST OF ABBREVIATION

ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
AUC	Area under the Curve
CAR	Chimeric Antigen Receptor T cell
CD	Cluster of Differentiation
CK5/8	Cytokeratin 5/8,
CT	Computer Tomography
CTLA-4	Cytotoxic T Lymphocyte Associated protein-4
DFS	Disease Free Survival
END	Elective Neck Dissection
EpCAM	Epithelial Cell Adhesion Molecule
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
HNC	Head and Neck Cancer
HNSCC	Head Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
ICG	Indocyanine Green
IHC	Immunohistochemistry
IL-8	Interleukin 8
LMR	Lymphocyte to Monocyte ratio
MDSC	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MLR	Monocyte to Lymphocyte Ratio
MUC-1	Epithelial Mucin
NET	Neutrophil Extracellular Traps
NIR	Near Infra-Red light
NK	Natural Killer
NLR	Neutrophil to Lymphocyte Ratio
OPSCC	Oropharyngeal Squamous Cell Carcinoma
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cell
PD-1	Programmed Cell Death Protein-1
PD-L1	Programmed Cell Death Protein-Ligand 1
PET	Positron Emission Tomography
PI	Propidium Iodide
PLR	Platelet to Lymphocyte Ratio
PMN	Polymorphonuclear Leukocytes
ROC	Receiver Operating Characteristic
SLN	Sentinel Lymph Node
SLNB	Sentinel Lymph Node Biopsy
SPECT	Single Photon Emission Computed Tomography
TAN	Tumour Associated Neutrophil
TCR	T cell Receptor
TIL	Tumor Infiltration Lymphocyte

CONTENTS

AIMS

The overall aim of this thesis is to study the immune response in tumour tissue, lymph nodes and blood in patients with head and neck cancer with focus on leukocytes, flow cytometry detection of tumour cells and T cell activity. Specifically to:

- Correlate the leukocyte ratios in blood from patients with oropharyngeal cancer to relevant clinical findings.

- Characterize different neutrophil subsets in head and neck cancer and to investigate their role in the disease.

- Appraise flow cytometry as a method to detect cancer cells lymph nodes in oral cancer patients.

- To establish a reliable and clinically useful protocol for sentinel lymph node biopsy in elective neck dissections in patients with oral cancer

- Evaluate the immune response in lymph nodes of oral cancer patients with focus on T lymphocytes activation and linkage to PD-1/PD-L1 expression

BACKGROUND

Head and Neck Cancer

Head and neck cancer (HNC) is a diverse group of cancers that appears in the nose, mouth, throat, larynx, sinuses and salivary glands. The most common type is squamous cell carcinoma (HNSCC). Well known risk factors are smoking and alcohol consumption, in the tonsils and the base of tongue the development of squamous cell carcinomas are closely related to human papilloma virus (HPV). HNSCC is known to have immunosuppressive activity (1).

HNC is classified using the TNM system where, T significes the extent of the primary tumour, N the extent of regional lymph node metastasis M and the absence or presence distant metastasis. A subdivision is pTNM, pathological classification, which takes the histological analysis in consideration. Stage is a concept commonly used, where the categories has been condensed into groups, and each group is more or less homogeneous with regard to survival (2).

Oropharyngeal Cancer

Oropharyngeal squamous cell carcinoma (OPSCC) appears mainly in the area of the tonsils and the lingual tonsil at the base of the tongue and share morphological and histological similarities. The surface is covered with non-keratinized squamous stratified epithelium with underlying lymphoid tissue. HPV infection is the most common risk factor for oropharyngeal cancer (3,4). The local symptoms may be modest, and the tumours metastasize early to regional lymph nodes. Therefore, a lump in the neck is often the first symptom. The incidence of oropharyngeal cancer in 2012 was globally 1,4 per 100 000 inhabitants and year (2,3 men versus 0,5 women) (5). In Sweden the incidence is 3,6/100 000 inhabitants per year and 70% are men (6).

Human Papilloma Virus

There are many types of HPV divided into low risk and high risk viruses depending on their capacity to induce cancer. 12 HPV subtypes are oncogenic in humans and the most common in oropharyngeal cancers is HPV16. All HPV have a double-stranded DNA genome that encodes regulatory proteins. In high risk HPV the regulation proteins E6 and E7 are oncogenes and their intervention with the cellular tumour suppressor proteins, p53 and Rb leads to dysregulation of the cell cycle control. When this occurs the kinase inhibitor p16 will be overexpressed (7). HPV is found in 40-100 % of tonsillar and base of tongue cancers in the western world (8) and in about 75% in Sweden(6,9). The incidence for HPV associated OPSCC has increased in the last decades (10,11) and has settled at a high level in Stockholm during the last decade (9). HPV is a strong favourable prognostic marker for patients with tonsillar or base of tongue cancer (4,12,13).

Treatment of Oropharyngeal Cancer

The protocol for treatment includes radiotherapy frequently combined with targeted therapy/ Epidermal Growth Factor Receptor antibodies (EGFRab) and sometimes chemotherapy. Base of tongue cancer patients in some regions of Sweden also receive brachytherapy. If there is a suspicion of residual lymph node metastasis after the oncological treatment a neck dissection is performed (14). PD1/PD-L1 therapy could also have a role in oropharyngeal cancer (15). Since patients with HPV positive OPSCC, especially non-smokers, have such a good prognosis it has

been suggested- and trails are ongoing- where the treatment could be de-escalated for selected patients (16). There are vaccines against HPV and in Sweden they are included in the general vaccination program for girls since 2010 (17) and may be so for boys in the near future (18).

Oral Cancer

Oral cancer may arise in the buccal mucosa, the upper and lower gum, the hard palate, the mobile tongue and the floor of the mouth. Tongue cancer located in the mobile part of the tongue, i.e. the frontal 2/3 and are referred to as mobile tongue cancer or oral tongue cancer. The mobile tongue consists of several muscular layers and the surface is covered by stratified squamous epithelium with papillae, taste buds and mucus glands.

Squamous cell carcinoma of the oral tongue is the most common oral cancer. The cancer frequently appears as a painless lesion on the lateral border of the tongue. It is correlated to smoking, alcohol and betel nut use. HPV infection is rare (19–22). The majority of the mobile tongue cancers are discovered at an early stage, T1-T2 N0. However loco regional recurrence is common and the prognosis is poor, with an overall survival of only 50-65 % (23,24) The estimated incidence 2012 of oral cavity cancer globally was 2.7 per 100,000 inhabitants and year (3.7 in men and 1.8 in women) and differs substantially with location, age and sex (5). In Sweden mobile tongue cancer presents with 140 cases each year (6). The incidence of tongue cancer has increased in women and all age groups except young men the last decades (25).

Treatment of Oral Cancer

The standard treatment of oral cancer is surgical resection of the primary tumour. If there are no detectable regional lymph node metastases at diagnosis, N0, there are two treatments options for the lymph nodes; elective neck dissection (END) or watchful waiting with therapeutic neck dissection at regional relapse of the cancer. Occult metastases are common in the neck of T1-T2, N0 patients (classified with radiology, before surgery) and have led to inconsistency of best option to treatment (26). A large prospective study showed a higher overall survival (OS) and disease free survival (DFS) for patients treated with an END as compared to watchful waiting (27). At our hospital only 6% of stage I (T1 N0) patients had a positive node in the END whereas stage II (T2 N0) patients had 41% occult metastases in their neck dissections specimens (28).

If a metastasis is found the patient usually receives external radiation towards the neck and depending on the surgical resection margin of the primary tumour and the Brandwein score (29) the patient can receive external radiation and/ or Brachycatheter radiation therapy at the local site (30).

Metastasis Detection

Prior to surgery all oral cancer patients are investigated with a CT scan covering the skull base, the neck and lungs to check for metastases and if needed PET-CT and MRI. If any suspicious neck nodes are found, ultrasound guided fine needle aspiration cytology is performed. According to the results a plan for the surgery is set up. Histopathological analysis of resected lymph nodes can confirm or exclude metastatic spread. A typical neck dissection specimen contains 15 to 30 lymph nodes. Usually only one cross-section of each individual node is screened by a pathologist.

Lymph Nodes Metastases

Many head and neck cancer patients present with regional metastases in the neck whereas distant metastases are rare. Lymph node metastases have a major impact on treatment as well as on the outcome for the patient. Neck dissection has been performed since the beginning of the twentieth century. In 1991 the American Society of Head and Neck developed a classification system, upgraded twice, that is widely used today. It is based on a radical neck dissection procedure. Today a modified neck dissection procedure, in which you preserve one or more of important non-lymphatic structures, is more common. A selective neck dissection procedure preserves one or more groups of lymph nodes compared to a radical neck dissection (31,32).

The lymphatic flow from the mobile part of the tongue drains mainly into the deep cervical glands lying between the posterior belly of the digastric muscle and the superior belly of the omohyoid muscle. The frontal part and the caudal part of the tongue drain to the submental glands through the mylohyoid muscle (33,34).

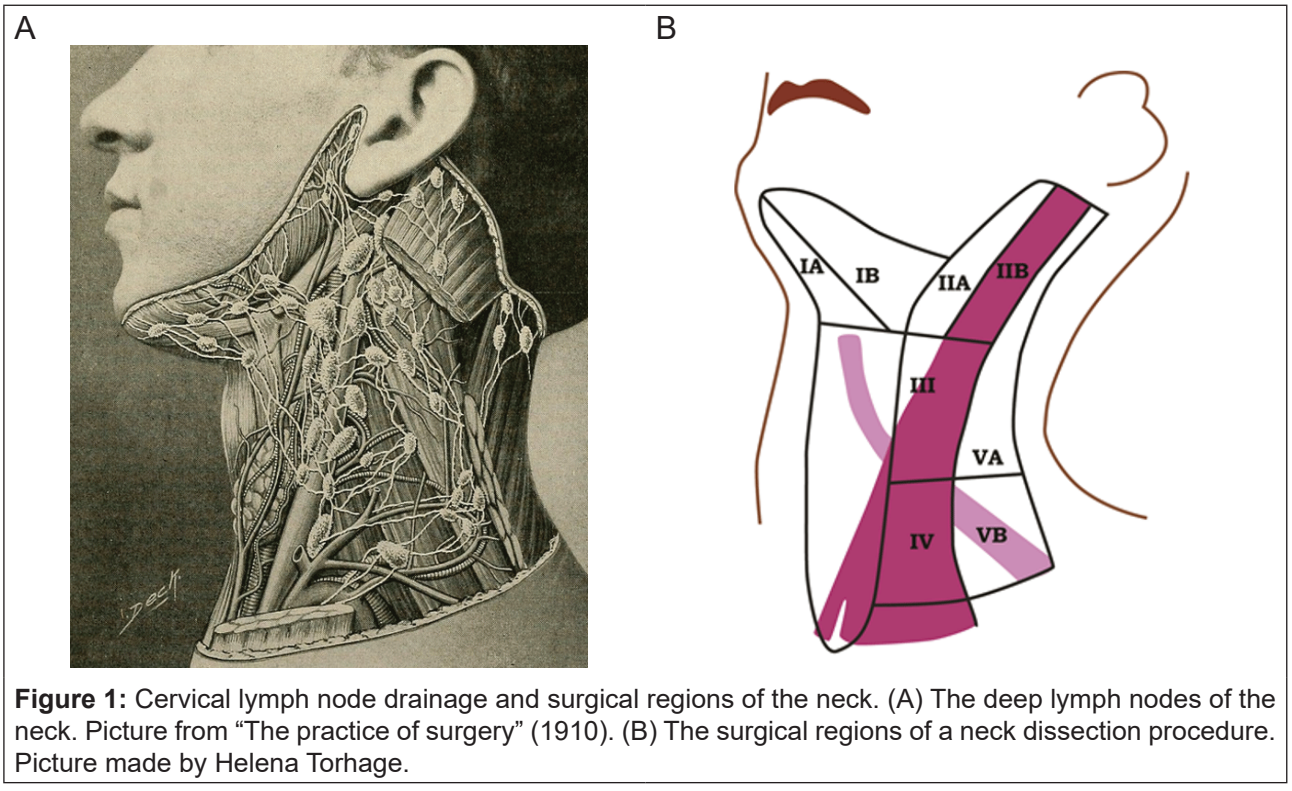


Figure 1: Cervical lymph node drainage and surgical regions of the neck. (A) The deep lymph nodes of the neck. Picture from "The practice of surgery" (1910). (B) The surgical regions of a neck dissection procedure. Picture made by Helena Torhage.

The Immune System

In the last decades more attention has been raised towards the role of inflammation in cancer diseases and it is now considered a hallmark of cancer. Immunotherapy is a potent treatment in many forms of cancer diseases (35,36).

The immune system consists of cells and molecules in our body that defend us against foreign harmful pathogens. In some situations the normal immune mechanisms are able to cause injury and disease, so-called autoimmune responses. Traditionally it has been divided into the innate immune system, an early, rapid and mechanic defence against pathogens and the adaptive immune system, which is slow but more specific and effective. There is a close interaction between the systems (37).

Innate Immunity

The first barrier of the innate system is the skin or the mucosa. The cellular defence of the innate systems includes monocytes, macrophages, granulocytes (neutrophils, basophils and eosinophils) and dendritic cells. The Natural Killer cell (NK cell) is a T lymphocyte with killer skills, without binding to the Major Histocompatibility Complex (MHC) I complex and counted as an innate cell. The innate immunity is an early fast defence against foreign pathogens.

Neutrophils

Neutrophils play an important role for the acute inflammation and are essential for the innate immune responses. They react rapidly upon intruding pathogens and the production of neutrophils from the bone marrow is fast. Neutrophils are normally the most frequent leucocyte in the blood circulation. They migrate to the area of infection to phagocytose and kill bacteria. Their half time in blood is about 6-8 h but this might be extended when they migrate into inflamed tissues (38). After destroying bacteria, neutrophils undergo apoptosis and are cleared from the tissue by resident macrophages. Until recently neutrophils have been looked upon as a homogeneous group of cells, but new research has demonstrated different neutrophil subsets based on their expression of CD16 and CD62L. These are $CD16^{dim}CD62L^{high}$ - considered less mature since they have banded nuclear morphology characteristic of neutrophils derived from the bone marrow, $CD16^{high}CD62L^{high}$ - phenotypically normal mature neutrophils and $CD16^{high}CD62L^{dim}$ - thought to be activated and have a hyper segmented nuclei (39,40).

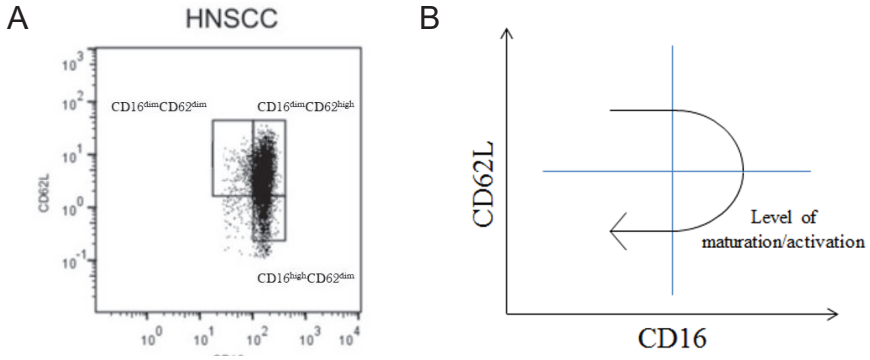


Figure 2: (A) Flow cytometry dot plot of HNSCC. (B) Schematic figure illustrating the maturation or activation process of neutrophils on a flow cytometry dot plot. Upper left quadrant; immature neutrophils express CD62L but hardly CD16, upper right quadrant; normal neutrophils express both CD62L and CD16. In the lower right quadrant; activated neutrophils express CD16 but has lost CD62L and in the left lower quadrant; end state neutrophils are displayed. Picture B is republished with the kind permission from Julia Arebro.

Adaptive Immunity

The adaptive immune system develops later than the innate immune system and is more specific. The adaptive immune response requires reactive B or T lymphocytes. The antigen presentation cell (APC), most often a dendritic cell from the innate immunity, displays the captured antigen on the cell surface for recognition of the lymphocyte. The lymphocytes become activated, proliferate and differentiate into an effector cell that may eliminate the foreign pathogen. The B-lymphocytes create an antibody specific response, create a memory and have their effector function through antibodies. T lymphocytes are responsible for cell mediated immunity.

T Cell Mediated Immunity

Roughly 30% of the circulating leukocytes are lymphocytes. A vast majority of the lymphocytes are situated in the lymphatic system. T and B lymphocytes develop from the same multipotent stem cell in bone marrow; the precursor to T lymphocyte migrates to the thymus where they mature to T lymphocytes. CD8+ or cytotoxic T lymphocytes are killer cells. The T cell receptor (TCR) on the cytotoxic T lymphocyte binds to the peptide presented on a MHC class I receptor of the APC and activates and releases cytokines that will kill the target or trigger apoptosis. CD4+ or T helper lymphocytes assist in many cellular and humoral immune reactions. The TCR of the T helper cell binds to the peptide bound MHC class II. T helper cells produce cytokines and coordinate the immune response.

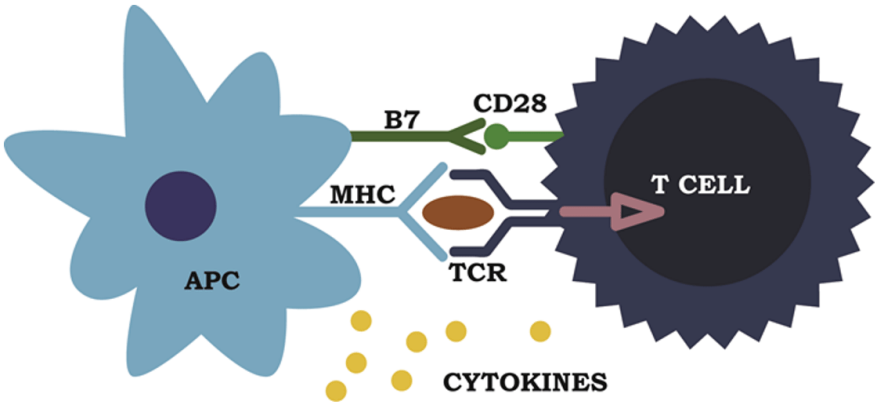


Figure 3: T cell activation The APC presents an antigen in its MHC, recognized of the TCR at the T-cell (signal 1). Co-stimulation occurs through binding of B7-CD28 (signal 2) and cytokines from the APC to the T-cell (signal 3). The T-cell becomes activated, (the pink arrow in the picture). Illustration made by Helena Torhage.

Neutrophils and Lymphocytes

A differential count of leukocytes is an easy and fast routine blood sample. The ratio between different leucocytes gives more information about the type of immune response than an absolute count of a specific cell type. A neutrophil lymphocyte ratio (NLR) is counted and has been suggested to serve as a biomarker for cancer prognosis. In several subtypes of cancer a high pre-treatment NLR has been associated with a poor overall survival (41–43).

Lymphocytes in Cancer

The lymphocyte reaction in oral and oropharyngeal cancers is well characterized at the tumour primary site and in the peripheral circulation but not in the lymph nodes. There is a decreased amount of lymphocytes in blood in patients with HNSCC (44). The lymphocyte host response in the primary cancer tissue is part of the Brandwein histological risk model used as a prognostic estimate, which influences treatment decisions. A strong lymphocyte response around the oral tumour is associated with better prognosis, presumably due to anti-tumoural cytotoxic T lymphocytes (45). A high level of CD4 and CD8 tumour infiltrating lymphocytes (TIL) improves overall survival in HNSCC (46). This phenomenon is seen in many solid tumours. It has further been shown that the morphological pattern of TILs are important and it differs between HPV positive and HPV negative cancers (47).

Cancer Immunology

The immune system capacity to recognize and destroy transformed cells before they have formed tumours called immune surveillance (48). This concept was set in the 1950s and today we have learned that the immune response is ineffective against human cancer cells but importantly it can be reactivated and kill tumour cells. So why does the immune system fail to eradicate tumour cells? The tumour has developed various mechanisms to avoid the immune response for example, they lose the antigen expression and can no longer be recognized by the immune system and the tumour cells grow fast and spread fast which overwhelm the host immune system.

At the tumour site dendritic cells pick up a tumour antigen and travel with the lymph draining system to the lymph node. At the lymph node the dendritic cells display present the antigen with a MHC I which is recognized by a TCR on a $CD8^+$ T cell. Co-stimulators, from APC cells or the $CD4^+$ T Cells are essential at the same time for the $CD8^+$ T cell to be able to become antigen-specific activated. The activated $CD8^+$ T cell then travels with the blood stream back to the primary tumour site and can fullfill its tumour destroying mission (49). (Figure 4) The $CD4^+$ T cells are as mentioned above important in the activation of $CD8^+$T Cells, they secrete tumour necrosis factor (TNF) and interferon – γ (INF- γ). The latter increases the MHC I expression on the tumour cell and can make it more sensitive for lysis by $CD8^+$ T cells.

Regulation of the Immune Response

The tumours have ability to evade the host immunity and cause the cancer disease. To be able to prevent immune evasion it is important to understand the mechanisms behind it. If we could understand these evasion manoeuvres of the tumour would find a way for immunotherapy (50).

The tumour evades antitumor responses from the T lymphocytes by engaging inhibitory molecules, which normally function to prevent autoimmunity and regulate immune response to microbes. Cytotoxic T lymphocyte- associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1) are inhibitory pathways that are upregulated on TILs (51). The inhibitory mechanisms establish checkpoints in the immune response. (Figure 5) Exactly how these inhibitory pathways are used by the tumour cell is not clear, but PD-L1 (PD-Ligand-1) is commonly expressed on human cancer cells, they sometime have a PD-L1 gene amplification. TILs found to be in an exhausted, dysfunctional form first described in chronic viral infection. The exhausted cell have diminished functions and amplified CTLA-4, PD-1 and other inhibitory molecules expressed on their surface. Tumour cells can secrete Tumour Growth Factor- β (TGF-β) which may inhibit T cells and macrophage proliferation and effector functions. The tumour can also evade the host immunity by antigen loss, failure to produce antigens or mutations in the genes of the MHC, so the tumour antigen will not be presented to the T cell. These functions make the T cell unable to recognize the tumour.

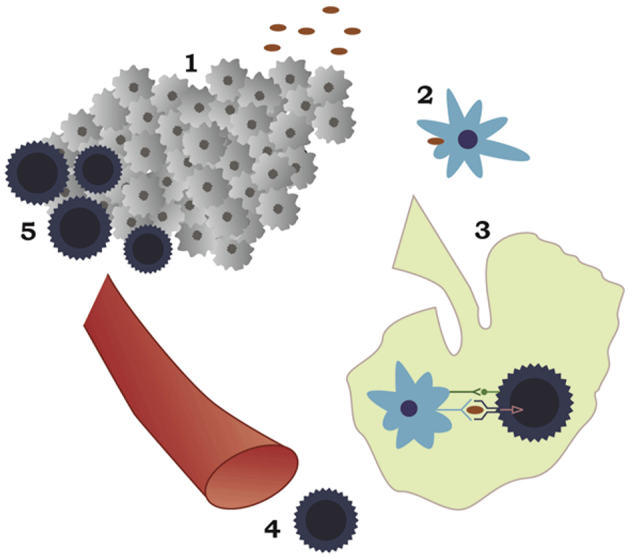


Figure 4: The tumour immunology circle. (1) At the tumour site the APC pick up a tumour antigen and (2) travel with the lymph drainage to the (3) lymph node was the activation of the T lymphocyte occurs. T lymphocyte travels with the blood stream (4) to the primary tumour site (5) were the can function as killer $CD8^+$ T cells. Illustration made by Helena Torhage

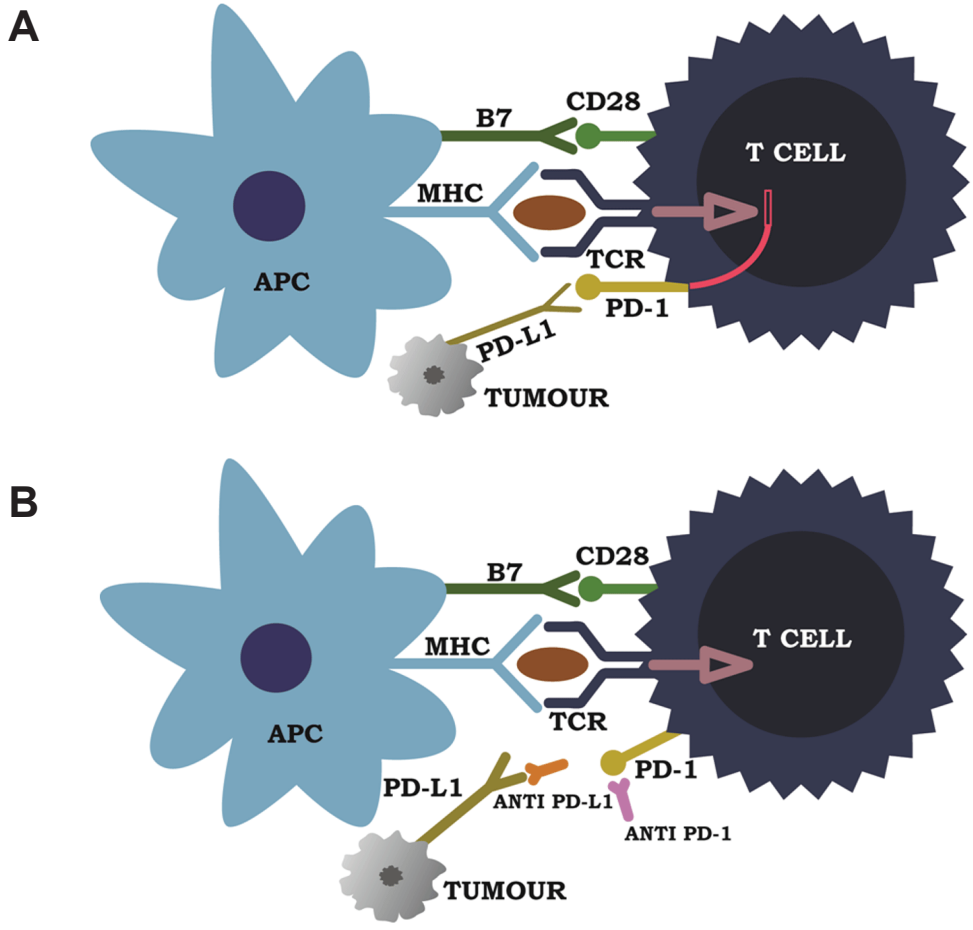


Figure 5: (A) PD-1 from the T cell and PD-L1 from the APC or a tumour cell block T-cell activation. (B) Anti PD-1 or anti-PD-L1 block the connection between the PD-1 and the PD-L1 and the inhibition bondage breaks and the T-cell become activated and can destroy tumour cells. Illustration made by Helena Torhage

Immunotherapy

The inhibitory mechanisms establish checkpoints in the immune response. Antibodies that block these inhibitory pathways have been developed and exhibit promising results as immunotherapy against the tumour cells. The drugs are therefore called checkpoint inhibitors. CTLA-4 normally binds to B7, a receptor at the APC necessary for co-stimulation to get a T lymphocyte activated, and disturbs the co-stimulation. When you add an anti CTLA-4 it binds to the CTLA-4 and the functional co-stimulation leads to activated $CD8^+$ cells, ready to destroy tumour cells (51). PD-1 at the T cell and the PD-L1 at the tumour cell normally bind to each other which lead to inhibition of the $CD8^+$ cell. When you add anti-PD-1 or anti-PD-L1 you break the bondage of PD-1/PD-L1 and the activated $CD8^+$ cell can regain its function to destroy tumour cells (Figure 5). There is a lot of research going on about tumour vaccination strategies; so far the only tumour prophylactic vaccination is the HPV vaccination (52). Chimeric antigen receptor (CAR) T cells are the new immune therapy at the market for lymphoma and lymphatic leukaemia and studies are ongoing for solid tumours. The patient's own T cells are used and in the lab a genetically engineered tumour antigen receptors together with co stimulators are added, then the whole vector is returned back to the patient. The T cell can now recognize the tumour antigen, with its new receptor and thereafter attack the tumour cell (53).

Immunology and Chemo Radiotherapy

The amount of lymphocytes in the circulation of HNSCC patients is reduced to less than half of the normal levels after chemoradiotherapy and remains decreased up to one year after treatment. Importantly, these low lymphocyte levels are associated with poor prognosis (54). A plausible explanation is the sensitivity of $CD4^+$ helper cells to cisplatin, aggravated by cisplatin-resistance observed in regulatory T lymphocytes. This imbalance between regulatory T cells and tumour-reactive T cells persists for several years after therapy (55).

MATERIALS AND METHODS

This section contains a brief overview of the materials and methods used in the studies. More details can be found in the papers I-V.

Human Study Populations (Paper I-V)

Paper I: Blood from 58 patients with oropharyngeal cancer, (base of tongue or tonsil) was obtained prior to treatment. Blood from 90 healthy donors served as control.

Paper II: A total of 46 HNSCC patients have contributed with tumour biopsies and/or blood for this study (Figure 6). Blood from 20 patients before initiation of treatment, along with 19 controls were used (A). Granulocytes were isolated from blood obtained from 12 untreated patients (C, eight of them were the same patients as in D, and four patients contributed only with blood). Tumour biopsies along with a blood sample were taken from 22 patients (D). Twelve biopsies were embedded in paraffin, sliced in 3 mm sections, and mounted on glass slides, and ten biopsies were used for flow cytometry analysis. Blood was also collected from 12 healthy donors to determine the impact of activated neutrophils on the antitumor immune response (B).

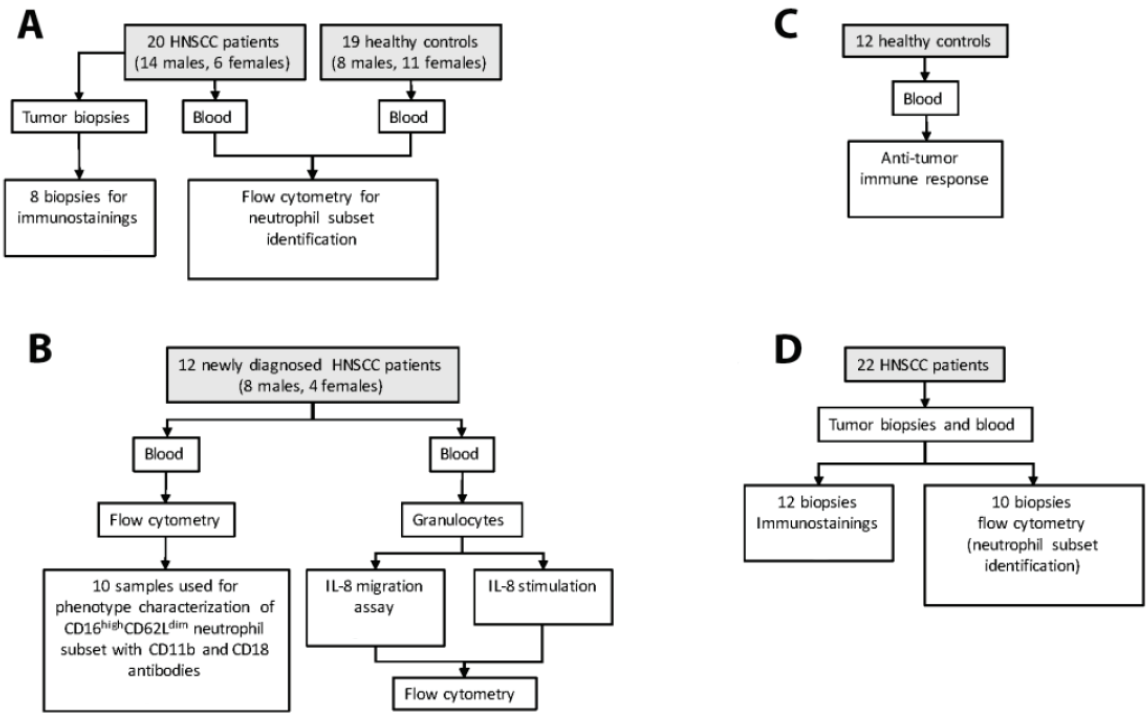


Figure 6: Patient samples the involvement in the different experiments in Paper II. (A) Tumour and blood, neutrophil subsets identification and immunostaining (B) Blood, anti-tumour response (C) Blood characterization of subsets with CD11b and CD18 and IL-8 assay and IL-8 stimulation (D) Tumour and blood, immunostainings and subset identification

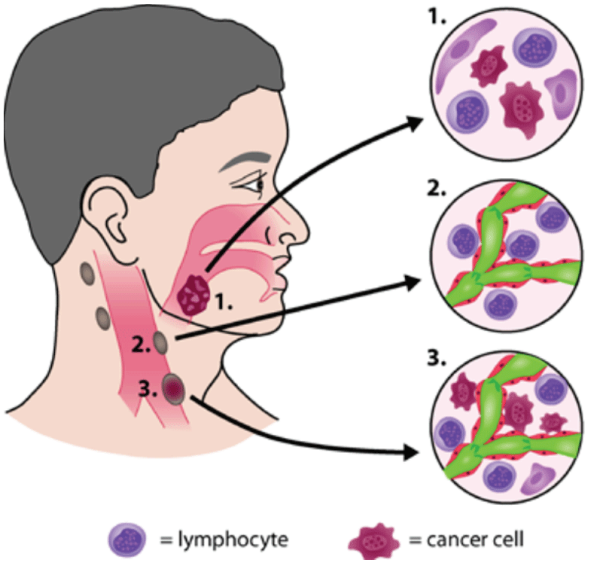
Paper III: Tumour biopsies from 19 patients with tongue cancer were analysed. Sections of lymph nodes from their neck dissections were obtained at the same time, in six of them sentinel nodes were identified. Four non-cancer donors contributed with healthy lymph nodes when having benign neck surgery (Figure 7).

Paper IV: 14 patients were investigated with sentinel node technique during surgery, 24 sentinel nodes were collected.

Paper V: Blood, tumour and lymph nodes from 30 patients with oral squamous cell carcinoma were included in this study. In 17 neck dissections sentinel lymph nodes were identified and 27 sentinel nodes were found.

The studies were mainly performed at the Ear-, Nose- and Throat department at Karolinska Institutet and Karolinska University Hospital in Stockholm, a part of study II was performed at Skåne University Hospital in Malmö, after approval from the Ethics committee at Karolinska Institutet and/or Lund University. Written informed consent was obtained from all participants in these studies.

Figure 7:
Tissue samples in Paper III and V were collected from several loci from each patient, from the primary tumour in the oral cavity (1), from a lymph node (2) and from a known metastasis, when present (3). If a sentinel node procedure was done, samples were taken from first receiving lymph node, together with a lymph node further down in the lymphatic draining system. When no lymph node metastases were suspected, a second lymph node from a different region of the neck than the first node was selected.



Cell Isolation (Paper II)

For cell isolation, Ficoll-Paque™ was used to separate polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) in blood. PMN and erythrocytes with high density sedimented to the bottom of the tube, and PBMC with low density could be found at the interface of plasma (above) and Ficoll- Plaque™ (below). To recover pure PMN the erythrocytes were lysed with ammonium chloride buffer. The cells were then cultured in RPMI-1640 supplemented with 0.3 g/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% autologous plasma to a concentration of 4 X 106 PMN at 37°C in humidified 5% CO2. In paper II PMN was isolated from blood of cancer patients and used in the granulocyte activation.

Tumour Cell Isolation (Paper III and V)

Tumour Dissociation KIT was used to mechanically and enzymatically dissociate surgical specimen in order to achieve a single cell suspension. A single cell suspension was needed in FACS. The tissue samples were cut into small pieces and treated with enzymes at 37°C in a special dissociator(Gentle MACS). After dissociation, the cells were filtered through a 100µm cellstrainer. A wash step was performed, samples were centrifuged and the cells were re-suspended in stainbuffer.

Flow Cytometry (Paper II-V)

Fluorescence activated cell sorting (FACS) is a method that analyses the physical and chemical properties of individual cells based on how they scatter light from a laser beam. Through different detectors flow cytometry gives information about cell size (displayed by forward scatter; FSc) and granularity (displayed by side scatter; SSc). Fluorescence intensity of flourochrome conjugated antibodies against extra- or intra cellular antigens provides information about the cell phenotype. It can simultaneously do multi-parametric analyses of physical and chemical characteristics in up to thousands of cells per second. Generated data from the flow cytometers can be plotted as a single dimension in a histogram, or as a two dimension dot plot based on two different parameters on the x and y axis. The regions on this plot can be separated based on fluorescence intensity and are called "gates" (Figure 8). The cells of a gate, a population, can be presented in relation to a specific group of cells, in absolute counts or as mean fluorescent intensity (MFI). In paper II we used Beckman Coulter Navios or a BD LSR Fortessa flowcytometer and data were analysed with either CXP analysis software or FlowJo software. In paper III and V the blood was lysed with ammonium chloride solution and washed with PBS and stained by antibodies before analysis in the FACS. We used FACS to distinguish the homogeneous mixture of cells into different groups according to their specific light scattering and fluorescent characteristics. It provides a physical separation of cells of a particular interest. Flow cytometry was performed on a BD Accuri cytometer and FACS data were analysed using FloJo software.

In paper I we used the routine leucocyte differential counts, the blood was analysed at the Karolinska University Hospital clinical lab. The leucocytes were differentially counted and a flow cytometric analysis was performed using Sysmex XE 5000.

In paper II neutrophil subsets were characterized based on the expression of CD16 and CD62L, neutrophils were identified as $CD16^+$ granulocytes and T-cells as $CD4^+$ lymphocytes in flow cytometry.

In paper III and V antibodies CK 5/8, EpCAM and (MUC-1) were fluorescein isothiocyanate (FITC) conjugated and used in the flow cytometry analysis. The FITC fluorescent signal, obtained from each of the antibodies separately, and all three in combination, are demonstrated in figure 8A. To separate intact tumour cells from debris, propidium iodide (PI) was used.

In paper V cells were first gated to exclude debris with the FACS. Cells were gated based on expression of cell poulation$CD3^+CD4^+$ and $CD3^+CD8^+$ to detect T-cells. CD69, CD71, HLA-DR were analysed individually on CD4 and CD8 T-cells. PD-1 expression was analysed with mean fluorescence intensity (MFI-mean).

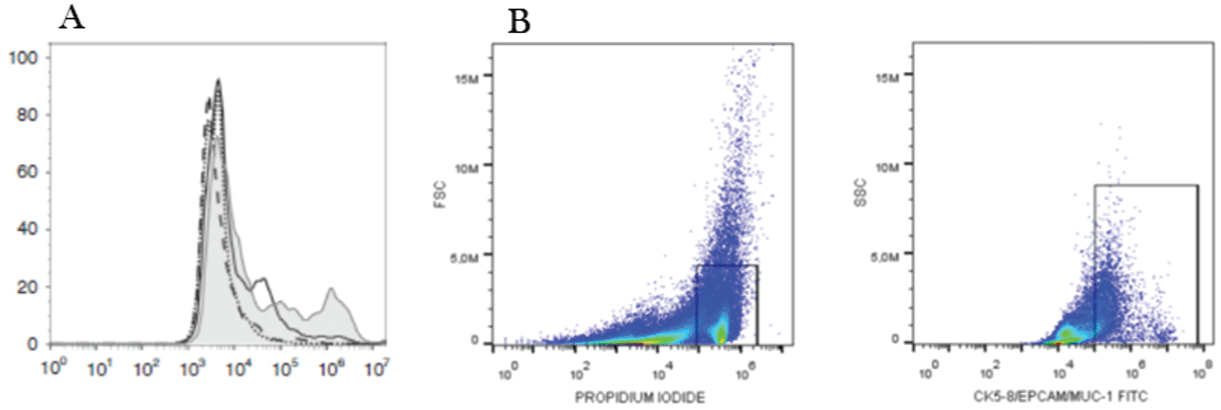


Figure 8: (A) Histogram of FITC signal fluorescence from a tumour sample stained either with anti CK5/8 (solid black line), anti EpCAM (dotted line) or anti MUC-1 (dashed line) and all three markers stained together (solid black line). (B) The gating strategy applied in FACS data analysis: first PI-positive cells, i.e. nucleated cells, were gated and from this population the frequency of FITC positive cells was quantified. The first plot shows a PI gate and the second a tumour sample with FITC positive cells.

Sentinel Node (Paper III-V)

The first lymph node or group of nodes that drains a cancer site is called the sentinel node(s). If a metastasis occurs this is where they are usually found. To identify them; Technetium Tc99m were labelled with one of the tracers (Nanocoll®, GE-Healthacare) or Lymphoseek®, Tilmanosept, Cardinal Health) and was injected around the tumour 16 hours before surgery. A SPECT/CT was performed at the morning of the surgery. Blue dye or Indocyanine green (ICG) was injected immediately preoperativel around the tumour. A camera with near infra-red light detected the fluorescein light from the ICG which makes the lymph node visible in the wound. A handheld gamma probe was used to localize the sentinel nodes according to their radioactivity. In paper III six patients were investigated with the sentinel node technique, in paper IV the sentinel node technique was gradually developed and evaluated and all included patients were invested with this technique. 17 patients were investigated with sentinel node in paper V. The sentinel node procedure was under development at our department during the studies, therefore the procedure slightly varied slightly between patients.

Histopathology and Immunohistochemistry (Paper II-V)

Immunohistochemistry is an antibody-based method to identify proteins in tissues and cells. In our studies, horseradish peroxidase (HRP) was used as detection enzyme. After incubation with a substrate diaminobenzidine (DAB), a positive immunoreactivity occurs and a brown colour can be seen. To provide contrast to the sections and to visualize the nuclei, the slides are usually counter strained with haematoxylin. To rule out unspecific background staining, negative controls for mouse and/or rabbit primary antibodies were used. This was done in paper II. In paper III and V the parts of the tumour specimens and neck dissection resections, that were investigated with flow cytometry, were submitted to routine clinical pathological examination and diagnosed according to the guidelines of our institution. All non-metastatic lymph nodes (N0) analysed with flow cytometry in paper III had additional histological and immunohistochemistry staining to rule out false negative results of standard clinical pathological examination.

The sentinel nodes in paper III, IV, V were fixed in formalin and submitted to routine paraffin section microscopic examination. Three hematoxilin-eosin (H&E) stained sections were taken at three levels, 200 micrometre between the levels. Between level one and two the specimen was investigated with a broad spectrum cytokeratin (CKMNF116). After fixation of the tumour in the tongue biopsy, one paraffin section of the tumour fraction was examined with H&E-stained section and one with immunohistochemically for PD- L1 (clone SP263 - Ventana). The PD-L1 stained section included an external control. The immunoreactivity for PD-L1 in the tumour cells was quantified by visual assessment and categorized first in 3 intervals (<1%, >1% and <50%, >50% according to the clinical guidelines) by 2 pathologists. Secondly all the slides were revaluated and re-categorized in 10%-groups.

Statistical Analyses (Paper I-V)

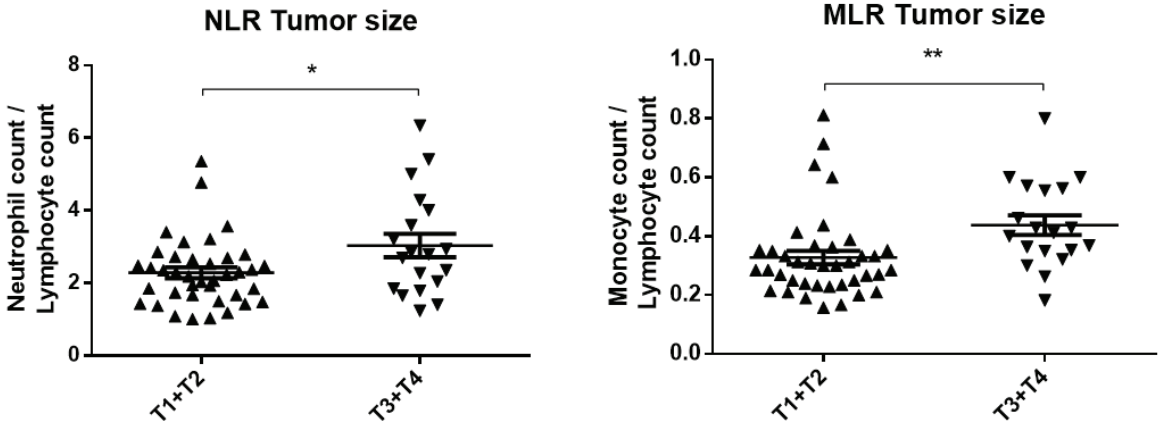
All statistical analyses were done with GraphPAd Prism version 6.01 (GraphPAd Softwere, LA Jolla, CA, USA) A p-value of < 0.05 was considered statistically significant and n equals the number of individual subjects. All plots show values with mean or mean ± standard error of the mean (SEM). In paper I a non-parametric Mann Whitney test and Fisher´s exact test were used and the area under curve (AUC) of a receiver operating characteristic (ROC) plot was calculated. In paper II for normally distributed unpaired data, the unpaired Student's t-test, with Welch correction if the variance was non-homogenous, was used. One-way repeated measures analysis of variance (ANOVA) with Tukey's post-hoc test was used for comparison of more than two sets of paired data that were normally distributed and when two sets of paired data were compared the Student's t-test was utilized. The survival function from survival data was estimated using Kaplan–Meier analysis, and a log-rank test was utilized to examine the significance of the different survival distributions between the two groups. In paper III and V the D'Agostino-Pearson normality test was used to determine if data sets were normally distributed, and one-way ANOVA or the Kruskall-Wallis tests were chosen, depending on the distribution of the data. The Brown-Forsythe test was conducted to evaluate homoscedasticity. ROC curves were plotted to calculate the area under the curve and evaluate sensitivity and specificity. In paper V paired t test was used to compare paired groups of data. Wilcoxon signed rank test for non-parametric paired data. Linear regression and correlation have been done.

RESULTS AND COMMENTS

Oropharyngeal Cancer Induces an Innate Inflammation (Paper I)

Blood from OPSCC patients displayed an increased number of neutrophils and monocytes whereas the lymphocytes were suppressed compared to in healthy controls. The neutrophil to lymphocyte ratio (NLR) and the monocyte to lymphocyte ratio (MLR) were calculated and patients with large tumours (T3-T4) exhibited a higher NLR and MLR compared to patients with small tumours (T1-T2) (Figure 9A). This was also true for the platelet to lymphocyte ratio (PLR). Patients with regional lymph node spread (N+) exhibited a lower NLR and MLR than patients with only local disease (N0) (Figure 9B). No correlation was seen between T and N stages when a Fisher's exact test was applied to control for confounding factors. NLR was found to be lower in the HPV positive group than in the HPV negative group.

A



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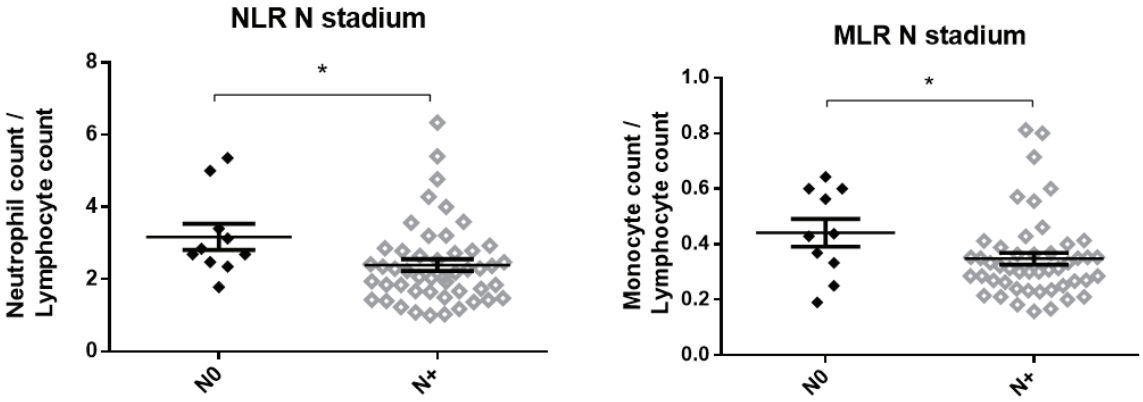

Figure 9: The NLR and the MLR in blood from 58 patients with OPSCC. The patients were divided according to the size of (A) the primary tumour (T1+T2 versus T3+4); and according to (B) their lymph node metastatic status (N0 versus N+).

Comments

NLR is used as a prognostic marker for many types of cancer (56,57). An elevated NLR before treatment has in several head and neck cancer studies been linked to poor overall survival (58,59). In one study an elevated NLR also exhibited a shorter disease specific survival (59).

In a study on HPV induced oropharyngeal cancers, the overall survival (OS) for the high NLR group was 85.3% compared to the low NLR group with 96,3%. A high NLR was associated with advanced disease but did not reach significance. The high NLR group had poor disease free survival (DFS) compared to the low NLR group (60). The reports cited above were published after our manuscript was published and due to differences in the endpoint selection it is hard to compare their results with our data. The 5-years overall survival in our study population was high (Figure 10). 47 out of 56 were still alive after 60 months. (Two patients were lost during the follow up period). The high NLR group had a survival rate at 84% and the low NLR group at 80 %. In our study patients with N2b-N2c neck nodes seemed to have an increased amount of lymphocytes, resulting in a low NLR, compared to the patients with N0-N2, but the difference did not reach significance. This could be compare to a study where the patients with high NLR showed a reduced survival, especially among those with higher N stage. However, it is important to recognize that no N0 was reported in the study (60).

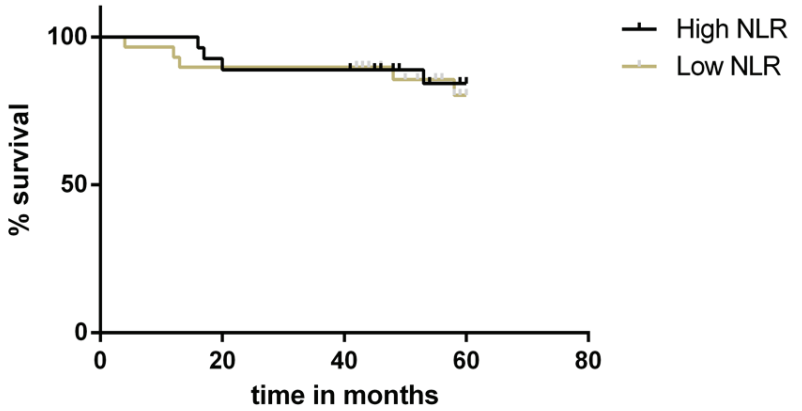

Figure 10: 5 years overall survival curve of oropharyngeal cancer patients with high NLR (n=23) and low NLR (n=23). High were above the mean (= 2,52) and low below mean.

Further, in a study where NLR, PLR and LMR (lymphocyte to monocyte ratio) in laryngeal, oropharyngeal, and hypopharyngeal cancer were investigated it was found, similar to our study, that a high NLR (and a low LMR) correlated with large tumours (42). In contrast to our results they did not find any correlation between high PLR and large tumours. Kano et al divided the N-stage into groups of N0-N2a and N2b-N3 and found that NLR were higher with greater N stage which is exactly opposite compared to what we found. In our study the patients with lymph node metastasis had a lower NLR than the group without metastases. In the study by Kano et al. only LMR was independently correlated to overall survival. It is important to notice that the hypopharyngeal cancer patients usually were diagnosed at an advanced stage and had a higher mortality compared to patients with oropharyngeal cancer. The study included tumour specimens from 102 hypopharyngeal, 116 oropharyngeal and 67 laryngeal cancer patients (42). High PLR (in this case due to high platelets accounts) has been correlated to poor prognosis and anti-platelet treatment has been reported to improve the prognosis (61).

Lymphocytopenia corresponds to a high NLR and is more common in large tumours, something that has been linked to poor outcome (44,62,63). Previous studies from our group have demonstrated suppressed and anergic T lymphocytes in HNSCC (64). These lymphocytes could not produce a proper immune response to avoid tumour progression. An increased amount of neutrophils in the blood is known to be related to a bad outcome (65) The same appears to be true for high levels of intra tumoural neutrophils (66). A likely explanation for this might be that high levels of neutrophils result in a systemic activation of neutrophil extracellular

traps (NETs). NETs bind pathogens extracellularly in networks of fibres, primary composed from neutrophil elastase (58).

To summarize, NLR is easy to measure with a blood test and can be used as a prognostic marker. The role of neutrophils in blood shows signs of systemic innate inflammation. But since the OS in HPV^{+} oropharyngeal cancer is high and the range of NLR is broad, the clinical use of the NLR in the individual patient is somewhat limited.

Neutrophil Subset can be used to Predict Survival (Paper II)

The three different neutrophil subsets CD16^{high}CD62L^{dim}, CD16^{dim}CD62L^{high} and CD16^{high}CD62L^{high} were found in the circulation of patients with HNSCC (Figure 11). The patients exhibited a higher percentage of CD16^{high}CD62L^{dim} neutrophils compared to healthy controls. The CD16^{high}CD62L^{dim} subset displayed a distinct phenotype with high expression of CD11b and CD18.

CD16^{high}CD62L^{dim} was prone to migrate into the tumour, this was facilitated by tumour-derived IL-8. IL-8 was also found to activate neutrophils and to promote subset transition. Activated CD16^{high}CD62L^{dim} neutrophils had the ability to inhibit migration, proliferation and to induce apoptosis of FaDu cells. Neutrophil elastase detected in activated CD16^{high}CD62L^{dim} neutrophils and tumour biopsies suggested that CD16^{high}CD62L^{dim} neutrophils impart anti tumoural activity via NETs. Patients with a high number of CD16^{high}CD62L^{dim} neutrophils had an increased survival rate (Figure12).

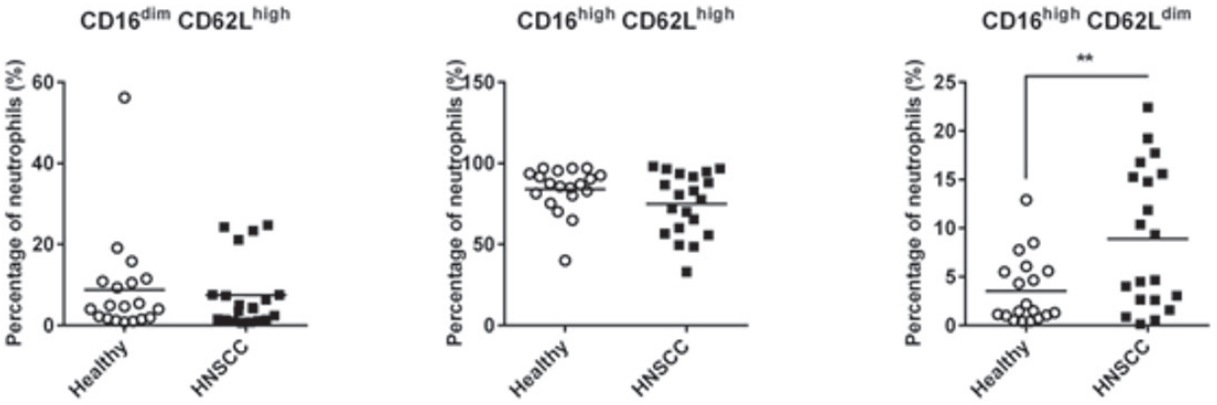

Figure 11: Neutrophil subsets in patients with HNSCC. Blood was obtained from patients with cancer; (n=20) and healthy controls (n=19), and incubated with antibodies against CD16 and CD62L. The neutrophil subsets were characterized with flow cytometry. *p <0.05; **p <0.01; ***p <0.001.

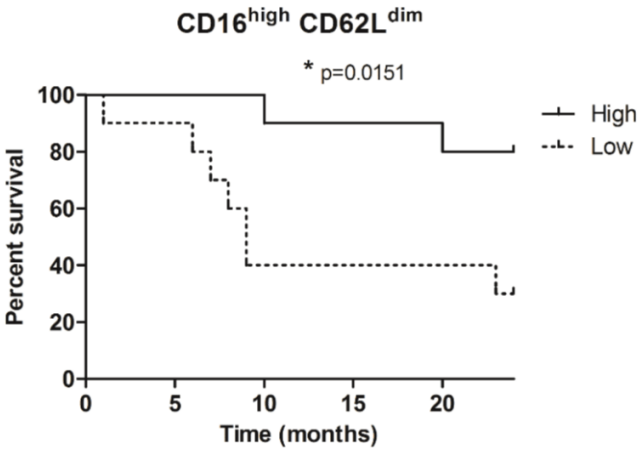

Figure 12: A Kaplan–Meier analysis of HNSCC patients divided into two groups, based on their levels of CD16^{high}CD62L^{dim} neutrophils in blood. A high level of CD16^{high} CD62L^{dim} neutrophils indicated an improved survival. *p=0.05. n= 20

Comments

Neutrophils have a complex and diverse role in inflammation and in cancer immunology. They exhibit both pro- and anti-tumorigenic roles which seem to differ depending on the cancer type and the stage of the disease (67,68). In many cancer forms, including HNSCC neutrophils can promote metastases (68–70).

There are many pro-tumorigenic mechanisms, initiation of tumour angiogenesis is one of them. Matrix metalloproteinase-9 (MMP-9) is secreted from neutrophils and leads to release of vascular endothelial growth factor (VEGF) from the extra cellular matrix (ECM) and VEGF induces angiogenesis. In lung cancer MMP-9 has been shown to inhibit apoptosis of cancer cells. Tumour associated neutrophils (TAN) release cytokines to induce chronic inflammation and arginase 1 which inhibits $CD8^+$ T lymphocytes and weakens the tumour defence. High numbers of TAN are therefore usually associated with poor prognosis and advanced disease. Finally, reactive oxygen species (ROS) are normally used by the neutrophils to destroy microorganisms, but can also indirectly promote tumour growth.

The neutrophils also possess anti-tumour functions. Since the mechanisms involved in the anti–tumour function often are the same as in pro-tumour activity it is easy to become confused. However, the difference is related to disparities of timing and tumour form. Neutrophils are fast and have the potential to kill tumour cells, involve anti-microbial functions and immune regulatory functions. N1 is an anti-tumour phenotype of neutrophils, well studied in murine models. N1 cells can recruit and activate $CD8^+$ T cells. Likewise, ROS appears to have the ability to eliminate tumour cells during their early development. Antibody Dependent Cell–mediated Cytotoxity (ADCC), is an effective part of the anti-tumour defence of the immune system. It represents an antibody directed towards tumour cells that binds to the immune cell often an NK cell but could also be a neutrophil. The antibody activates the cell to destroy the tumour cell. Neutrophils could express Tumour necrosis factor Related Apoptosis Inducing Ligand (TRAIL) on its surface and induce apoptosis of the tumour cell (68,69). The neutrophil seems to regulate the tumour to grow or to decline using more or less the same mechanisms but in different environments.

The presented work details the role of the different neutrophil subsets in HNSCC (40). It is our belief that the different subsets seem to represent different stages of activation or maturation. $CD16^{high}$-$CD62L^{dim}$ neutrophils were found to be elevated in patients compared to healthy individuals. They represent a mature and activated form, capable to affect the tumour cells. The subgroup $CD16^{dim}CD62L^{high}$ neutrophils have the characteristics of immature cells, from the bone marrow and $CD16^{high}CD62L^{high}$ are normal mature neutrophils. $CD16^{dim}CD62L^{dim}$ neutrophils were not found in this study (Figure 2). We saw an association between IL-8 activated $CD16^{high}CD62L^{dim}$ neutrophils and the formation of NETs. The latter seem to be responsible for the anti-tumour function. However, knowledge about NET formation and its role in tumour microenvironment is still very limited. In a recent review they described the function of NETs as part of a tumour progression rather than anti-tumour function (68).

In our study patients with a numerous $CD16^{high}CD62L^{dim}$ neutrophils had a better survival. The included patient tumours were all squamous cell carcinomas but located in different parts of the head and neck area, this might influence the survival of the patients and is a weakness of our study. A statistical analysis was performed to search for biases but none were found between, high and low $CD16^{high}CD62L^{dim}$ neutrophils. To summarize, neutrophils have an important role in the development of tumours. The subgroup $CD16^{high}CD62L^{dim}$ seems to increase migration and NET-formation with anti-tumour activity. In addition, $CD16^{high}CD62L^{dim}$ neutrophils correlate with increased survival in HNSCC patients.

Cancer Cells Detection and Sentinel Lymph Node Procedure (Paper III- IV)

Paper IV establishes a protocol for sentinel node identification. To facilitate the planning of the surgery a preoperative (SPECT-CT) was performed. Nine out of 14 patients received nanocoll, a colloid commonly used in sentinel node detection, and four patients received tilamanocept, a new mannose based diagnostic radioactivity carrier. The carriers, nanocoll and tilmanocept were equipped with a radioactive, Techneticum99m isotop. One or the other was injected the day before surgery and in the morning the surgery day a SPECT-CT was done. In nine of the patients a 3D volume analysis was achieved using the SPECT-CT data, to better visualise the sentinel node. In contrast to nanocoll the use of tilmanocept resulted in a stabile nodal uptake with the radioactivity staying in the node for a long period. Hence, the latter was preferred by both the nuclear medicine physicist, making the 3D reconstructions and the surgeon.

Visual marking

In breast cancer SLN procedure the routine for detection is to inject blue dye in the lymphatic system. However, in the neck blue dye has not given the same good results. Only one patient was included where blue dye was used in our study. In the other 13 patients we used fluorescence (ICG) for staining of the sentinel node. ICG was injected around the tumour at the start of surgery and during the surgery a near-infrared (NIR) light, a telescope and a camera specially adapted for the purpose was used to visualize the fluorescence from the sentinel node (71). Since the light from ICG only travels 10 mm in the tissue a hand-held gamma probe was used to localize the radioactivity. After identification of the sentinel nodes an END was performed. The sentinel nodes were divided in two by a pathologist. One part was analysed with immune histopathology in the routine pathology lab whereas the other part was processed using flow cytometry.

The concept we arrived at for identifying the sentinel node starts with the presurgical injection of radioactive technetium Tc99m carried on tilmanocept (Lymphoseek®) around the tumour. The radioactive uptake is visualized in the node the following morning, preoperatively with (SPECT-CT). In the beginning of the surgical procedure ICG is then injected around the tumour and visualized with infrared light. To further support the sentinel node detection the surgeon, uses a hand held gammaprobe.

Markers for analysis

To be able to use flow cytometry for detection of tumour cells in the lymph node we needed to find the most reliable markers. We tried many different markers and combination of markers to get a reliable result. We needed to be sure that we could detect all the tumour cells that we put in the FACS. Many pre-studies were done before we applied for ethical permission to study patients. The conclusion was to use CK5/8, MUC-1 and EpCAM as markers since they are

epitopes common to the epithelial cells. Normal lymph nodes do not contain epithelial cells which are why and these markers can serve as markers for squamous cells including carcinoma cells. Using the three markers under the same fluorescence channel provided a good distinction between positive and negative events.

Two different FACS methods were tried, the first was based on universally applied signal strength and the second on an individually determined signal strength based on the background. The two strategies correlated well with each other. Therefore, we concluded that flow cytometry is a reliable method for analysing positive lymph nodes using predetermined cut off levels (Figure 13). We calculated the level of detection based on the results from cancer-free lymph nodes from the control patients. The median rate of EpCAM/Ck8/MUC-1 positive cells was 8, 1% in oral tumours and 3,9 % in histologically confirmed metastatic lymph nodes. Metastatic lymph nodes could be reliably distinguished from non-metastatic nodes.

Propidium iodine(PI), was used to mark the nucleus to distinguish tumour cells from debris in the flow cytometer (Figure 14). In this proof of concept study, lymph nodes from healthy individuals, resected while undergoing surgery for benign reason were used as controls. The control lymph nodes were analysed in the same way as the lymph nodes from tumour patients (Figure 14)

While this study was running the clinic decided to use the sentinel lymph node (SLN) technique. We detected and investigated nine sentinel nodes and four of them scored above the level of detection. In the lymph nodes, where no sentinel node procedure was done, four lymph nodes had above level of detection out of 21.

Comments

Flow Cytometry

Flow cytometry is routinely used in the investigation of haematological cancers (72). In renal cell carcinoma flow cytometry has been effectively used in the search for micro metastases in sentinel lymph nodes (73). So far flow cytometry has not been used in the search for metastases in lymph nodes in head and neck cancer patients. In this study we identified the tumour cells in the lymph nodes that we received after surgery. Since epithelial cells not normally are present in lymph nodes we used epithelial markers in the FACS to represent squamous carcinoma. There are rare examples of brachial arch remnants and cytokeratin positivity of interstitial reticular cells that can be seen in the lymph nodes and may be positive to any of the markers (74,75). During the process of metastasis carcinoma cells undergo epithelial-to-mesenchymal transition and lose their expression of epithelial marker such as E-cadherin (76). The choice of markers is a balance between high sensitivity and an increase in false positive findings.

Before we settled for CK5/8, EpCAM, MUC-1 we tried several other markers and combination of markers to find the best composition of antibodies. The best results were obtained when the antibodies were marked with the same colour and united into one channel in the FACS. This also gave us the opportunity to analyse the cells with additional markers at the same time in FACS. The antibodies used in the flow cytometer decide and limit the result of the investigation; with right antibodies the analysis is fast and distinct. The speed of flow cytometry enables

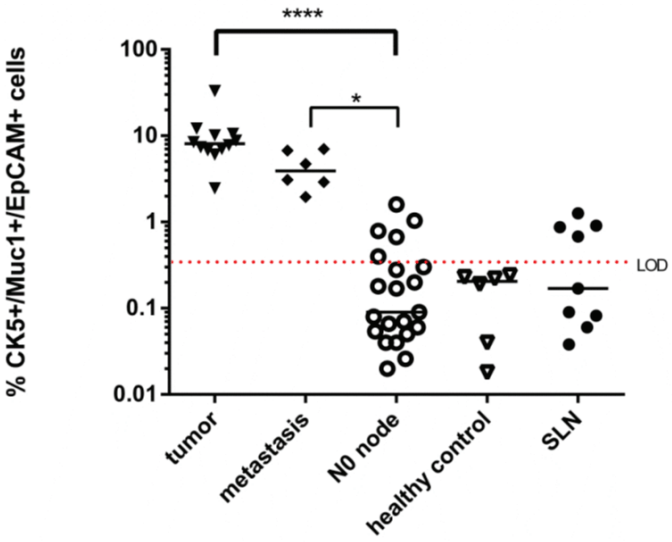

Figure 13: The frequency of epithelial-marker-positive cells in primary tumour samples, metastases, histologically non-metastatic nodes (N0 node), control lymph nodes from cancer-free individuals and sentinel lymph nodes (SLN). They are evaluated according to individually determined cut off levels for each sample. LOD= level of detection (3SD of healthy controls median). Y-axis is a log10 scale.

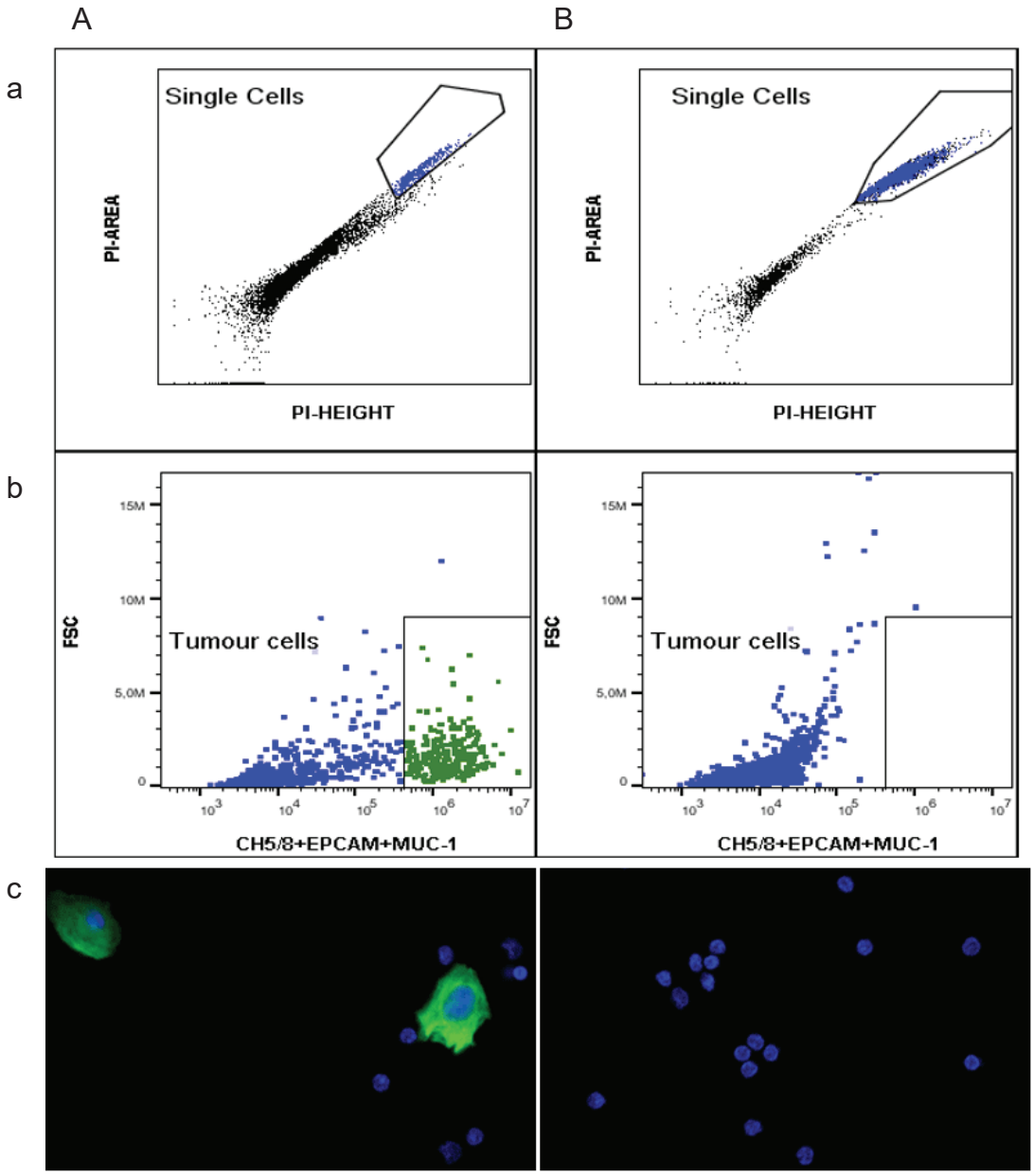

Figure 14: Details of flow cytometry method (A) Positive metastasis (B) Negative lymph node. (a) FACS gated for propidium iodide (PI), (b) FACS gated for epithelial markers, CK5/8, EpCAM, MUC-1 (c) Photomicrograph of cells, triple-stained with CK5/8, EpCAM and MUC-1 FITS- conjugated antibodies (green) and (blue)

an analysis of the material from surgery within 3 h. The FACS analysis could be automatized which is an advantage.

In our study the pathologist divided the lymph node, a metastasis was usually seen without microscope, and could be cut in two and shared, one section was analysed with FACS and the other section was sent for regular histopathology. Micro metastases (>0,2 mm in diameter) or isolated tumour cells are small and could therefore be present in any of two the sections (2). Hence, the result from the histopathology and the flow cytometry could differ and both of them be true.

Finding Occult Metastases

In our clinic, stage II patients have occult metastases in 41 % and stage I patients in 6% (28). (In oral cancer Stage I equals to T1N0, Stage II equals to T2N0). Treatment recommendations for this group of patients diverge between END and watchful waiting with therapeutic neck dissection if regional relapse of the disease. In a large study of patients were randomized to either END or watchful waiting with therapeutic surgery. Both overall survival and disease-free survival were better in elective neck dissection group (27). The rate of metastases that is undetected by imaging and clinical investigation in T1-T2 oral cancers is about 20 % (26). Thus, about 80% have then received unnecessary surgery. Many studies indicate that a search for sentinel node could be an appropriate way to investigate the neck for metastases (78,79). In our study, we found a raised number of tumour cells in the sentinel nodes. This result stress the importance to focus on the sentinel node.

Sentinel Node

In breast cancer sentinel lymph node biopsy (SLNB) is a routine investigation and has been used successfully for a long time (80). The anatomy implies to more concerns in the SLNB procedure of the neck compared to the axillary area. The Sentinel European Node Trial (SENT) is a large European study including 415 oral cancer patients from 14 centres. In this study, the sensitivity for SLNB was 86% and the negative predictive value 95 % with only minor complication (3%). The authors encourage the clinical use of the SLNB technique (78). The post-operative complications were investigated after SLNB and END, the overall result favoured the first, even when the prognosis had been taken into consideration (81).It takes time to learn how to do a sentinel node detection, to attain a good result and to use the technical devices in an optimal way. ICG penetrates only 10 mm and thus has the drawback of not being visible deep in the tissue. Our experiences with ICG are that it penetrates different depths in different tissues, never more than 10 mm. Many lymph nodes in the neck region are situated in behind the sternocleidomastoid muscle and a gamma probe may be needed in the initial stage of the procedure.

We have used tilmanocept a new diagnostic tracer in this study. With tilmanocept a more distinct picture of the radioactive uptake can be obtained and it is possible to exclude the light of the radioactivity at the injection site when you visualize it in the computer, to get a better 3D picture of the sentinel node. This was important when tumours in the oral cavity had sentinel nodes in region I, which were hidden in the light of the injection site. We believe the future is to combine ICG with nanocoll Tc99m in one injection (82). In this study we wanted to describe how to get a reliable result with a reasonable logistics to find the sentinel nodes. The regular endpoint with sentinel node biopsy studies is to detect a positive or a negative lymph node and

thereafter decide on further surgery. All our sentinel node detection was followed by a neck dissection and our focus was to find the sentinel nodes and thereafter identify the metastatic cells with flow cytometry and in parallel search for immune markers to be able to select patients for immunotherapy (Figure 15).

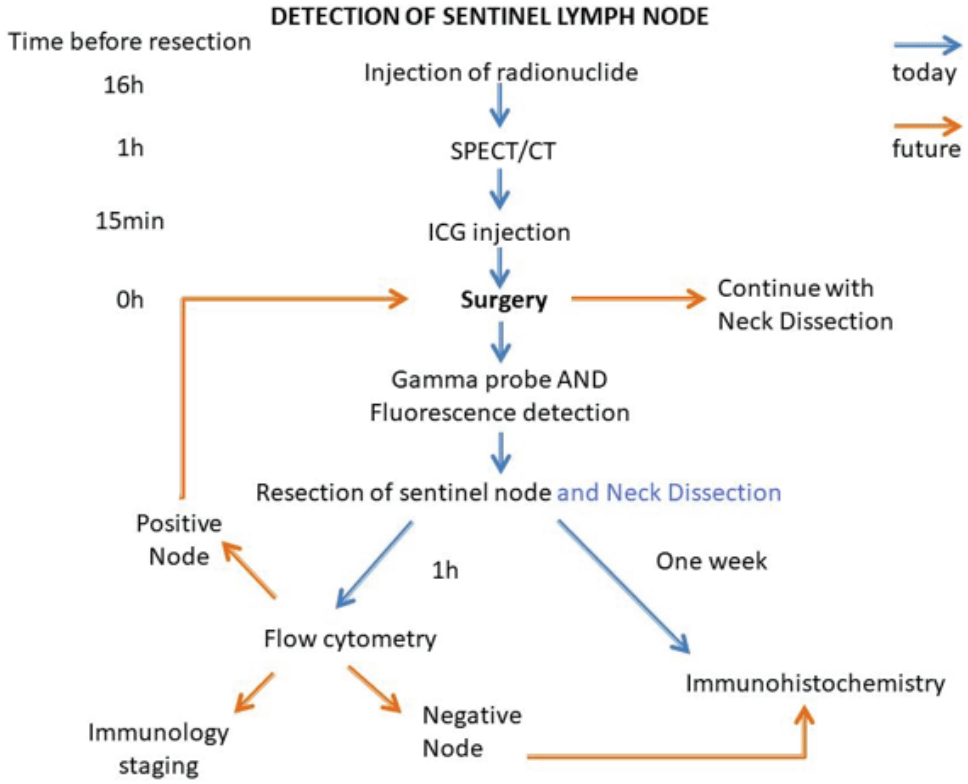

Figure 15: A flow chart describing the sentinel node flow of today (blue) and the sentinel node flow of the future (orange).

In the future we hope to indentify and bring out the sentinl node, analyse it with FACS, in the operating room, while the surgeron continue to resect the primary tumour of the tounge. The result of the FACS will then guide us, if we find a positve lymph node we will continue with a neck disection, and if a negative lymph node is found, we will analyse the rest of the node with IHC. At the same FACS analyse we will stage the patient according to their individual immunology.

Immunology staging using sentinel nodes (Paper V)

Tumour antigens activate T lymphocytes in the lymph node, which indicates the possibility to boost the immune response in the individual patient. The T cell activation varies with the investigated locations and tumour antigen exposure. The activation markers studied were chosen according to their differences in time for expression and thus their ability to indicate a time variance (83–86).

Lymphocyte Activation

The mean activation of T cells in our patients was high in tumours and low in blood, this pattern was seen for all markers tested. The level of activated lymphocytes varied between patients in tumours and lymph nodes. The intermediate activation marker $CD71^+$ was rare compared to the other markers studied. We correlated the $CD4^+$ T cells with the $CD8^+$ T cells in the

different locations. $CD4^+CD69^+/CD8^+CD69^+$ in the tumour had a good correlation as well as $CD4^+CD69^+/CD8^+CD69^+$ and $CD4^+CD71^+/CD8^+CD71^+$ in the lymph node. $CD4^+CD71^+/CD8^+CD71^+$ in the tumour had a significant positive correlation but $CD8^+CD71^+$ were generally low in number. The percentage positive CD4 and CD8 T- cells in blood and positive to any of the markers tested was low compared to positive T cells in tumour and in lymph nodes.

The Role of Sentinel Node

The sentinel nodes exhibited significantly higher percentage of $CD69^+$ T lymphocytes compared to the regular lymph nodes but the metastasis group had the highest levels. We found more $CD4^+CD71^+$ T cells in the metastatic lymph nodes than in the non-metastatic lymph nodes (Figure16). When the sentinel lymph nodes were compared with non- sentinel lymph nodes in the same patient, no patient in the sentinel node group had less than 20% of $CD69^+$ T cells and all except two patients had a higher level in the sentinel nodes compared to non-sentinel lymph nodes (Figure 17). Further, the sentinel node seems to reflect the tumour, since a high level of CD71+ in the sentinel node resulted in a high activation of the $CD4^+CD71^+$ T lymphocytes in the corresponding tumour.

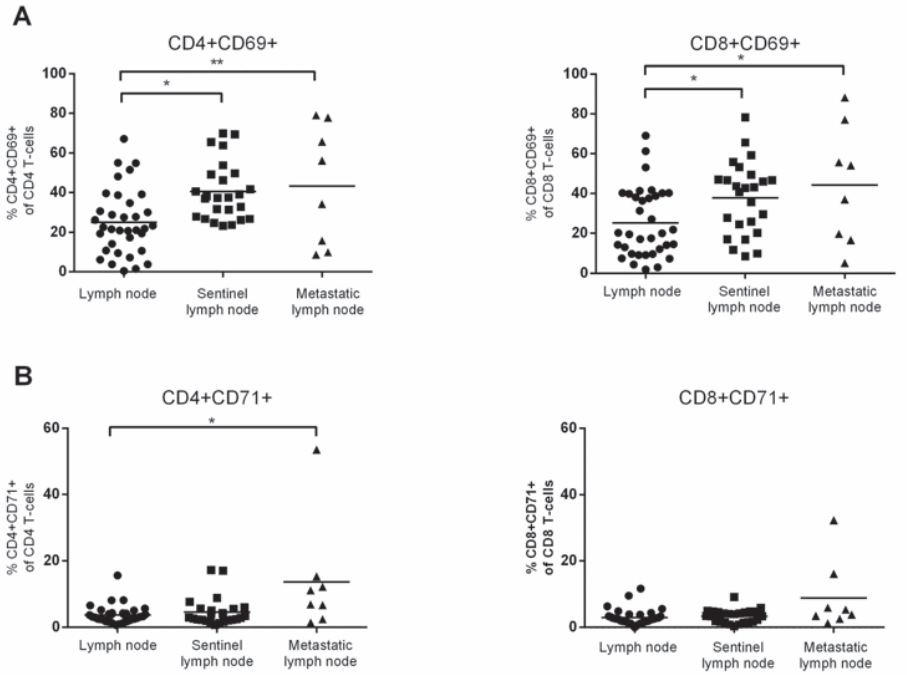

Figure 16: Activated $CD4^+$ or $CD8^+$ T lymphocytes (A) $CD69^+$ and (B) CD71+ in regular lymph nodes, sentinel lymph nodes and lymph nodes with metastases. Sentinel nodes with metastases according to histopathologic analysis were placed in the metastasis group. In the group named lymph nodes includes non-sentinel nodes and all lymph nodes examined when no sentinel node procedure was performed. When activated with HLA-DR we did not see any differences between the groups (not shown). Mean is represented by a solid line. *p< 0.05, ** p<0.01, *** p<0.001, ****p<0.0001

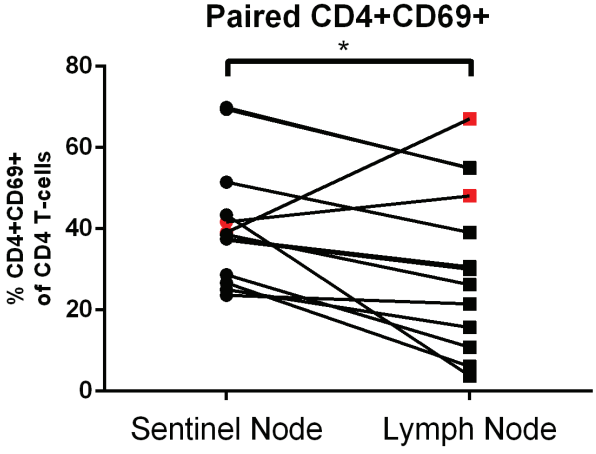

Figure 17: The percentage $CD4^+CD69^+$ cells of total $CD4^+$T lymphocytes in sentinel nodes in relation to regular lymph nodes of the same patient. When data from more than one node, in the groups, were available a mean value was calculated. Other $CD4^+$ T cells as well as $CD8^+$ T cells, studied with the activation markers CD71 and HLA-DR, did not differ between the groups (not shown). *p<0.05

PD1/PD-L1 Pathway

The PD-1/PD-L1 pathway is in focus for immunotherapy in head and neck cancers (87–90). Tumour cells express PD-L1 on their surface. We divided the tumours into groups containing 10% positive PD-L1 tumour cells, 20% positive cell and so on. We could not find any correlation between PD-L1 positive tumour cells and the activated T lymphocytes in the same tumour. We further investigated Tlymphocytes for PD-1 expression in six patients. The $CD8^+CD71^+$ lymphocytes in the tumours were positive to PD-1 and significant higher than in the $CD4^+CD71^+$ lymphocytes. In the sentinel lymph nodes of the same patients, we could not find any difference between $CD4^+$ and $CD8^+$ T lymphocytes (Figure 18).

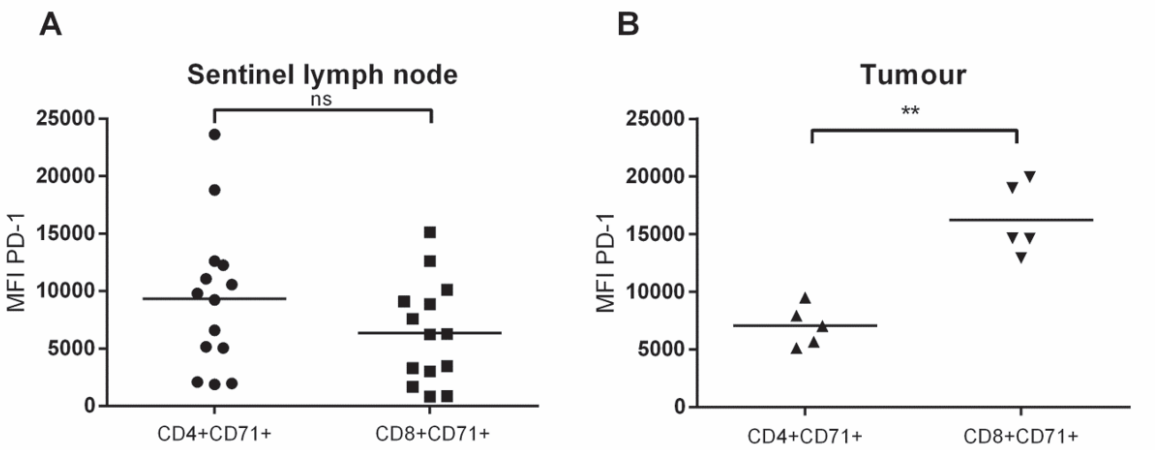

Figure 18: Levels of MFI PD-1 in $CD4^+CD71^+$ T cells and $CD8^+CD71^+$ T cells in (A) the sentinel nodes and in (B) the tumours of the same patients. n=6, one of the tumour samples was not analysed due to technical reasons *p<0.05, ** p<0.01

Comments

In this study we focused on T cells activation in oral cancer patients and the use of the lymph nodes as a source of information. The essential role of the lymph node in tumour immunology has been described in many studies (49,89). The T cell response and the T cell activation occur in the lymph node. The micro milieu for the tumour is central and the balance between co-inhibitory (eg PD-1, CTLA-4) and co-stimulatory (eg OX-40,CD28) factors is important for the individual immune response (90). In a persistent tumour antigen exposure the $CD8^+$ cell can be exhausted and express increased levels of inhibitory receptors such as PD-1 (91,92). It is hard to know if the $CD8^+$ cells in the tumour have preserved functions or if they were exhausted and had lost their normal capacities.

Patients with many TIL mainly $CD8^+$ cells, in the tumour has been shown to have a good prognosis in many types of cancer as well as in head and neck cancer (46,93–95). In tumours, the lymphocyte activation profile demonstrated a high early (CD69) activation of $CD8^+$ T cells which results in a low CD4CD8 ratio (Figure 19 B) and thereafter a shift towards a low number of later (CD71) activated CD8 T cells (corresponding to a high ratio of CD4/CD8 T cells). This could be a result of a reduced development of $CD8^+$ cells in the tumour or infiltration of $CD4^+$ cells to the tumour from the lymph nodes.

Starska et al studied the T cell activity (CD69, CD71) with flow cytometry in blood and laryngeal cancer tissue and their results resemble ours, the T cell activity was intense in both $CD4^+$ and $CD8^+$ cells when adding laryngeal tumour antigens (85). They did not do lymph node studies.

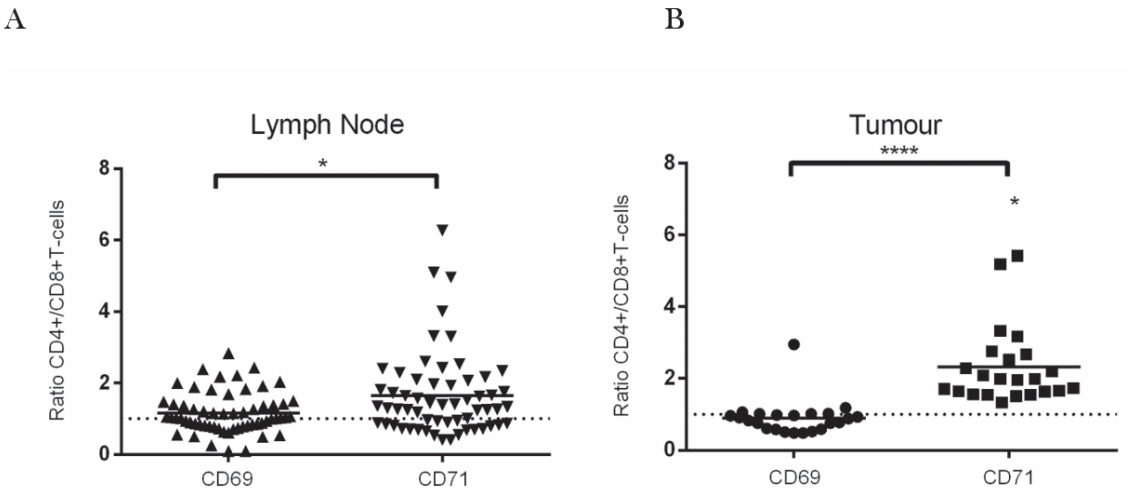

Figure 19: Ratio of $CD4^+/CD8^+$ T lymphocytes in relation to their expression of $CD69^+$ and $CD71^+$ in the lymph nodes (A) and the primary tumour (B). The dotted line represents a cut off (=1) where the number of $CD4^+$ equals the number of $CD8^+$ cells. Note: The significance was not affected by the outliers. Mean is represented by a solid line. *p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001.

Straka et al. have used the transferrin receptor 1, CD71 a marker involved in the metabolism of iron, in their studies for T lymphocyte activation and so did we. It takes about 12 h for the receptor to be expressed at the surface of the activated T lymphocyte (83,96). The choice to use CD71 in our study has several reasons. In immunology the timing of a process is important and we wanted to use markers that developed at different points of time, the CD69 marker takes about half the time to develop at the surface as the CD71 marker. The surface expression of CD71 facilitates the use of flow cytometry. In earlier studies CD71 has been considered to express cell proliferation, and has been correlated to Ki 67, a well known intracellular marker (86,97–99). It is tempting to hope that that CD71 could be an easy traced marker for activated T cells, proliferation and anti-tumour defence.

Immunology in Sentinel Nodes

We have focused on the sentinel nodes to investigate the immune responses to passing tumour antigens. All patients had a reaction in the sentinel nodes compared to the lymph nodes further down in the lymphatic draining system, none of the patients had less than 20% $CD69^+CD4^+$ cells in their sentinel nodes (Figure 17). It is conceivable that patients with a higher percentage activated T cells in the sentinel nodes than in the non-sentinel nodes (lymph nodes) have a better prognosis. We had two patients going in the opposite direction but none of them have one year after completed therapy shown any tendency to relapse.

PD-1/PD-L1 Pathway

PD-L1 expressed at the tumour cell, can be visualised with IHC and in our study positive cells were counted by the pathologist. The PD-L1 expression has been used to guide patient selection for PD-1/PD-L1 inhibitors. In many clinical studies the tumours were divided in groups of <1%, >1% and >50% of positive PD-L1 cells (88,100). This is the routine procedure at our pathology lab. We looked for an association between PD-L1 and the percentage positive $CD71^+$T cells we had in our TILs. We could not find any association with the positive PD-L1 cells divided as above. To be sure of no association we re-evaluated the groups into groups of 10% positive PD-L1 cells < 1%, 1-10%, 10-20 % and so on. No association between the CD71 activated T cells in tumours and the expression of PD-L1 at tumour cells could be found.

A link between PD-1 at the T lymphocyte and PD-L1 on the tumour cell is needed to get a response of checkpoints inhibitors. Today PD-L1 is used for patient selection for treatment and in clinical trials. In one recent study 36% of the tumours included were positive for PD-L1(101) A tumour was classified in this study as positive when >5% of the tumour cells were PD-L1 positive. This result was comparable to other studies (18.5-45%)(102,103) all of them were using the 5% limit and only oral cavity cancers were included. Interestingly, some studies reported of a higher number of PD-L1 positive tumours in oral cancer in females than in men (102,104). In a study of HPV negative oral cancers they looked at PD-L1 gene expression. 45% of the tumours were positive to PD-L1 in IHC and in 19% of the cases a CD274/PD-L1 gene amplification was detected. CD274/PD-L1 gene amplification and positive PD-L1 immunostaining was only associated in 73% of the cases and concordance of PD-L1 expression in tumour and metastasis were only 72% (103).

In our study we could not find any correlation between CD71 expression and PD-L1 positivity in IHC. It is important to remember the there is a lot of differences in the various methods for PD-L1measurements today (87). On the immunostained slides there is often a heterogenic picture, and the intensity of the staining may differ in different sections of the tumour and between patients (102). However, in many studies there is a correlation between high level of PD-L1 and worse outcome (101,103,104).

Taken together, PD-L1 in IHC is today an available marker for patient selection, it has a lot of short comings and better markers are needed. The activation pattern in T lymphocytes in the sentinel nodes differs from other lymph nodes and the T cell activation in the sentinel node seems to follow the activation pattern of the tumour. Investigation of the T cell activation pattern in the sentinel nodes gives us an immunology stage of the individual patient and it might reflect the ability of the patient to respond to immunotherapy. Flow cytometry could therefore have an important role in the analysis of PD-1/PD-L1 pathway.

FUTURE PERSPECTIVES

Therapies that affect the immune system to eradicate a cancer disease are here to stay (88). In last years the so-called checkpoints inhibitors have emerged as an important strategy for treating relapses of head and neck cancer, but not all patients respond to therapy (101). The need for better patient selection is clear. In this thesis we have tried to elucidate the role that lymph nodes play in the immune response to head and neck cancers.

It is clear that the microenvironment is involved in the tumour progression. Recent research shows that HPV positive oropharyngeal tumours have a different pattern of progression then HPV negative tumours. Hence, it could be of interest to study these two groups separately according to their different immunology. It is unclear whether neutrophils promote or prevent the development of cancer. It is also unclear if the aggressiveness of the tumour leads to changes of the immune status or the neutrophils and the monocytes contribute to the aggressiveness of the tumour.

A neutrophil lives for a limited time and a cancer disease takes months or maybe years to develop. Since the neutrophils cohort change, it is also possible for the role of the neutrophil to change during the tumour progression and maybe that could explain the pro-tumorigenic and the anti-tumorigenic features the neutrophil can take (68). The activated $CD16^{high}CD62L^{dim}$ neutrophils showed anti-tumorigenic characteristics and patients with high levels of this subtype showed increased survival. It would be interesting to verify this with a large cohort of homogenous cancer patients.

To use of FACS to detect tumour cells in lymph nodes of oral squamous cell carcinoma has not previously been done. Before clinical implements can be realized, the FACS gating has to be set and standardized with a large cohort. If flow cytometry analysis of lymph nodes becomes routine, many kinds of immunology studies can be done and immunology staging be a possibility.

The techniques of sentinel node are constantly improved. Dynamic investigations with SPECT–CT could reveal if a sentinel node is a single first receiver of tumour antigens or if there are several nodes in a paralleled system. Our studies showed many SN far from the primary tumour area in region IV and V or at the contralateral side. If a limited neck dissection without SN had been applied, several of these distant nodes would probably have been missed.

The future is in personalized medicine. To further investigate the correlations between activated T cells and the inhibitory pathways will be needed. To stage the oral cancer patients according to their immunology status in the lymph node would equippe us with a new diagnostic tool.

CONCLUSIONS

- Patients with oropharyngeal cancer displayed signs of increased systemic inflammation. Generally, large tumours seemed to be associated with a high neutrophil to monocyte ratio whereas patients with metastatic node spread showed a low corresponding value.

- A specific neutrophil subset, $CD16^{high}CD62L^{dim}$ appeared to have anti-tumour properties, with the ability to inhibit cancer cell migration and proliferation and to induce apoptosis. Further, the elastase in the activated neutrophils created neutrophil extracellular traps (NET) and a high rate of $CD16^{high}CD62L^{dim}$ neutrophils corresponded to a better survival.

- Flow cytometry could be used to detect cancer cells in lymph nodes. The results could be presented within 6 hours from the time of biopsy and the data correlated with the clinical histopathologic investigation performed in parallel. The results indicated that flow cytometry could be a very sensitive tool to find micro metastases.

- Various techniques for identification of sentinel nodes in oral cancer were evaluated in a clinical setting. A combination of techniques was found to constitute a reliable, clinically feasible work concept. An injection of radioactive technetium Tc99m carried on tilmanocept started the process. The lymph nodes were visualized with SPECT/CT before surgery and with ICG fluorescence dye in combination with a hand-held gamma probe during surgery.

- Lymph node metastases in oral cancer exhibited a higher level of activated T cells than cancer free lymph nodes. CD69, a marker of T cell activity was generally higher in sentinel lymph nodes than in regular nodes. PD-L1 on tumour cells did not correlate to the expression of activation markers on T lymphocytes in the tumour. $CD8^+$ T lymphocytes with high $CD71^+$PD-1 expression were more abundant in the tumours than in the sentinel nodes. Altogether this indicates that immunologic activity of the sentinel node might prove useful to predict response and select patients for immunological treatment.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Antalet patienter med huvud- och halscancer ökar i Sverige och omkring 1300 personer insjuknar årligen. Cancern kan behandlas framgångsrikt om de upptäcks i tid. I ungefär en tredjedel av fallen har tumören spridit sig redan vid första läkarbesöket. Behandlingen styrs av tumörens storlek, lokalisation och eventuell spridning. Det är viktigt att få korrekt diagnos. Spridningen till halsens lymfkörtlar kan vara svår att upptäcka, hörnstenen är mikroskopisk undersökning av bortopererade lymfkörtlar. Metastaser som inte upptäcks leder till återfall av cancern, kan vara svåra att behandla och leda till kraftig funktionsnedsättning eller död.

Vårt immunförsvar reagerar på cancerceller och sedan några år tillbaka finns läkemedel som stärker förmågan för vårt immunoförsvar att döda cancerceller. Behandlingen är dyr, biverkningarna många och endast en mindre del av alla patienter svarar på behandlingen. Det krävs därför nya diagnostiska verktyg för att avgöra vilka patienter som bör behandlas. Stora resurser läggs ned för att hitta lämpliga markörer i blod och tumör för att göra denna selektion. Få tycks arbeta med lymfkörtlarna, den plats där den egentliga immunologiska reaktionen äger rum.

Denna avhandling undersöker det immunologiska samspelet mellan huvud-halscancer och immunologiskt verksamma celler som återfinns i blod, tumör och lymfkörtlar. Första delen fokuserar på neutrofiler, vita blodkroppar viktiga för vårt medfödda snabba immunförsvar. Vi utreder om flödescytometri, en cellanalys som länge använts för diagnostik av blodcancer också kan användas för att hitta skivepitelcancerceller, lymfkörtelmetastaser vid munhålecancer mer specifikt. Avslutningsvis undersöks om det går att utnyttja det immunologiska svaret i lymfkörtlarna för att avgöra vem som är lämplig för nya former av immunologisk cancer terapi.

Det första delarbetet visar att antalet neutrofiler i blodet ökar när man drabbas av cancer i munhålan. Detta kan tolkas som att patienten utvecklat ett inflammatoriskt svar på cancern. Det neutrofila inflammationsvaret visade sig vara tydligt relaterat till tumörens storlek.

Neutrofila celler kan vara inaktiverade eller aktiverade. De senare har en betydligt större förmåga att påverka omgivningen än de förstnämnda. Det andra delarbetet visar att en speciell typ av aktiverade neutrofiler, $CD16^{high}CD62L^{dim}$, förekommer vid cancer. Patienterna delades upp i två grupper, en med höga värden $CD16^{high}CD62L^{dim}$ neutrofiler och en med låga nivåer. Patientgruppen med höga nivåer visade sig leva längre. Det är därför frestande att anta att $CD16^{high}CD62L^{dim}$ på något sätt hämmar cancerutvecklingen.

Delstudie tre visar hur flödescytometri kan användas för diagnostik av lymfkörtel metastaser på halsen vid cancer i munhålan. Fördelen med denna metod är att den går snabbt, kan automatiseras och att den samtidigt ger möjlighet att analyserar lymfkörtelns immunologiska profil. Vi valde att använda markörer för epitelceller som normalt inte finnas i lymfkörtlarna och som därmed väl representerar förekomst cancerceller. Det gick att visa att den nya metoden var minst lika bra på att hitta metastaser som den tidskrävande standardmetod används som idag används rutinmässigt.

De lymfkörtlar som mest intimt kommunicerar med en tumör via lymfbanor benämns vanligen portvaktskörtlar eller sentinel nodes. De är de första körtlar som tar emot metastatiska celler som sprider sig från tumören är de vanligen där som de första metastaserna etableras och återfinnes. Det är också i portvaktskörtlarna som immunförsvaret först reagerar på förekomst av tumörceller. De är därför viktiga av diagnostiska skäl. Arbete fyra beskriver hur man rent praktiskt kan gå till väga för att identifiera dessa portvaktskörtlar före och under operation.

I det avslutande arbetet kartläggs det immunologiska svaret i lymfkörtlar jämfört med det i blod och tumör vid munhålecancer. Vi har speciellt fokuserat på skillnader mellan portvaktskörtlar, körtlar som innehåller metastaser och övriga lymfkörtlar. De data som redovisas kan ge ledtrådar, inte bara om prognos utan också om vilka patienter som lämpar sig för immunologisk cancerterapi.

Sammanfattningsvis kan sägas att denna avhandling ser på huvud-halscancer ur ett immunologiskt perspektiv, där cancersjukdomen bedöms som en inflammatorisk sjukdom som engagerar vårt immunförsvar. De resultat som redovisas öppnar vägen för nya möjligheter till diagnostik och terapi. Det är vår förhoppning att vi genom denna forskning bidragit något till att cancer i huvud-halsregionen skall kunna gå samma gynnsamma utveckling till mötes som många former av blodcancer redan gjort.

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REFERENCES

1. Mandal R, Senbabaoglu Y, Desrichard A, Havel JJ, Dalin MG, Riaz N, et al. The head and neck cancer immune landscape and its immunotherapeutic implications. JCI Insight [Internet]. [cited 2018 Sep 21];1(17). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5070962/
2. TNM Classification of Malignant Tumours (8th Edition) [Internet]. Hoboken, NJ, USA: Wiley-Blackwell; 2017.
3. Licitra L, Bernier J, Grandi C, Merlano M, Bruzzi P, Lefebvre J-L. Cancer of the oropharynx. Crit Rev Oncol Hematol. 2002 Jan 1;41(1):107–22.
4. Mellin H, Friesland S, Lewensohn R, Dalianis T, Munck-Wikland E. Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. Int J Cancer. 2000 May 20;89(3):300–4.
5. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R, Chaturvedi AK, Bray F, et al. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. CA Cancer J Clin. 2017 Jan 1;67(1):51–64.
6. National Quality Registry for Head and Neck Cancer - Nationella Kvalitetsregister [Internet]. [cited 2017 Aug 25]. Available from: http://kvalitetsregister.se/english-pages/findaregistry/registerarkivenglish/nationalqualityregistryforheadandneckcancer.2287.html
7. Tommasino M. The human papillomavirus family and its role in carcinogenesis. Semin Cancer Biol. 2014 Jun 1;26:13–21.
8. Dalianis T. Human papillomavirus (HPV) and oropharyngeal squamous cell carcinoma. Presse Medicale Paris Fr 1983. 2014 Dec;43(12 Pt 2):e429-434.
9. Näsman A, Nordfors C, Holzhauser S, Vlastos A, Tertipis N, Hammar U, et al. Incidence of human papillomavirus positive tonsillar and base of tongue carcinoma: A stabilisation of an epidemic of viral induced carcinoma? Eur J Cancer. 2015 Jan;51(1):55–61.
10. Chaturvedi AK. Epidemiology and clinical aspects of HPV in head and neck cancers. Head Neck Pathol. 2012 Jul;6 Suppl 1:S16-24.
11. Hammarstedt L, Lindquist D, Dahlstrand H, Romanitan M, Dahlgren LO, Joneberg J, et al. Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer. Int J Cancer. 2006 Dec 1;119(11):2620–3.
12. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, et al. Human Papillomavirus and Survival of Patients with Oropharyngeal Cancer. N Engl J Med. 2010 Jul 1;363(1):24–35.
13. Ragin CCR, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. Int J Cancer. 2007 Oct 15;121(8):1813–20.
14. Wang MB, Liu IY, Gornbein JA, Nguyen CT. HPV-Positive Oropharyngeal Carcinoma: A Systematic Review of Treatment and Prognosis. Otolaryngol Neck Surg. 2015 Nov 1;153(5):758–69.
15. Lyford-Pike S, Peng S, Young GD, Taube JM, Westra WH, Akpeng B, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res. 2013 Mar 15;73(6):1733–41.
16. Mirghani H, Amen F, Blanchard P, Moreau F, Guigay J, Hartl DM, et al. Treatment de-escalation in HPV-positive oropharyngeal carcinoma: Ongoing trials, critical issues and perspectives. Int J Cancer. 2015 Apr 1;136(7):1494–503.
17. Markowitz LE, Tsu V, Deeks SL, Cubie H, Wang SA, Vicari AS, et al. Human Papillomavirus Vaccine Introduction – The First Five Years. Vaccine. 2012 Nov;30:F139–48.
18. Gottvall M, Stenhammar C, Grandahl M. Parents' views of including young boys in the Swedish national school-based HPV vaccination programme: a qualitative study. BMJ Open [Internet]. 2017 Feb 28 [cited 2018 Sep 21];7(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5337740/
19. Gandini S, Botteri E, Iodice S, Boniol M, Lowenfels AB, Maisonneuve P, et al. Tobacco smoking and cancer: A meta-analysis. Int J Cancer. 2008 Jan 1;122(1):155–64.
20. Petti S. Lifestyle risk factors for oral cancer. Oral Oncol. 2009 Apr 1;45(4):340–50.
21. Reidy J, McHugh E, Stassen LFA. A review of the relationship between alcohol and oral cancer. The Surgeon. 2011 Oct 1;9(5):278–83.
22. Sturgis EM, Wei Q, Spitz MR. Descriptive epidemiology and risk factors for head and neck cancer. Semin Oncol. 2004 Dec 1;31(6):726–33.
23. Rusthoven K, Ballonoff A, Raben D, Chen C. Poor prognosis in patients with stage I and II oral tongue squamous cell carcinoma. Cancer. 2008 Jan 15;112(2):345–51.
24. Listl S, Jansen L, Stenzinger A, Freier K, Emrich K, Holleczek B, et al. Survival of patients with oral cavity cancer in Germany. PloS One. 2013;8(1):e53415.
25. Annertz K, Anderson H, Palmér K, Wennerberg J. The increase in incidence of cancer of the tongue in the Nordic countries continues into the twenty-first century. Acta Otolaryngol (Stockh). 2012 May;132(5):552–7.
26. Psychogios G, Mantsopoulos K, Bohr C, Koch M, Zenk J, Iro H. Incidence of occult cervical metastasis in head and neck carcinomas: development over time. J Surg Oncol. 2013 Mar;107(4):384–7.
27. D'Cruz AK, Vaish R, Kapre N, Dandekar M, Gupta S, Hawaldar R, et al. Elective versus Therapeutic Neck Dissection in Node-Negative Oral Cancer. N Engl J Med. 2015 Aug 6;373(6):521–9.
28. Kamali A, Gahm C, Palmgren B, Marklund L, Halle M, Hammarstedt-Nordenvall L. Regional recurrence in early stage I-II oral tongue cancer: a single institutional study and review of the literature. Acta Otolaryngol (Stockh). 2017 Jul;137(7):755–61.

29. Brandwein-Gensler M, Teixeira MS, Lewis CM, Lee B, Rolnitzky L, Hille JJ, et al. Oral Squamous Cell Carcinoma. Am J Surg Pathol. 2005;29(2):12.
30. Lee SU, Cho KH, Moon SH, Choi SW, Park JY, Yun T, et al. Clinical outcome of high-dose-rate interstitial brachytherapy in patients with oral cavity cancer. Radiat Oncol J. 2014 Dec;32(4):238–46.
31. Robbins KT, Clayman G, Levine PA, Medina J, Sessions R, Shaha A, et al. Neck dissection classification update: revisions proposed by the American Head and Neck Society and the American Academy of Otolaryngology-Head and Neck Surgery. Arch Otolaryngol Head Neck Surg. 2002 Jul;128(7):751–8.
32. Robbins KT, Shaha AR, Medina JE, Califano JA, Wolf GT, Ferlito A, et al. Consensus statement on the classification and terminology of neck dissection. Arch Otolaryngol Head Neck Surg. 2008 May;134(5):536–8.
33. Poirier P-J (1853-1907) A du texte, Charpy A (1848-1911) A du texte, Cunéo B (1873-1944) A du texte. Abrégé d'anatomie. Coeur, artères, veines, lymphatiques, centres nerveux, nerfs craniens, nerfs rachidiens / par P. Poirier,... A. Charpy,... B. Cunéo,... [Internet]. Paris: Masson; 1908 [cited 2017 Aug 25]. Available from: http://gallica.bnf.fr/ark:/12148/bpt6k6208158q
34. Gray H. Anatomy of the human body. [Internet]. 20th ed., thoroughly revised and re-edited by Warren H. Lewis. Philadelphia,; 1918. Available from: http://hdl.handle.net/2027/mdp.39015052215780
35. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar 4;144(5):646–74.
36. Miller JFAP, Sadelain M. The Journey from Discoveries in Fundamental Immunology to Cancer Immunotherapy. Cancer Cell. 2015 Apr 13;27(4):439–49.
37. Clark R, Kupper T. Old Meets New: The Interaction Between Innate and Adaptive Immunity. J Invest Dermatol. 2005 Oct;125(4):629–37.
38. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013 Mar;13(3):159–75.
39. Kamp VM, Pillay J, Lammers J-WJ, Pickkers P, Ulfman LH, Koenderman L. Human suppressive neutrophils CD16bright/CD62Ldim exhibit decreased adhesion. J Leukoc Biol. 2012 Nov;92(5):1011–20.
40. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers J-W, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J Clin Invest. 2012 Jan;122(1):327–36.
41. Hu K, Lou L, Ye J, Zhang S. Prognostic role of the neutrophil-lymphocyte ratio in renal cell carcinoma: a meta-analysis. BMJ Open. 2015 Apr 8;5(4):e006404.
42. Kano S, Homma A, Hatakeyama H, Mizumachi T, Sakashita T, Kakizaki T, et al. Pretreatment lymphocyte-to-monocyte ratio as an independent prognostic factor for head and neck cancer. Head Neck. 2016 Sep 1;n/a-n/a.
43. Li M-X, Liu X-M, Zhang X-F, Zhang J-F, Wang W-L, Zhu Y, et al. Prognostic role of neutrophil-to-lymphocyte ratio in colorectal cancer: a systematic review and meta-analysis. Int J Cancer J Int Cancer. 2014 May 15;134(10):2403–13.
44. Kuss I, Hathaway B, Ferris RL, Gooding W, Whiteside TL. Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. Clin Cancer Res Off J Am Assoc Cancer Res. 2004 Jun 1;10(11):3755–62.
45. Maleki S, Schlecht NF, Keller C, Diaz J, Moss J, Prystowsky MB, et al. Lymphocytic Host Response to Oral Squamous Cell Carcinoma: An Adaptive T-Cell Response at the Tumor Interface. Head Neck Pathol. 2011 Jun;5(2):117–22.
46. Nguyen N, Bellile E, Thomas D, McHugh J, Rozek L, Virani S, et al. Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. Head Neck. 2016 Jul;38(7):1074–84.
47. Poropatich K, Hernandez D, Fontanarosa J, Brown K, Woloschak G, Paintal A, et al. Peritumoral Cuffing by T cell Tumor Infiltrating Lymphocytes Distinguishes HPV-Related Oropharyngeal Squamous Cell Carcinoma from Oral Cavity Squamous Cell Carcinoma. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2017 Jun 20;
48. Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res. 1970;13:1–27.
49. Chen DS, Mellman I. Oncology Meets Immunology: The Cancer-Immunity Cycle. Immunity. 2013 Jul;39(1):1–10.
50. Ferris RL. Immunology and Immunotherapy of Head and Neck Cancer. J Clin Oncol Off J Am Soc Clin Oncol. 2015 Oct 10;33(29):3293–304.
51. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015 Apr 13;27(4):450–61.
52. Arbyn M, Xu L, Simoens C, Martin‐Hirsch PP. Prophylactic vaccination against human papillomaviruses to prevent cervical cancer and its precursors. Cochrane Database Syst Rev [Internet]. 2018 [cited 2018 Sep 21];(5). Available from: https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD009069.pub3/abstract
53. Abken H. Adoptive therapy with CAR redirected T cells: the challenges in targeting solid tumors. Immunotherapy. 2015 Jun 1;7(5):535–44.
54. Campian JL, Sarai G, Ye X, Marur S, Grossman SA. Association between severe treatment-related lymphopenia and progression-free survival in patients with newly diagnosed squamous cell head and neck cancer: Treatment-Related Lymphopenia in Head and Neck Cancer. Head Neck. 2014 Dec;36(12):1747–53.
55. Schuler PJ, Harasymczuk M, Schilling B, Saze Z, Strauss L, Lang S, et al. Effects of Adjuvant Chemoradiotherapy on the Frequency and Function of Regulatory T Cells in Patients with Head and Neck Cancer. Clin Cancer Res. 2013 Jan 12;19(23):6585–96.

56. Tang X, Du P, Yang Y. The clinical use of neutrophil-to-lymphocyte ratio in bladder cancer patients: a systematic review and meta-analysis. Int J Clin Oncol. 2017 Oct 1;22(5):817–25.
57. Wang Y, Peng C, Cheng Z, Wang X, Wu L, Li J, et al. The prognostic significance of preoperative neutrophil-lymphocyte ratio in patients with hepatocellular carcinoma receiving hepatectomy: A systematic review and meta-analysis. Int J Surg. 2018 Jul 1;55:73–80.
58. Mascarella MA, Mannard E, Silva SD, Zeitouni A. Neutrophil-to-lymphocyte ratio in head and neck cancer prognosis: A systematic review and meta-analysis. Head Neck. 2018 May;40(5):1091–100.
59. Tham T, Bardash Y, Herman SW, Costantino PD. Neutrophil-to-lymphocyte ratio as a prognostic indicator in head and neck cancer: A systematic review and meta-analysis. Head Neck [Internet]. [cited 2018 Sep 9];0(0). Available from: http://onlinelibrary.wiley.com/doi/abs/10.1002/hed.25324
60. So YK, Lee G, Oh D, Byeon S, Park W, Chung MK. Prognostic Role of Neutrophil-to-Lymphocyte Ratio in Patients with Human Papillomavirus–Positive Oropharyngeal Cancer. Otolaryngol Neck Surg. 2018 Aug 1;159(2):303–9.
61. Rachidi S, Wallace K, Day TA, Alberg AJ, Li Z. Lower circulating platelet counts and antiplatelet therapy independently predict better outcomes in patients with head and neck squamous cell carcinoma. J Hematol OncolJ Hematol Oncol. 2014 Sep 27;7:65.
62. Chikamatsu K, Sakakura K, Toyoda M, Takahashi K, Yamamoto T, Masuyama K. Immunosuppressive activity of CD14+ HLA-DR- cells in squamous cell carcinoma of the head and neck. Cancer Sci. 2012 Jun;103(6):976–83.
63. Ray-Coquard I, Cropet C, Van Glabbeke M, Sebban C, Le Cesne A, Judson I, et al. Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. Cancer Res. 2009 Jul 1;69(13):5383–91.
64. Millrud CR, Hylander T, Kumlien Georen S, Kågedal Å, Winqvist O, Cardell LO. Inverse immunological responses induced by allergic rhinitis and head and neck squamous cell carcinoma. PloS One. 2014;9(1):e86796.
65. Caldeira PC, Vieira ÉLM, Sousa AA, Teixeira AL, Aguiar MCF. Immunophenotype of neutrophils in oral squamous cell carcinoma patients. J Oral Pathol Med. 2017 Oct 1;46(9):703–9.
66. Trellakis S, Bruderek K, Dumitru CA, Gholaman H, Gu X, Bankfalvi A, et al. Polymorphonuclear granulocytes in human head and neck cancer: enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. Int J Cancer J Int Cancer. 2011 Nov 1;129(9):2183–93.
67. Treffers LW, Hiemstra IH, Kuijpers TW, Berg TK van den, Matlung HL. Neutrophils in cancer. Immunol Rev. 2016 Sep 1;273(1):312–28.
68. Uribe-Querol E, Rosales C. Neutrophils in Cancer: Two Sides of the Same Coin. J Immunol Res. 2015;2015:983698.
69. Brandau S, Dumitru CA, Lang S. Protumor and antitumor functions of neutrophil granulocytes. Semin Immunopathol. 2013 Mar;35(2):163–76.
70. Dumitru CA, Gholaman H, Trellakis S, Bruderek K, Dominas N, Gu X, et al. Tumor-derived macrophage migration inhibitory factor modulates the biology of head and neck cancer cells via neutrophil activation. Int J Cancer. 2011;129(4):859–869.
71. Polom K, Murawa D, Rho Y-S, Nowaczyk P, Hünerbein M, Murawa P. Current trends and emerging future of indocyanine green usage in surgery and oncology: a literature review. Cancer. 2011 Nov 1;117(21):4812–22.
72. Woo J, Baumann A, Arguello V. Recent advancements of flow cytometry: new applications in hematology and oncology. Expert Rev Mol Diagn. 2014 Jan 1;14(1):67–81.
73. Hartana CA, Kinn J, Rosenblatt R, Anania S, Alamdari F, Glise H, et al. Detection of micrometastases by flow cytometry in sentinel lymph nodes from patients with renal tumours. Br J Cancer. 2016 Oct 11;115(8):957–66.
74. Gould VE, Bloom KJ, Franke WW, Warren WH, Moll R. Increased numbers of cytokeratin-positive interstitial reticulum cells (CIRC) in reactive, inflammatory and neoplastic lymphadenopathies: hyperplasia or induced expression? Virchows Arch Int J Pathol. 1995;425(6):617–29.
75. Triantafyllou A, Williams MD, Angelos P, Shah JP, Westra WH, Hunt JL, et al. Incidental findings of thyroid tissue in cervical lymph nodes: old controversy not yet resolved? Eur Arch Oto-Rhino-Laryngol Off J Eur Fed Oto-Rhino-Laryngol Soc EUFOS Affil Ger Soc Oto-Rhino-Laryngol - Head Neck Surg. 2016 Oct;273(10):2867–75.
76. Vig N, Mackenzie IC, Biddle A. Phenotypic plasticity and epithelial-to-mesenchymal transition in the behaviour and therapeutic response of oral squamous cell carcinoma. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2015 Oct;44(9):649–55.
78. Schilling C, Stoeckli SJ, Haerle SK, Broglie MA, Huber GF, Sorensen JA, et al. Sentinel European Node Trial (SENT): 3-year results of sentinel node biopsy in oral cancer. Eur J Cancer Oxf Engl 1990. 2015 Dec;51(18):2777–84.
79. Schilling C, Shaw R, Schache A, McMahon J, Chegini S, Kerawala C, et al. Sentinel lymph node biopsy for oral squamous cell carcinoma. Where are we now? Br J Oral Maxillofac Surg. 2017 Oct;55(8):757–62.
80. Galimberti V, Manika A, Maisonneuve P, Corso G, Salazar Moltrasio L, Intra M, et al. Long-term follow-up of 5262 breast cancer patients with negative sentinel node and no axillary dissection confirms low rate of axillary disease. Eur J Surg Oncol EJSO. 2014 Oct 1;40(10):1203–8.
81. Hernando J, Villarreal P, Álvarez-Marcos F, Gallego L, García-Consuegra L, Junquera L. Comparison of related complications: sentinel node biopsy versus elective neck dissection. Int J Oral Maxillofac Surg. 2014 Nov;43(11):1307–12.

82. KleinJan GH, Werkhoven E van, Berg NS van den, Karakullukcu MB, Zijlmans HJM a. A, Hage JA van der, et al. The best of both worlds: a hybrid approach for optimal pre- and intraoperative identification of sentinel lymph nodes. Eur J Nucl Med Mol Imaging. 2018 Apr 25;1–11.
83. Ponka P, Lok CN. The transferrin receptor: role in health and disease. Int J Biochem Cell Biol. 1999 Oct;31(10):1111–37.
84. Starska K, Glowacka E, Kulig A, Lewy-Trenda I, Brys M, Lewkowicz P. Prognostic value of the immunological phenomena and relationship with clinicopathological characteristics of the tumor--the expression of the early CD69+, CD71+ and the late CD25+, CD26+, HLA/DR+ activation markers on T CD4+ and CD8+ lymphocytes in squamous cell laryngeal carcinoma. Part II. Folia Histochem Cytobiol. 2011;49(4): 593–603.
85. Starska K, Glowacka E, Kulig A, Lewy-Trenda I, Brys M, Lewkowicz P. The role of tumor cells in the modification of T lymphocytes activity--the expression of the early CD69+, CD71+ and the late CD25+, CD26+, HLA/DR+ activation markers on T CD4+ and CD8+ cells in squamous cell laryngeal carcinoma. Part I. Folia Histochem Cytobiol. 2011;49(4):579–92.
86. Wieland E, Shipkova M. Lymphocyte surface molecules as immune activation biomarkers. Clin Biochem. 2016 Mar 1;49(4):347–54.
87. De Meulenaere A, Vermassen T, Aspeslagh S, Huvenne W, Van Dorpe J, Ferdinande L, et al. Turning the tide: Clinical utility of PD-L1 expression in squamous cell carcinoma of the head and neck. Oral Oncol. 2017 Jul 1;70:34–42.
88. Dogan V, Rieckmann T, Münscher A, Busch C-J. Current studies of immunotherapy in head and neck cancer. Clin Otolaryngol. :n/a-n/a.
89. Kim JM, Chen DS. Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). Ann Oncol. 2016 Aug 1;27(8):1492–504.
90. Outh-Gauer S, Alt M, Le Tourneau C, Augustin J, Broudin C, Gasne C, et al. Immunotherapy in head and neck cancers: A new challenge for immunologists, pathologists and clinicians. Cancer Treat Rev. 2018 Apr;65:54–64.
91. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 2015 Apr;36(4):265–76.
92. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. Immunology. 2010 Apr;129(4):474–81.
93. Fang J, Li X, Ma D, Liu X, Chen Y, Wang Y, et al. Prognostic significance of tumor infiltrating immune cells in oral squamous cell carcinoma. BMC Cancer [Internet]. 2017 May 26 [cited 2018 Mar 8];17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5446725/
94. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012 Apr;12(4): 298–306.
95. Wolf GT, Chepeha DB, Bellile E, Nguyen A, Thomas D, McHugh J. Tumor Infiltrating Lymphocytes (TIL) and Prognosis in Oral Cavity Squamous Carcinoma: A Preliminary Study. Oral Oncol. 2015 Jan;51(1):90–5.
96. Reddy M, Eirikis E, Davis C, Davis HM, Prabhakar U. Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. J Immunol Methods. 2004 Oct;293(1–2):127–42.
97. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol. 1984 Oct 1;133(4):1710–5.
98. Motamedi M, Xu L, Elahi S. Correlation of transferrin receptor (CD71) with Ki67 expression on stimulated human and mouse T cells: The kinetics of expression of T cell activation markers. J Immunol Methods. 2016 Oct 1;437:43–52.
99. Schuurman HJ, van Wichen D, de Weger RA. Expression of activation antigens on thymocytes in the 'common thymocyte' stage of differentiation. Thymus. 1989;14(1–3):43–53.
100. Ferris RL, Blumenschein GJ, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. N Engl J Med. 2016 Nov 10;375(19):1856–67.
101. Schneider S, Kadletz L, Wiebringhaus R, Kenner L, Selzer E, Füreder T, et al. PD-1 and PD-L1 expression in HNSCC primary cancer and related lymph node metastasis – impact on clinical outcome. Histopathology [Internet]. [cited 2018 Jul 1];0(ja). Available from: http://onlinelibrary.wiley.com/doi/abs/10.1111/his.13646
102. Satgunaseelan L, Gupta R, Madore J, Chia N, Lum T, Palme CE, et al. Programmed cell death-ligand 1 expression in oral squamous cell carcinoma is associated with an inflammatory phenotype. Pathology (Phila). 2016 Oct;48(6):574–80.
103. Straub M, Drecoll E, Pfarr N, Weichert W, Langer R, Hapfelmeier A, et al. CD274/PD-L1 gene amplification and PD-L1 protein expression are common events in squamous cell carcinoma of the oral cavity. Oncotarget. 2016 Feb 22;7(11):12024–34.
104. Lin Y-M, Sung W-W, Hsieh M-J, Tsai S-C, Lai H-W, Yang S-M, et al. High PD-L1 Expression Correlates with Metastasis and Poor Prognosis in Oral Squamous Cell Carcinoma. PLoS ONE [Internet]. 2015 Nov 12 [cited 2018 Jul 10];10(11). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4642967/