Faculty of Health Science, UiT – The Arctic University of Norway

CD57 as a prognostic marker in Oral squamous cell carcinoma

A study within the NOROC project

Cathrine Elise Warvik

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Supervisor: Elin-Synnøve Hadler-Olsen, Professor, Department of Medical Biology, UiT

Preface

I have always been interested in pathology, and both my 2nd year assignment and my project as a student in the medical research program have consisted of projects on the histopathological assessment and diagnosis of cancer. In the research program I have worked on the immunohistological scoring of markers in oral squamous cell carcinoma (OSCC), specifically D2-40, a marker of lymph vessels. In my master thesis I decided to ask my main supervisor on my research program project if there were any projects on OSCC that were adjacent to this, but with enough difference to evolve my knowledge on the assessment of histological markers.

The choice fell on CD57, a marker of a subset of NK-cells, that has been found to be a possible prognostic marker in a systematic review of articles written by my main supervisor Elin-Synnøve Hadler-Olsen and Tumorbiology group member Anna Maria Wirsing.

I would like to thank my supervisor, Elin Synnøve Hadler-Olsen for her patience and guidance, always giving concise and clear advice. I would also like to thank Pathologist Ruth Schwienbacher for advice on the implications of observations in the tumor tissue samples.

Lastly, I would like to thank the tumorbiology research group at the Department of Medical Biology at the university of Tromsø. This thesis is built on the invaluable knowledge and experience I have received as a student in their research group.

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Cathrine Elise Warvik

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is a cancer arising from the epithelium lining the oral cavity. Despite improvement in both detection of disease and treatment protocols, the survival has not increased as expected. The survivors of OSCC also report a high grade of debilitating side-effects of the treatment. Prognosis and choice of treatment is mainly decided by the TNM-status. This classification does not consider the biological processes in the tumor microenvironment. It is thought that with the inclusion of a battery of histopathological markers, the prediction of prognosis and choice of treatment could further improve. This can cause both an increase in survival, as well as a decrease in unwanted side effects of treatment. In this thesis, CD57; an immunohistochemical marker of a subset of cytotoxic NK-cells is assessed as a possible prognostic marker for OSCC.

Method: This is a retrospective study of patients included in the Norwegian Oral Cancer Study (NOROC). A subset of 131 patients from the cohort were included. Inclusion criteria were tumor arising from the mobile part of the tongue, tumor tissue available for immunohistological staining and recipient of curative treatment. Tumor tissue samples were sent to the Pathology department at the University Hospital of Northern Norway in Tromsø for CD57 immunohistological staining. The slides were scanned and assessed digitally using the Olympus Olyiva data program. Assessing the marker was done using a hot-spot method, where areas of higher density of CD57 positive cells were scored. CD57 expression was divided in high and low expression groups. Kaplan-Meier analysis was performed to assess association between CD57 expression and disease specific 5-year survival.

Results: Patients with a higher density of CD57 positive cells had a higher 5-year survival rate than those with a low density, but the results were not statistically significant. While CD57 is defined as a specific marker for NK-cells, a subset of cells not compatible with NK-cell morphology, as well as background staining in some areas of tissue can have affected the CD57 scoring.

Conclusion: We could not validate CD57 as a reliable prognostic marker for OSCC. However, due to some unspecific immunohistochemical staining, the scoring method should be reassessed before a definite conclusion is drawn.

List of Abbreviations

- CD: cluster of differentiation
- DSS: disease specific survival
- ENT doctor: Ear, nose, and Throat doctor
- HE stains: Hematoxylin & Eosin stain
- HN can cancer: head and neck cancer
- ICD-10: International Classification of Diseases 10^{th} revision
- MHC class I and II: major histocompatibility complex class I and II
- N-CAM: numeral adhesion molecules
- NK-cell: Natural killer cell
- OSCC: Oral squamous cell carcinoma
- SCC: Squamous cell carcinoma
- TLR receptor: Toll-Like Receptor
- TNM-Status: Tumor, node, and metastasis status

1 Background

1.1 Oral Squamous cell carcinoma

1.1.1 Epidemiology

Oral squamous cell carcinoma (OSCC) is a subtype of head and neck cancer (1). Cancer in the head and neck region is a rather small group of cancer, in 2019 2,5 % of all new reported cancer were in the head and neck region(1). Of approximately 800 cases of cancer in this region in 2019, 240 of them were OSCC(1). This is caused by a difference in exposure to risk factors in an international setting. As figure 1 shows, the rate per 100 000 of cancer cases rise for both genders around the age of 50(2). The incidence is highest in the age group 40-60 in the population (1, 3, 4). As figure 2 shows, the rate per 100 000 is higher among men than women in Norway, but there has been an increase in the incidence among women(1). This is caused by gender norms contributing to the differences in exposure to the risk factors(1). Changes in what has been deemed culturally appropriate for the genders have decreased the difference in incidence(1). It is important to keep in mind that the prevalence of OSCC differs between countries and regions globally, due to a different exposure to risk factors. In India for example, cancer in the head and neck region comprise 25 % of all cancer cases annually(1, 3).

1.1.2 Risk factors

The two main risk factors for development of OSCC is smoking and intake of alcoholic beverages(1, 3, 4). It has been shown that if these two factors coexist, there will be a synergetic risk present, rather than an additive risk(4). These two factors are the most common in the western world. Other risk factors more prevalent in other regions include; other forms of tobacco and chewing of betel leaves, and intake of other oral carcinogens(1). Another form of smoking, “reverse smoking” where the lit part of the cigarette is inserted in the oral cavity is also more common in other parts of the world and correlates with a higher incidence of OSCC in the hard palate(1). HPV-virus infection was long thought to be another risk factor for OSCC. This was caused by the merging of OSCC with oropharyngeal cancer in research. By separating cancer cases in the oral cavity from cases in the oropharynx in research, it has been shown that the HPV-virus is relevant for oropharyngeal cancer, not OSCC(5).

1.1.3 Survival rates

Earlier discovery and diagnosis of OSCC, as well as improvement in treatment protocols have increased the 5-year survival for OSCC patients(1, 6). Figure 3 shows the 5- year survival for Norwegian patients in percentages. In the period 1980-1984, the 5-year survival of male patients was 43,1 %, and female patients 48,4 %, this has improved to 62,2 % for men and 64,4% for women in 2019(7). It is important to note that this is cumulative for all stages of OSCC, and the survival for more severe stages are much lower, in the NOROC cohort, median 5-year specific survival in total for all patients was 52 %, but divided by stage survival was 80 % for stage I and down to 33 % for stage IV(8). The methods used during diagnosis has not seen great changes during the last decades, and it is thought that with discovery of new tools for diagnosis and subclassifications, the treatment, survival and late effects after treatment could greatly improve.

1.1.4 Classification

The classification of OSCC is used to predict the severity of the case, and the choice of treatment. There are two systems in place for classification, the TNM- status and the grade of differentiation(1, 9).

1.1.4.1 TNM-status and stage

The TNM-status is a classification system used for all solid cancer (1, 9, 10). It consists of three factors, The T stands for primary tumor, and is a measure of the tumor size and invasion depth. The N stands for regional lymph nodes and denotes degree of cancer metastasis to lymph nodes in the region draining the cancer. M stands for distant metastasis and is either positive or negative, positive denotes presence of metastasis while negative denotes the lack thereof. The cutoffs for what sizes of tumors are defined as T1, T2 and T3 and what is considered N0, N1 and N2 nodal invasion is different for all cancers. The TNM for classification for OSCC specifically is shown in table 1 The TNM-status can be used to describe the stage of the cancer(1, 10). The stage division is shown in table 2. Stages are divided in 7 groups, 0-IVC(10). As seen in the table, nodal affection will give a more severe stage, while a patient with distant metastasis is automatically the most severe stage(10).

1.1.4.2 Level of differentiation

Level of differentiation is another way of classifying cancers(9). This classification assesses how well or poorly differentiated the cancer cells are, compared to the cells the cancer cells

are derived from. It normally assesses the cytologic differentiation, as well as the presence of mitosis in the tumor(9). A tumor will have a high grade of differentiation if the cancer cells have a high resemblance to their non-cancerous precursor cells. If they are moderately differentiated, they still hold some resemblance to the progenitor cells. A cancer with poorly differentiated cells has lost much of the resemblance to their progenitor cells. The level of differentiation is called *grading* to separate it from the TNM *staging*¸ and is usually divided into grade I-IV. A poor differentiation usually have a worse prognosis than a well differentiated tumor, but the TNM-status is a more important prognosticator(1).

1.1.4.3 Limitations of prognosis based on TNM-status and differentiation

The TNM status and level of differentiation can to some degree predict the prognosis of patients, but they give no insight into the biological processes causing the tumor development. Figure 4 shows two examples of outcome. In the first outcome, patient A and B are only diagnosed with a TNM-status which is identical, and they receive the same treatment (black arrow). Patient A is cured but experience late effects, but the cancer of patient B is not cured and the cancer develops further. In the second outcome, the TNM-status is paired with a marker showing that patient A have a more benign form of cancer (green color), while patient Bs cancer is more aggressive (orange color). Patient A receive less treatment, while Patient B receive increased treatment. The outcome is that Patient A gets less side-effects, while Patient B is also cured but with some side effects.

1.1.5 Clinical presentation

The clinical presentation of OSCC is usually either a chronic ulcer, erytroplakia or leukoplakia in the oral cavity(1, 3, 4). The cancer is usually discovered during clinical examination at the general practitioners office, at the dentist's office, or by an ENT specialist(11). If there is a clinical suspicion of OSCC, or it cannot be ruled out, the patient should be referred to the hospital by a cancer patient pathway(1, 11).

1.1.6 Histopathology

The normal tissue lining the oral cavity consists of squamous stratified epithelium(12). It is the cells in this epithelial lining that is the origin of OSCC. Figure 5 shows the most common localizations(1). The most common location is in the mobile part of the tongue (the anterior 2/3rds)(1). Other locations include; gum, floor of mouth, hard palate, buccal mucosa,

vestibule of mouth and retromolar area.(1, 8). OSCC make up 95 % of oral cancer cases, the last 5 % arises in the salivary glands and are adenocarcinomas, melanomas arising from mucosal melanocytes, sarcomas and lymphomas, these will not be discussed further in this thesis (1, 3). The development from normal histological tissue to cancer goes through several stages. Keratinocytes in the squamous epithelium are the cells of origin(6). It is thought that the malignant transformation is started when a chronic exposure to the risk factors will break the cells homeostasis(6). This will lead to a gradual change in the epithelium, starting with hyperplasia, developing to increasing grades of dysplasia until carcinoma in situ, which is the most severe grade of dysplasia. The transformation to cancerous tissue is complete when the cells break the basal membrane and invades the underlying tissue. Assessing a histological slide, the tumor and surrounding tissue is usually present. It is important to separate the cancer tissue from dysplastic epithelium that can usually be found on either side of the tumor. In research, it is often of importance to assess the invasive front, the place of the tumor that is actively invading the underlying tissue. Image 1 shows a typical tissue slide of OSCC, with a tumor and its invasive front, normal tissue and both normal and dysplastic epithelium.

1.1.7 Treatment

There can be two different intentions in the treatment of OSCC, curative and palliative. In this thesis we will only describe treatment with a curative aim, as this is one of the inclusion criteria in the study. The main treatment for patients with OSCC will be a surgical resection of the tumor(1, 3, 4, 11). Another form of treatment, that is often used as adjuvant to the surgical removal is radiation therapy. Radiation after surgery is considered if the tumor invades deeper than 3 mm, if it is impossible to get a clean resection, or the tumor has aggressive characteristics(11). Radiation therapy can also be considered as a curative monotherapy, if surgery is contraindicated(1). Chemotherapy can also play a part in the treatment of OSCC, but is less common(1). Treatment off OSCC in Norway is only performed at the ENT departments at the four regional hospitals in Norway(1). After treatment there is a routine follow up ever 2^{nd} month for the first year, every 3^{rd} month until the second year, and every 4^{th}-6^{th} month from the 2^{nd} year to the 5^{th} year(11). After the 5^{th} year patients will under normal circumstances be considered cured, and the routine follow up appointments will end unless there is individual factors indicating the need for continuation(11).

1.1.7.1 Unwanted effects of the treatment of oral squamous cell carcinoma

The head and neck area harbors many both important, and delicate anatomical structures of high importance for the daily function of a patient. Both surgery and radiation therapy can leave the patient whit several debilitating lasting side-effects(1, 3, 11). Surgery where the tumor and surrounding tissue must be removed, can severely impair function of the oral cavity(11). Difficulties with talking, mastication and swallowing are prevalent(11). Radiation therapy give rise to separate effects, the acute effects that present during treatment, and chronic effects that present months after treatment(11). The acute effects are soreness and dryness of the oral cavity and mucous saliva. Infections and inflammation can also present as acute effects(11). Late effects is long term destruction of tissue, such as fibrosis, and degeneration of the salivary glands (11). The degeneration of salivary glands leads to mouth dryness, an especially bothersome side-effect for patients. Prognostic markers can become an important tool to avoid this side effect, by better deciphering which patients that can be spared radiation after surgery and still be cured of their cancer. A rare, but severe late effect is osteoradionecrosis, the necrosis of bone tissue, usually affecting the mandibula(11). This is important to keep in mind during assessment of treatment protocols. Even if a patient is cured of their OSCC, there is a high change that the treatment has left them with lifelong debilitations that will severely affect their day to day life.

1.1.8 Limitations in research on OSCC

OSCC as a research subjects have long been at a standstill because of the quality of the research. Many papers have small cohorts of patients, with a high diversity in both cancer stage and localization(13). Results from such research is almost always inconclusive. This has caused a loop where the same possible markers will get reassessed time and time again, because the projects that have already been done give no certain answers(13). The hope with a larger cohort where it is possible to assess markers on more homogenous subsets of patients is that it is possible to say with more certainly if a cellular marker can be used prognostically, and if not, do so with enough certainty that it will not keep on being reassessed(13, 14).

1.2 Prognostic markers

A prognostic marker is any clinical measure that can describe a patients risk of a future outcome(15). The markers can be almost anything, for measurements such as size of tumor, level of protein or genetic mutations present in the DNA(15). In this thesis, the focus will be

on cellular markers in histological tissue, and the future outcome of interest is survival time after diagnosis with OSCC. In the case of OSCC, immunohistochemical markers have been a focus point.

1.3 Immunohistochemical staining

Immunohistochemical staining is a special form of histological staining identifying specific structures in tissue samples that would otherwise be hard to differentiate by a normal Hematoxylin & Eosin (HE) stain. It is based on the principles of antigen antibody recognition in the immune response (12). The target protein is defined as an antigen, it can be any cellular structure, usually a protein in for example in the nucleus, the cytoplasm or on the cell membrane of a cell. This will be the structure that we want to visualize(12). This is done by two antibodies, a primary antibody that bind to the antigens specific epitope, and a secondary antibody that can bind to the primary antibody(12, 16). The antibodies are made by two processes. First the antigen is injected into another species (common animals are mouse, goat, horse etc.) to make the primary antibody (16). The immune system of this animal will recognize the antigen of human origin as a foreign body and make antibodies to mark it for destruction. These antibodies are then isolated from the animal, and can afterwards be produced in the lab using monoclonal technology(16). To make the secondary antibody, the primary antibody is injected in yet another species, so that the same process repeats, making an antibody to mark the primary antibody. Again, this secondary antibody is isolated and mass produced using monoclonal technology (12, 16). The secondary antibody is modified with a form substance that can be visualized(12). This can either be fluorescein, gold, ferritin or the enzyme peroxidase(12). In this thesis peroxidase immunohistochemistry was used, the characteristic brown staining can be seen in images 1-3.

1.4 NK-cells

NK-cells are part of the innate immune system. Their name, Natural Killer cells, is derived from their ability to kill certain target cells(17, 18). NK-cells can be activated both as a part of the innate and the adaptive immune system(18, 19). Their main function is to be a part of the immediate immune response against infectious agents in the tissue, especially intracellular pathogens(12). A second function, that will not be further discussed here, is their ability to increase the state of inflammation, thus also contributing to the defense against extracellular pathogens(19) The NK-cell arise from the common lymphoid precursor, that also is the origin

of T- and B-lymphocytes(16, 17, 19). They make up approximately 5-10 % of the circulating lymphocytes in the body(16, 17). Their cytotoxic effects are the equivalent of CD8+ T-lymphocytes in the adaptive immune system.(17, 19). NK-cells cytotoxic function is not turned on and off by a singular receptor during the innate response(19). Over 30 receptors are known, and any NK-cell will have a subset of these. These 30 receptors have either an activating effect or an inhibitory effect, and the sum of signals from these decide if the NK-cell activates or not(19). Inhibitory NK-cell receptors tend to recognize MHC class I molecules, while receptors that activate the NK-cell tends to recognize other proteins(19). During an adaptive immune response, when IgG is present, the CD16 receptor alone will activate the cell (19).

NK cells release perforins and granzymes that create channels in the plasma membrane of the target cell, causing fragmentation of the DNA and cell death by apoptosis, or lysis(17). This cannot be done from afar, the NK-cell is dependent on close contact with the target cell(19). This also means that they can only kill one cell at a time(19). The perforins and granzymes present in the cell in granules that are quickly transported to the cell membrane surface by microtubule tracks(19). The surface of the granules fuse with the cell membrane surface, releasing the cytotoxic molecules in a synapse, named the NK-cell synapse(19). The target cell then undergoes apoptosis, a form of programmed cell death(19). The dead target cell is removed by macrophages(19). NK-cells express 3 Toll-like receptors, TLR3, TLR7 and TLR8(19). TLR3 recognizes viral RNA that is double stranded, while TLR7 and TLR8 recognize RNA that is single stranded(19). When the Toll-like receptors (TLR)bind RNA, they activate an intracellular pathway, causing an increase in the secretion of interferon type I, that in turn increases the cytotoxicity of NK-cells(19).

1.4.1 Role in cancer regulation

One of the Hallmarks of cancer is evasion of immune destruction(20). One way cancer cells do this is downregulation of MHC class I, evading the destruction by cytotoxic T-cells(19). However, as noted in the NK-cells general part of this paper, the receptors on NK-cells that recognize MHC class I Inhibit destruction, meaning that by not presenting MHC I to the NK-cell, the cancer cells are at higher risk to be detected. Int his way the NK-cell and the cytotoxic T-cell have complementary functions. Because of this, NK-cells are of interest in

cancer research, as their presence in tumor tissue could indicate that they are attacking the cancer cells.

1.4.2 Morphology and identifying immunohistochemical markers

In a normal HE stained tissue slide; NK-cells will be identical in morphology to other subsets of lymphocytes(12). They are typically tiny cells, with a diameter of approximately 7 µm(12). The nucleus is dark and compact and is surrounded by only a thin rim of eosinophilic cytoplasm. This makes the cells resemble tiny black dots in the tissue as seen in image 3(12).

NK-cells can be identified by several immunohistochemical markers. These markers are 3 that stain the NK-cells, and 3 that they are never stained by. These markers are: $CD3^-$, $CD4^-$ $CD8^-$ $CD56^+$, $CD16^\pm$ and $CD94^+$(17, 21). $CD3^-$,$CD4^-$and $CD8^-$ is used to separate them from T-cells, as these CD-markers are mainly expressed by them and interacts with either the T-cell receptor (TCR) on the T-cell surface, or MCH class I or II in target cells(17). CD16 is deemed a clinical marker of NK-cells, and function as a receptor for IgG, mediating the antibody-dependent cytotoxicity of the cell(17). CD56 is also deemed a clinical marker of NK-cells, and its function is isoforms of numeral adhesion molecules (N-CAM)(17).

1.4.3 CD57

The CD57 antigen has several other names, such as HNK-1, LEU-7 and L2, only CD57 will be used from this moment forward. The CD57 marker is defined as a marker of a subset of NK-cells(22). This subset of NK-cells is identified by their loss of proliferation and increase in cytotoxic potential(22). Moreover, as well as NK-cells, the marker also stains several neural structures, of importance in tongue tissue is the myelinated Schwann cells of motor neurons, implicating that some nerves will also be stained in the tissue(22). Comparing this marker with the classic NK-cell marker discussed earlier, cytotoxic NK-cells with a $CD16^{pos}CD56^{dim}$ or inflammatory NK-cells with a $CD16^{pos}CD56^{neg}$ expression is seen to also have expression of CD57, while regulatory NK-cells with $CD16^{neg}CD56^{bright}$ expression do not(22-24).

1.5 CD57 and OSCC

Several papers have already explored C57 as a possible prognostic marker for OSCC(25-27). As mentioned in the last part, CD57 is deemed a marker of a subset of NK-cells with a poor proliferative capacity, but an increased cytotoxic function(21). It is thought that it is favorable

to have these cells present in the tumor tissue. Studies with a mice model depleted for NK-cells have shown a higher incidence of cancer(18, 28).

1.6 Aim

The aim of the thesis is to assess if the number of CD57 positive NK-cells in tumor tissue from OSCC patients is associated to their survival, and thus if the marker has prognostic potential for OSCC.

2 Materials and Method

2.1 The Norwegian Oral Cancer Study

The Norwegian oral cancer study (NOROC) is a retrospective national multi-center study of oral cancer started in 2016. The study was approved by the Northern Norwegian Regional Committee for Medical Research Ethics (Protocol numbers REK Nord; 2013/1786 and 2015/1381). And is partially financed by Helse Nord.

2.1.1 Aim

The aim of the NOROC study is to optimize the treatment of patients with oral cancer.(14, 29). The research projects in this study focus on the discovery and verification of potential prognostic markers, that can be used to individualize the treatment for OSCC (14, 29). The possibility to better differentiate which patients will need radiation therapy after surgery is one of the main objectives(14). It is thought that by improving the decision of this treatment survival can be improved, and the side-effects that OSCC patients suffer after radiation therapy might decrease (14, 29, 30). Since OSCC is a subtype of head and neck cancer with a low incidence, another goal of this study is to collect a large cohort so that systematic and strong studies can be done on OSCC, giving more solid research results than those often seen in the OSCC research field (14, 29, 30). Four research centers were involved in the inclusion of patients and research: Haukeland University hospital in Bergen, St. Olavs university hospital in Trondheim, The university hospital of Oslo, and the university hospital of Northern Norway in Tromsø.

2.1.2 Cohort

The cohort collected for the NOROC study consists of all patient that were diagnosed with OSCC from 1st of January in 2005 to 31st of December 2009 at the four university hospitals in Norway. This gave a five-year period of inclusion(8). A total of 535 patients were

included(14). Inclusion criteria in the cohort were: first time head and neck (HN) cancer relevant International Classification of Diseases (ICD-10) codes: C02-C06k, Exclusion criteria were: codes C05.1 and C05.2, histopathology of tumor sample not corresponding to Squamous cell carcinoma (SCC), HN cancer second primaries (second time with primary cancer, not a relapse).5-year follow up was ensured for all patients, with an end date for follow up in 2015(8). A total of 535 patients were included(8). Before inclusion clinical information from the hospital journals as well as tumor tissue was reassessed to ensure that all patients were in fact OSCC patients(8). The tumors were reassessed with the updated TNM-status(10). 27 % of patients first thought to be OSCC patients were excluded on the basis of misdiagnosis with OSCC, upon reassessment it turned out they were in fact cases of oropharyngeal cancer(8).

2.1.3 Inclusion of patients from the NOROC cohort in this thesis

A subset of all the 535 patients included in the NOROC cohort was chosen for this project. In total 131 patients, from Bergen, Tromsø and Oslo were included in the preliminary planning of the project. Inclusion criteria was cancer located at the mobile part of the tongue and treatment with curative intent (patients only receiving palliative treatment were excluded). Of the 131 patients that were included 111 could be used. Of the 20 that could not be included in the analysis 17 were missing due to no available tumor tissue for staining and 3 had available tissue for staining, but no tumor and/or CD57 staining in the tissue slide upon evaluation. This is an expected loss of patients in a project like this.

2.2 preparation of the tissue samples

For these patients, archival paraffin embedded tissue samples from the time of diagnosis were collected and staining for CD57 was done in the automated slide stainer Ventana Benchmark, XT (Ventana, Tucson, AZ, USA) at the Diagnostic Clinic—Clinical Pathology, University Hospital of North Norway, accredited according to the ISO/IEC 15189 standard for the respective staining. It was decided to do this at the department because the cellular marker in question, CD57, is a routine marker that is stained for there, and the results would be more concise than a manual staining done in a research lab. When the samples were stained, they were all scanned by a bioengineer, and uploaded to the Olympus olyiva data program for assessment.

2.3 Hot-spot scoring

The hot spot method is a type of histological scoring often used in assessment of potential prognosic histologic markers in cancer. “Hot-spots” are defined as areas in a tissue with a higher density of the marker in question. A preset number of hot-spots are chosen per patient, in this case 5 pictures of hot-sports were scored per patient. There were some rules on where the hot spots could be in relation to the tumor. As the focus is NK-cells interaction with the tumor cells, and they need to be close to their target cells to execute their function, it was decided to only score hot-spots that were intra- or peritumoral. The limit was set as one power field at the 20x magnification away from tumor cells. An example of a hot-spot can be seen in image 2. 20x was chosen because this is the largest preset magnification in the Olyvia program. The next thing was what cells were to be counted. CD57 should be a specific immunohistochemical marker of NK-cells, but as image 3 shows, it could sometimes leave a unspecific stain in the tumor cells, and also stains nerves with motor neurons. Because of this, it was decided that all single cell structures stained were to be included in the count, denoted CD57 positive cells, while obvious deviations such as background staining of tumor cells and motor neurons in nerves were excluded. Lymphocytes have a specific appearance, as show in image 2 circled in green, it was decided to disregard this and count all single cells that were stained, regardless of lymphoid looking morphology or not. Small speckles of staining were also counted, as this can be cells cut in the periphery of their body during preparation of the slide. When all pictures were taken, the cells were counted. Seeing as some hot-spots can have up to several hundreds of positive cells, with a high density and difficult to get an exact number, it was decided to stop the counting in a hot-spot at 100 cells. After counting cells in all the 5 pictures per patient, the mean $CD57^+$ cell density for the 5 hot-spots was calculated for all tumors. As the cutoff for counting was set at 100 cells per picture, the highest possible mean score was 100.

2.4 Statistical analysis

Statistical analysis was performed using SPSS software version 26.0 for windows (IBM, Armonk, NY). Descriptive frequency analysis was performed to describe the clinopathological variables. Disease specific survival (DSS) was analyzed using univariate Kaplan-Meier analysis. The mean $CD57^+$ density per tumor was used to calculate new variables. The 25, 50 and 75 percentiles for mean density were calculated and tested as cutoff

for dividing the tumors into CD57 low and CD57 high. The 25-, 50- and 75- percentile cutoffs were all tested with Kaplan-Meier method. The P-value was evaluated, and Kaplan-Meier plots with survival based on 5-year DSS were plotted. P-values had to be <0,05 to be considered statistically significant.

3 Results

Clinicopathological characteristics of the 131 patients are shown in table 3. Of patients in the cohort, 61 % were men. The largest age group was 61-70-year-old at 30 %. At the time of diagnosis, 40 % were actively smoking, while 24 % were former smokers. Moderately differentiated tumors were the most common at 71 % of the tumors. By TNM-status, T2 was the most common at 40 %, while most patients did not have nodal affection (N0-status). Divided by stage, most patient had stage I or II, only 2 % of the patients were in the stage III group.

The 5-year DSS was 62% for men and 68% for women. By age, the 5-year DSS decreased with higher age, 83 % of patients 50 years or younger were alive after 5 years, while only 28 % of patients over 80 years after 5 years. Looking at the smoking status of the patients, the 5-year DSS was approximately the same, ranging around 60-70%. The 5-year DSS decreased with increasing T-stage at diagnosis. Invasion of lymph nodes gave a decrease in 5-year DSS, this difference was statistically significant. There is a decrease in 5-year DSS with higher stages. For stage I the 5-year DSS was at 93 %, but for stage III it was down to 48 %.

CD57 density was divided in high and low density by three different cut-offs, the 25, 50 and 75 percentiles. The frequency for high and low expression of CD57 with the all the percentiles as cut-off is shown in table 1. At the 25-percentile, 11 % of patients had low density of CD57, while 88 % had a high density. Five-year DSS is also shown in table 1. Survival was lower for patients with a low expression compared to those with a high expression. 45 % of patients with low expression was still alive 5-years after diagnosis, the same number for patients with a high density was 68,2%.

Figure 8, 9 and 10 show the Kaplan-Meier plots for cut-off of high and low density of CD57 set at the 25-, 50- and 75-percentiles. As shown, the cutoff with the largest difference in survival was the 25-percentile. As seen in table 3, all percentiles were tested for statistical

significance. None of them were statistically significant, the 25-percentile was the closest with a P-value at 0,095.

4 Discussion

The cohort used in this thesis have composition in accordance with the epidemiology of OSCC. There is a higher incidence of males, and most of the patients are in the older age groups. Smoking is one of the main risk factors, so the fact that most patients are either active or former smokers is in accordance with former knowledge. It is still important to note the 26 % of the patients had never smoked, showing that smoking is just one of the risk factors for OSCC. As shown in the results it is expected that patients with a larger tumor, nodal invasion and later stages have a lower DSS, as these are markers of worse prognosis.

The difference in survival between the CD57+ high- and low density groups were not statistically significant, but there was a difference. For the 25-percentile cut off there is an increased survival for patients with a high density of CD57+ cells. This is in accordance with what is expected based on the function of CD57+ NK-cells in the tumor microenvironment. They have an increased cytotoxic function compared to other NK-cells, and are inclined to attack cancer cells that try to evade immune responses by downregulating MCH class I.

CD57 is a defined in literature as a specific marker of a subset of terminally differentiated cytotoxic NK-cells. Observations in this thesis has shown that there were some discrepancies to this statement. NK-cells are part of the lymphoid leukocytes and should be small round cells consisting largely of a dark round nucleus and a thin rim of cytoplasm surrounding it. It is expected that cells fitting this description should be the ones that are stained with the CD57 marker in the tumors. Instead, the cells that had the strongest staining were larger cells with a lighter nucleus and larger and more irregular area of cytoplasm as circled in red in image 2. The results we expected was that structures like the one circled in blue should be present. This was presented to a pathologist at the department of pathology at UNN, she theorized that the larger cells might be macrophages. There have been no apparent reports on CD57 staining of macrophages published. It is uncertain if these large cells should be counted or not.

4.1 Limitations

When choosing the method of scoring, there will always be limitations and choices that can affect the results. One of the things that can have affected the result is the cut-off of 100 cells per power field. The results might be different if the cut-off is set higher, or the meticulous work of counting ALL cells in the power fields with high density is performed. Another weakness is the validation of scoring. In this project only one person has scored the cells alone, normally with semiquantitative scoring of immunohistochemical staining there should be testing for intra- and interobserver variations. This is to assess the reproducibility of the scoring.

Immunohistological staining is said to be specific, but there are almost always some other structures in the tissue that also get stained. In the case of the CD57 staining the discussion is if the large cells that no not resemble lymphocytes should be included in the scoring, and if not, how should they be identified and disregarded from the scoring?

One more thing is the amount of tumor tissue assessed, in this thesis only on histological slide per patient was assessed. This is only a small portion of a whole tumor, and the slide chosen might not represent the presence of CD57 cells in the whole tumor.

The CD57 marker was only analyzed with univariate analysis, not multivariate. Because of this the results are not controlled for confounding factors.

5 Conclusion

Based on the results in this thesis, CD57 cannot be considered a prognostic marker for OSCC. The results show that there is a difference in survival between patients with a high or low density of CD57 positive cells when the cutoff between high and low is set at the 25-percentile for the cases. Still, the difference is not statistically significant. Because of the uncertainty in the inclusion criteria of which cells should be counted and the lack of multivariate analysis to assess if the marker has prognostic value in a subset of the cohort, CD57 cannot fully be disregarded as a prognostic marker. The method of scoring and assessment should be further revised and tested on a larger part of the NOROC cohort with both intra- and interobserver variations documented, and both uni- and multivariate analysis should be performed.

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6 Figures, Images and Tables

Figure 1: Rate per 100 000 of OSCC in age groups among male and female patients, in the time period 2015-2019(2).

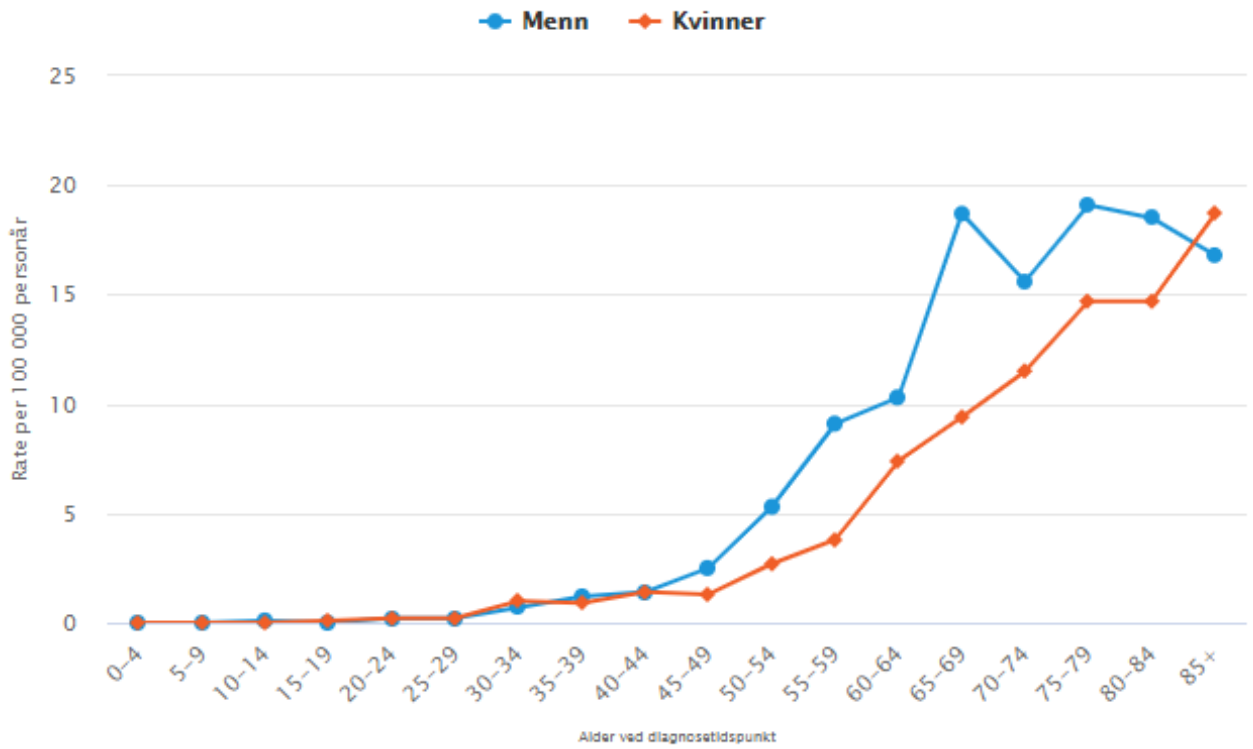


Figure 2: Rate per 100 000 of OSCC among male and female patients in the time period 1960-2019 (2).

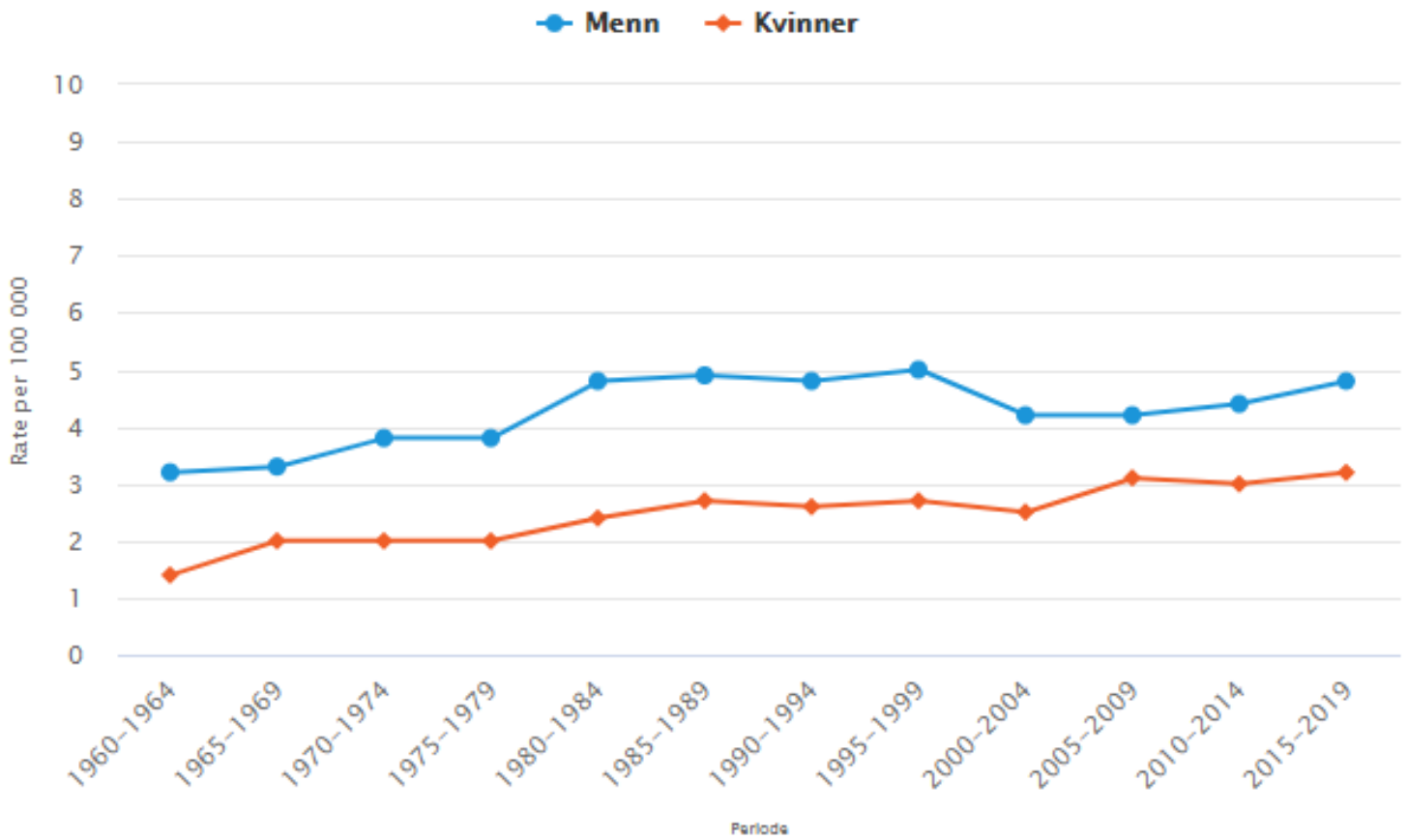


Figure 3: 5-year survival for male and female patients with OSCC in Norway in the period 1980 to 2019, shown in percentages. Survival has increased some for both genders in the time period from 43,1 % to 62,2 % for men and 48,4% to 64,4% for women(7).

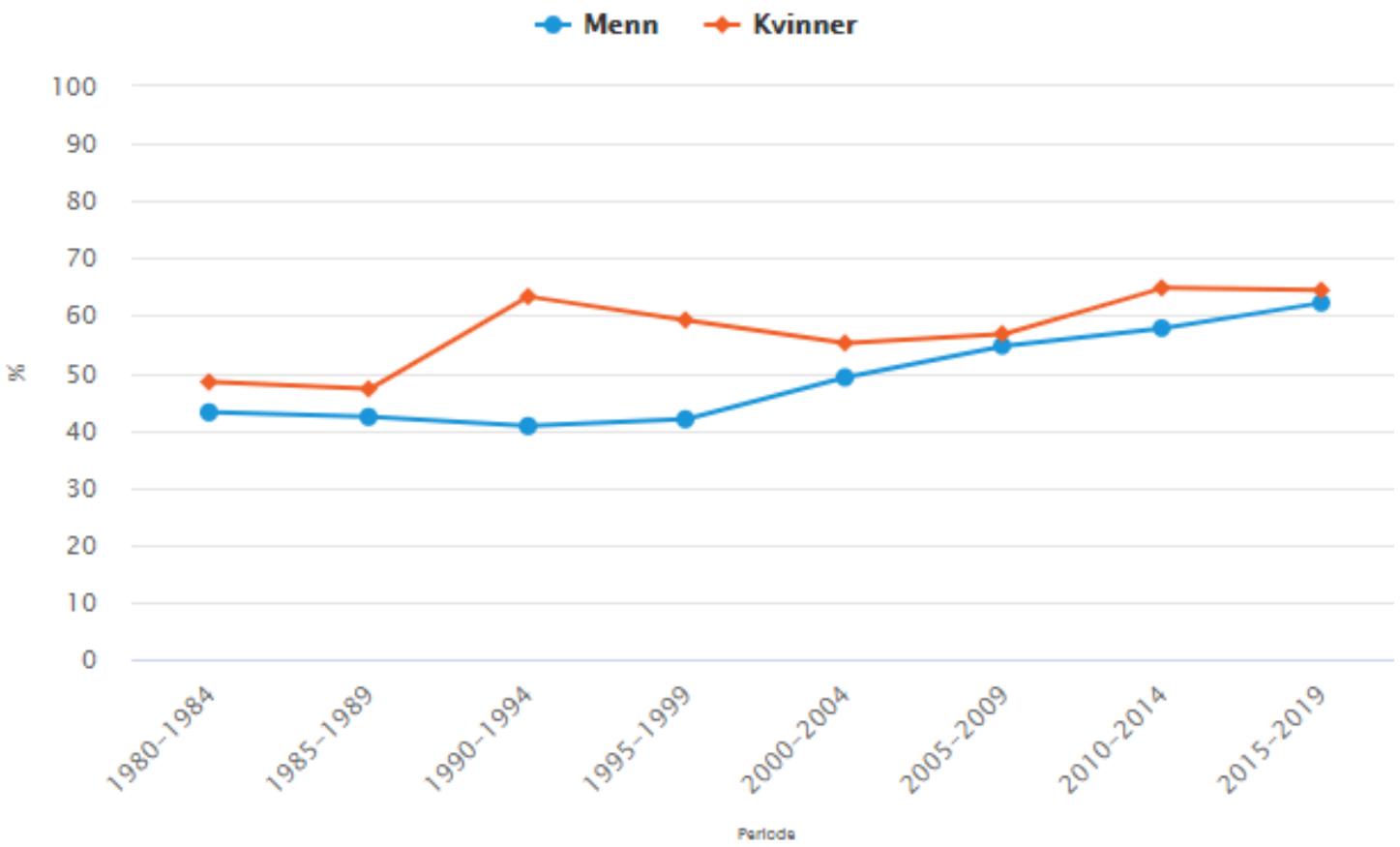


Figure 4: Patient A and B and different outcomes based on two examples: only consideration by TNM-status and TNM-status but with markers than indicate prognosis of the tumors. In the first example both patients get the same score only based on TNM-status (white circles) and receive the same treatment (black arrow), the consequence is curation but with side effects for patient A (red marks) and cancer progression for patient B. in the second example patient A and B receive diagnosis with both TNM-status and cellular markers (difference in these indicated by different colors). The outcome in this instance in a difference in treatment protocols used (indicated by difference in arrow size), the outcome is curation for both, but with some side effects. Patient A get less side effects than with the treatment in the first example because of a decrease in treatment.

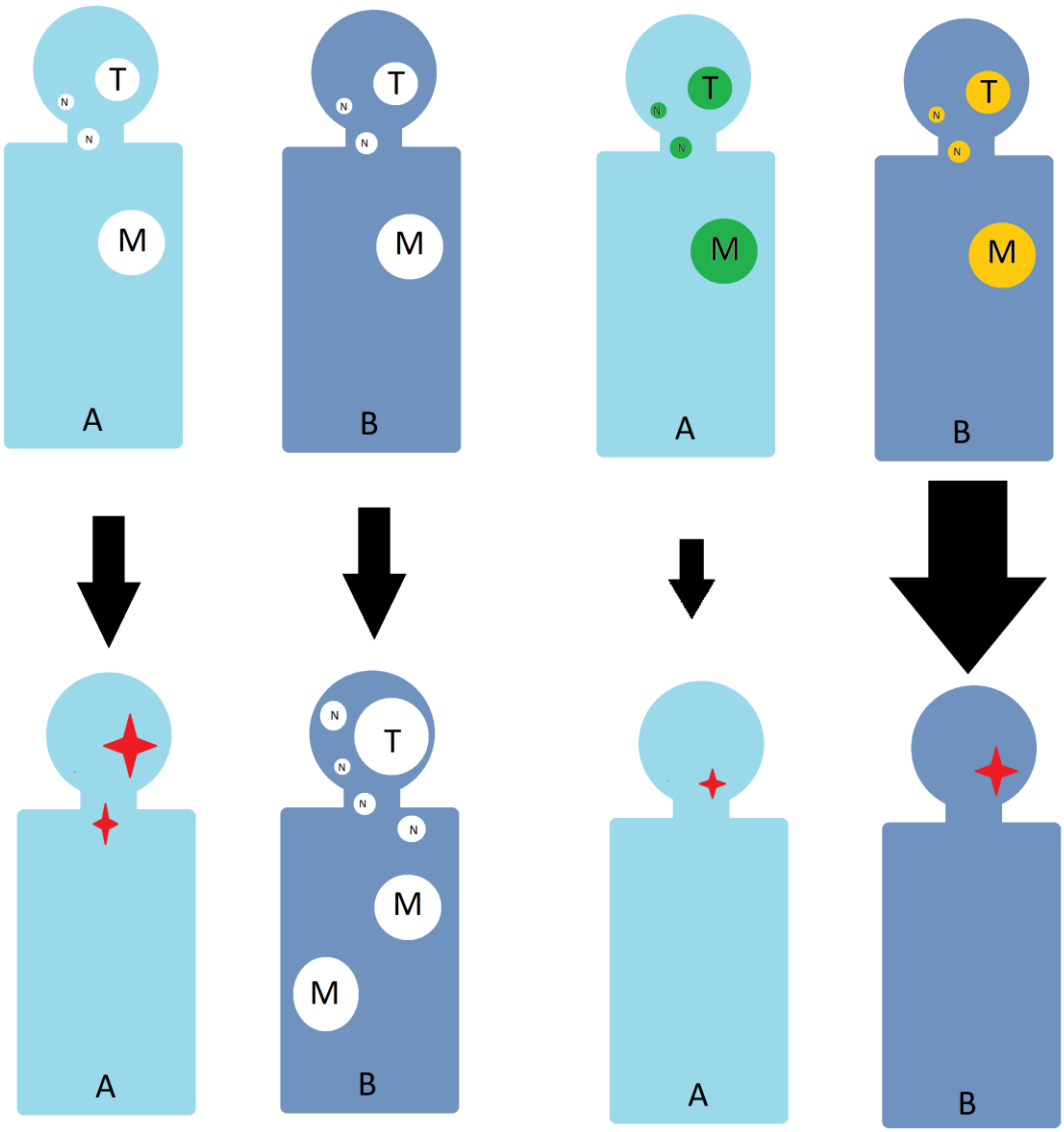
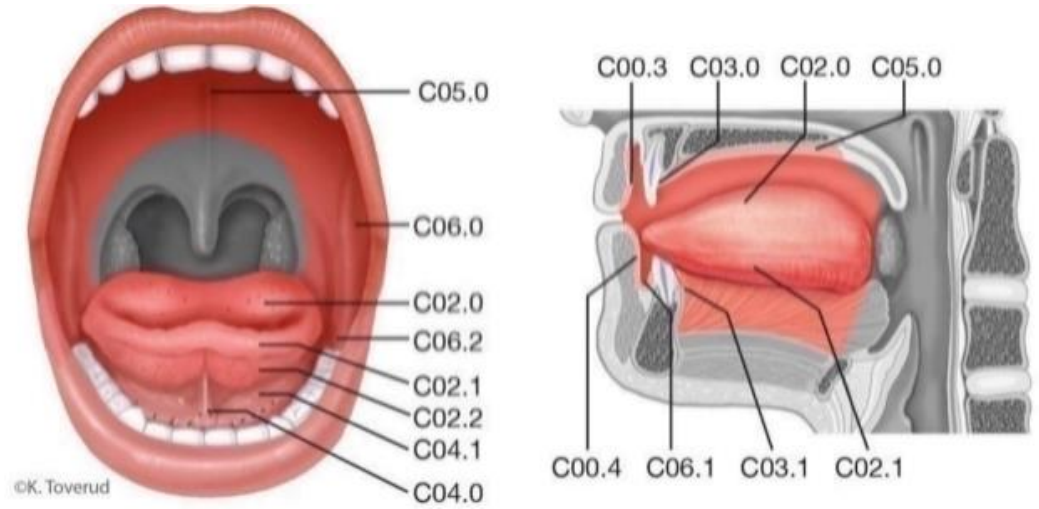


Figure 5: The most common localizations of OSCC(1).



Code	*Localization*
C02.0-02.9	Mobile part of tongue (anterior 2/3rds)
C03.0	Upper gum
C04	Floor of mouth
C05.0	Hard palate
C06.0	Buccal mucosa
C06.1	Vestibule of mouth
C06.2	Retromolar area

Figure 6: Example of how the Antigen on a cell surface is marked by a primary antibody, that is marked by a secondary antibody connected to a peroxidase that gives the characteristic brown staining. Based on illustrations in several references(12, 16, 17).

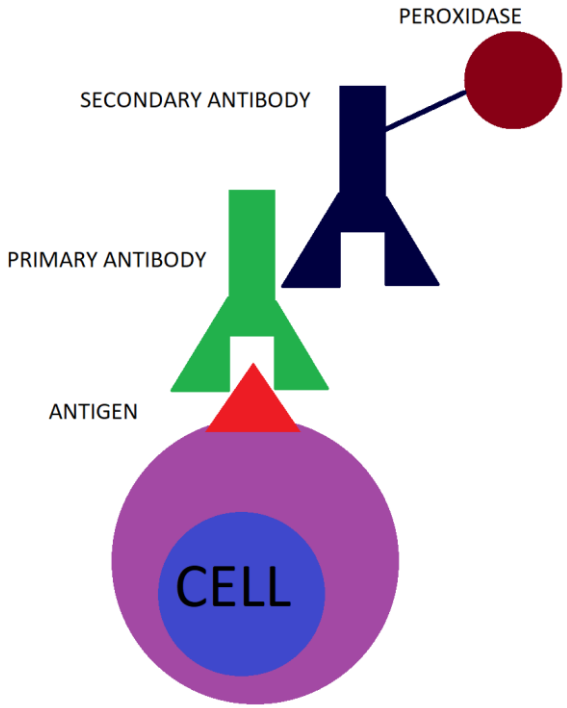


Figure 7: Box plot of the CD57 mean in the sample. The 25-, 50- and 75-percentiles at 13, 31 and 60 were tested as cut-offs for the high and low expression of CD57 in the sample.

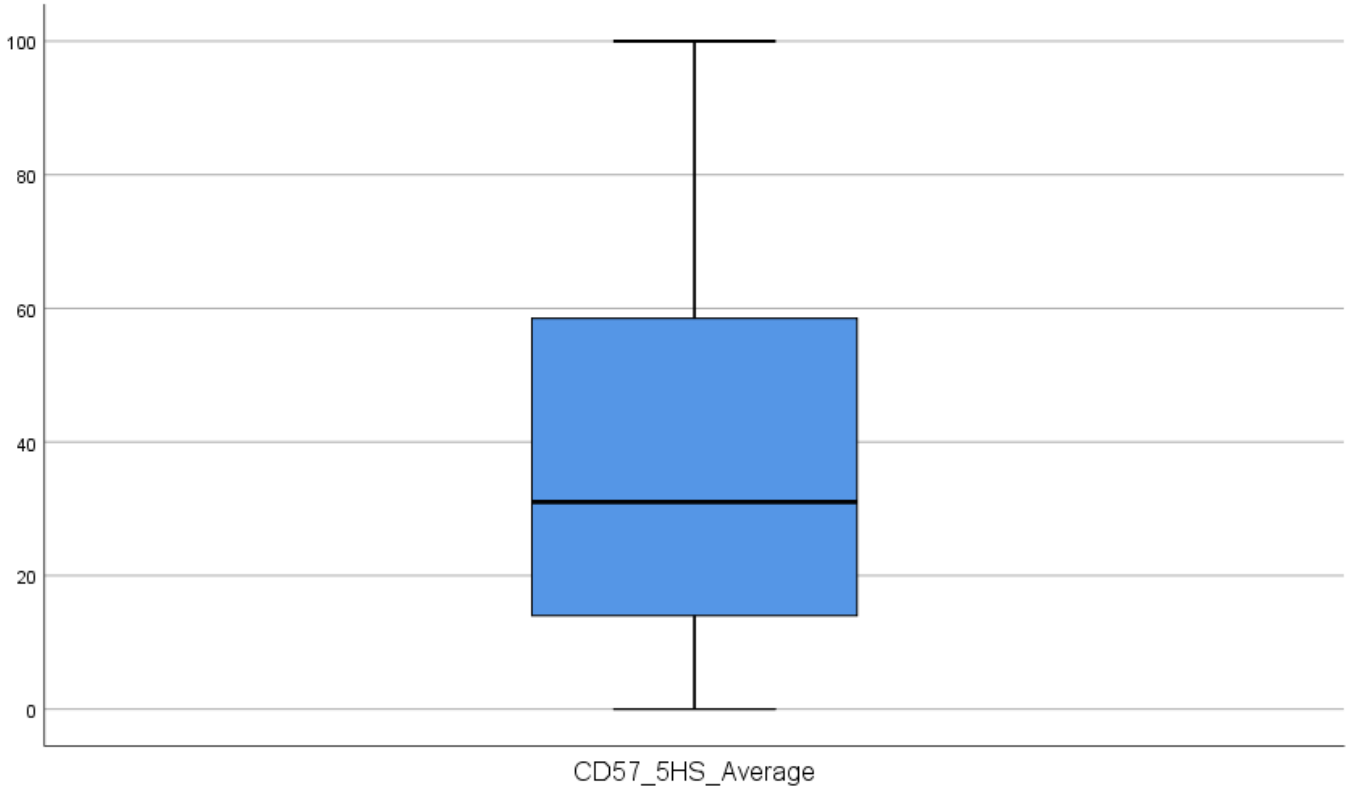


Figure 8: Kaplan-Meier graph for the survival rates of patients based on the expression of CD57 with a cut-off between high and low expression set at the 25-percentile.

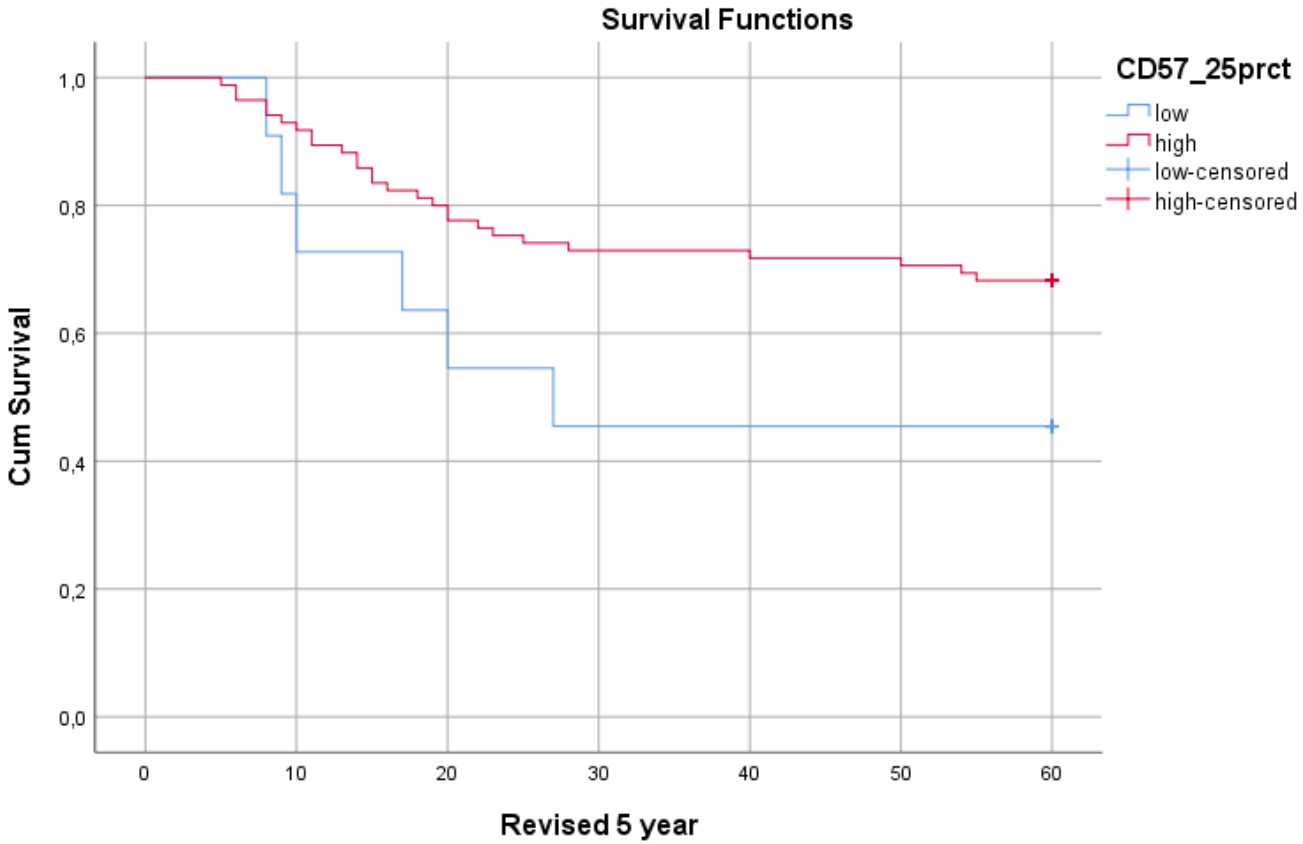


Figure 9: Kaplan-Meier graph for the survival rates of patients based on the expression of CD57 with a cut-off between high and low expression set at the 50-perncetile

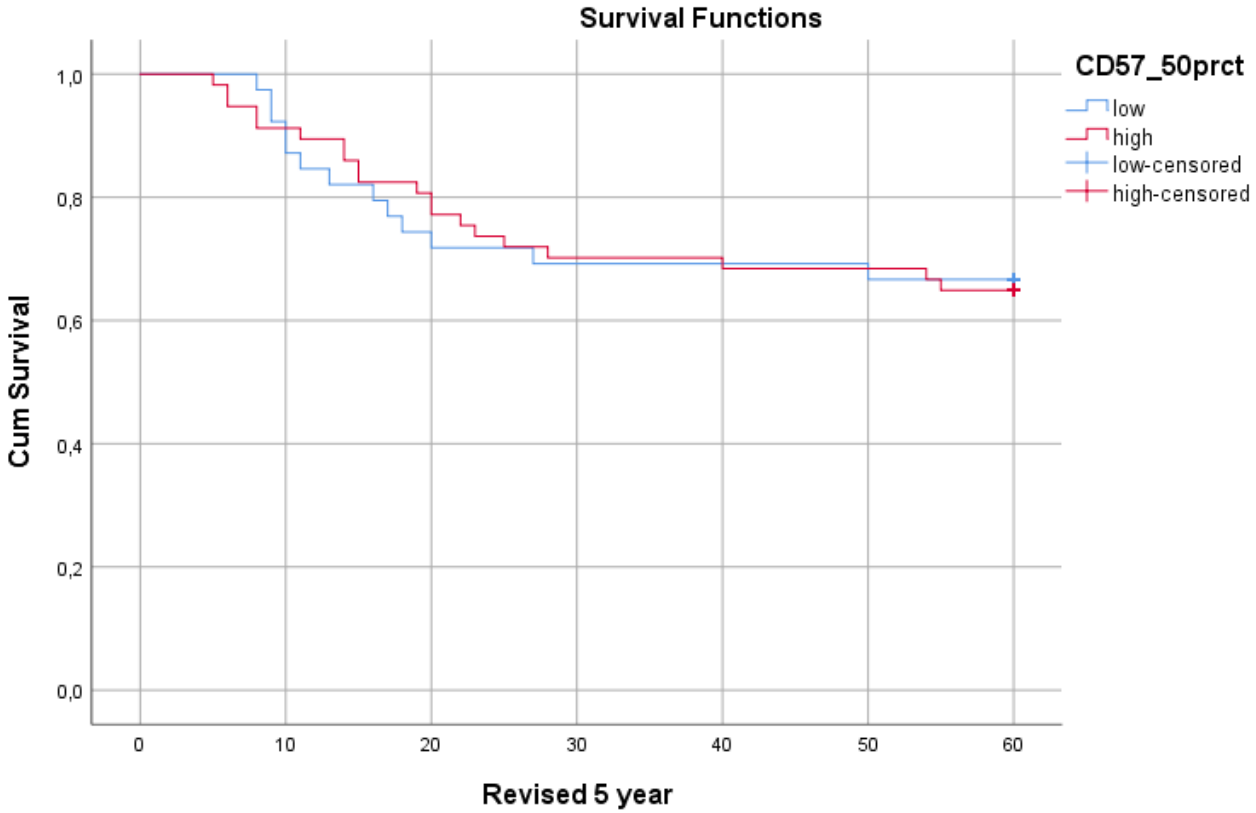


Figure 10: Kaplan-Meier graph for the survival rates of patients based on the expression of CD57 with a cut off between high and low expression set at the 75-percentile

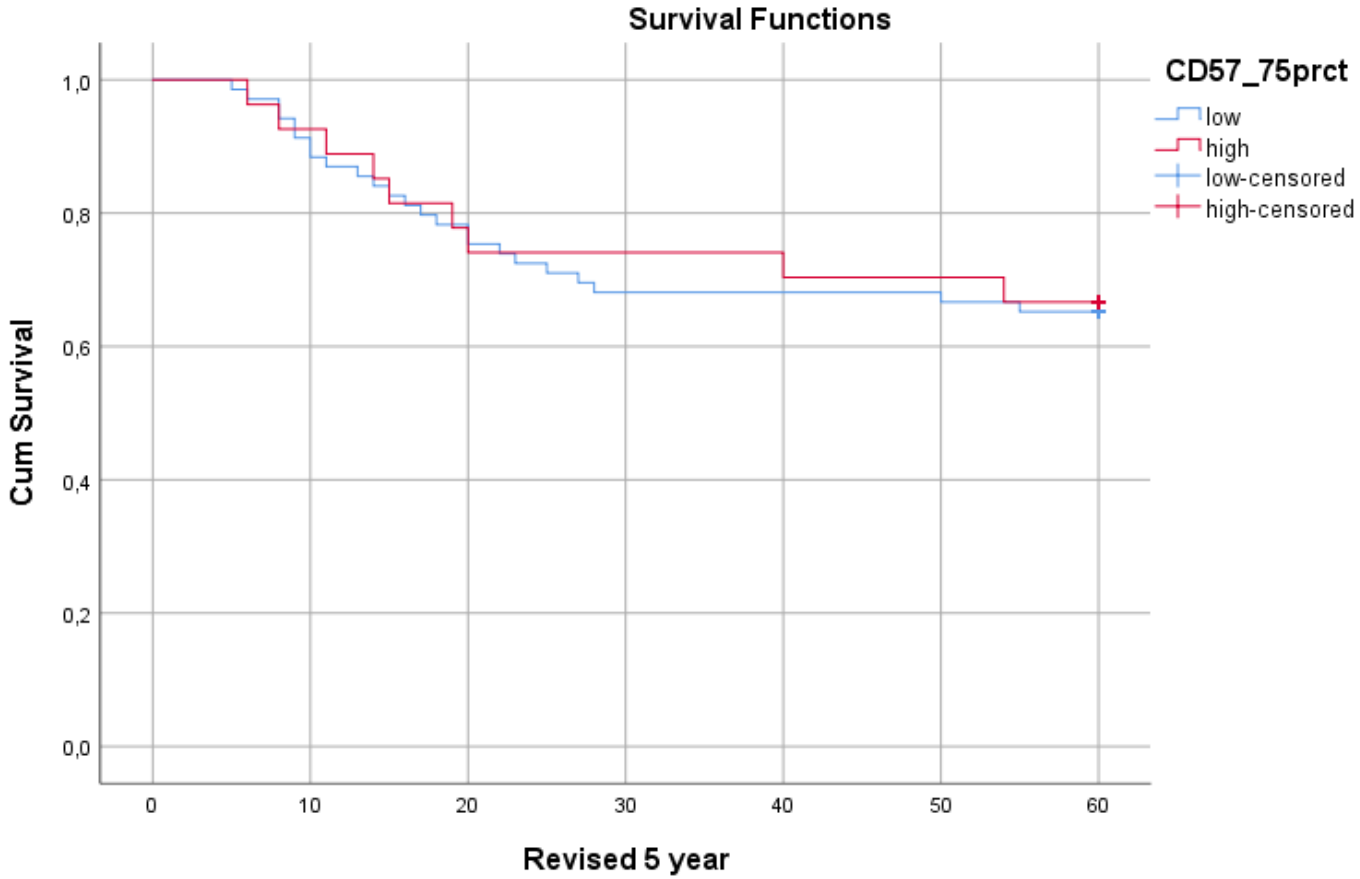


Image 1: A typical histological slide with an OSCC tumor. In the middle is the tumor, with an invasive front. Normal tongue tissue is surrounding the tumor. On the surface both normal and dysplastic epithelium is shown.

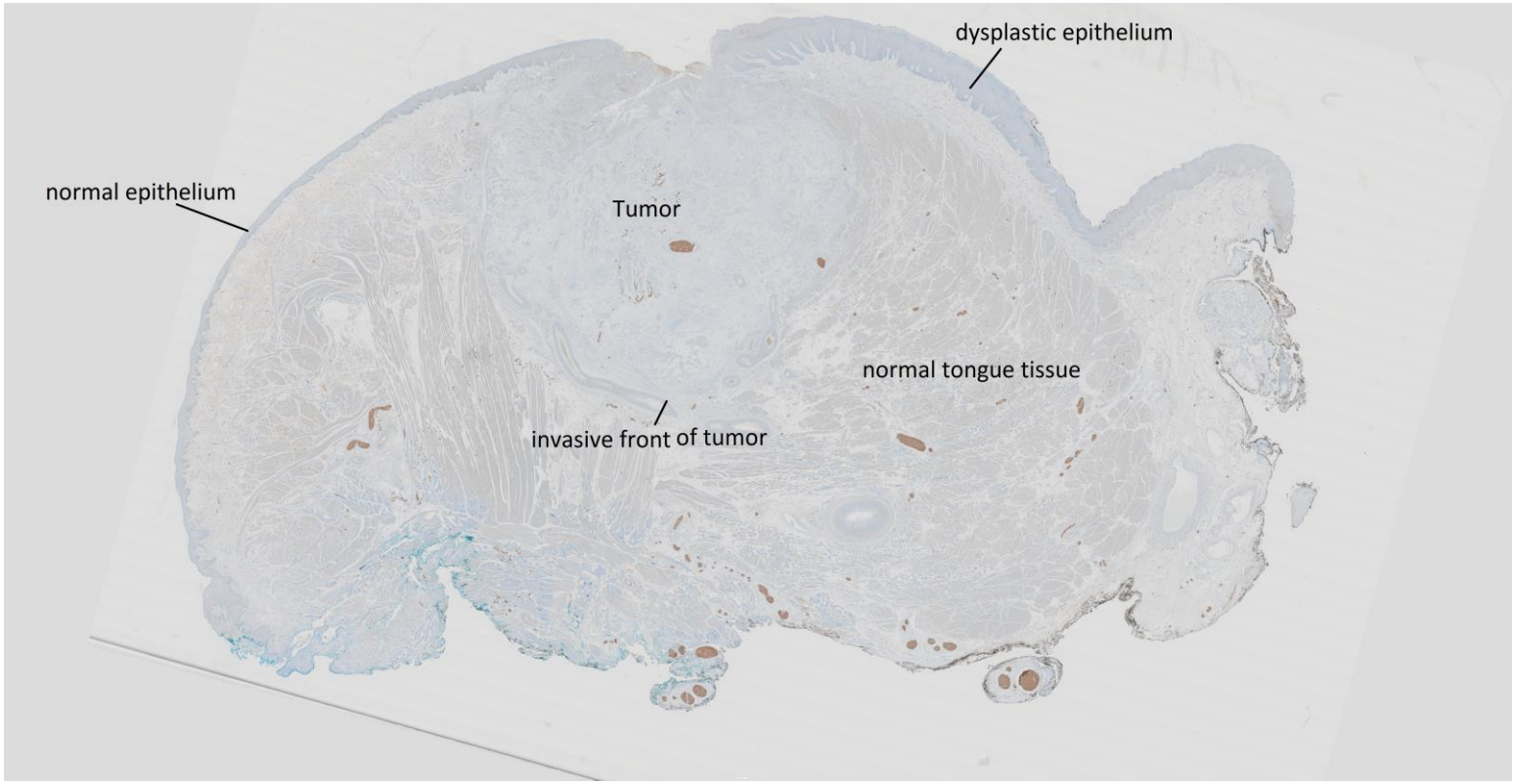


Image 2: Example of a hot-spot picture, note the areas of tumor tissue in the upper left and lower right part of the picture. Circled in red is a CD57 positive cell with a morphology deviating from the typical lymphoid morhpology, circled in blue is a smaller stained cell more aligned with the expected morhpology. Circled in green is an example of a non-stained lymphocyte, note the small size, thin rim og cytoplasm, and dot like apperance.

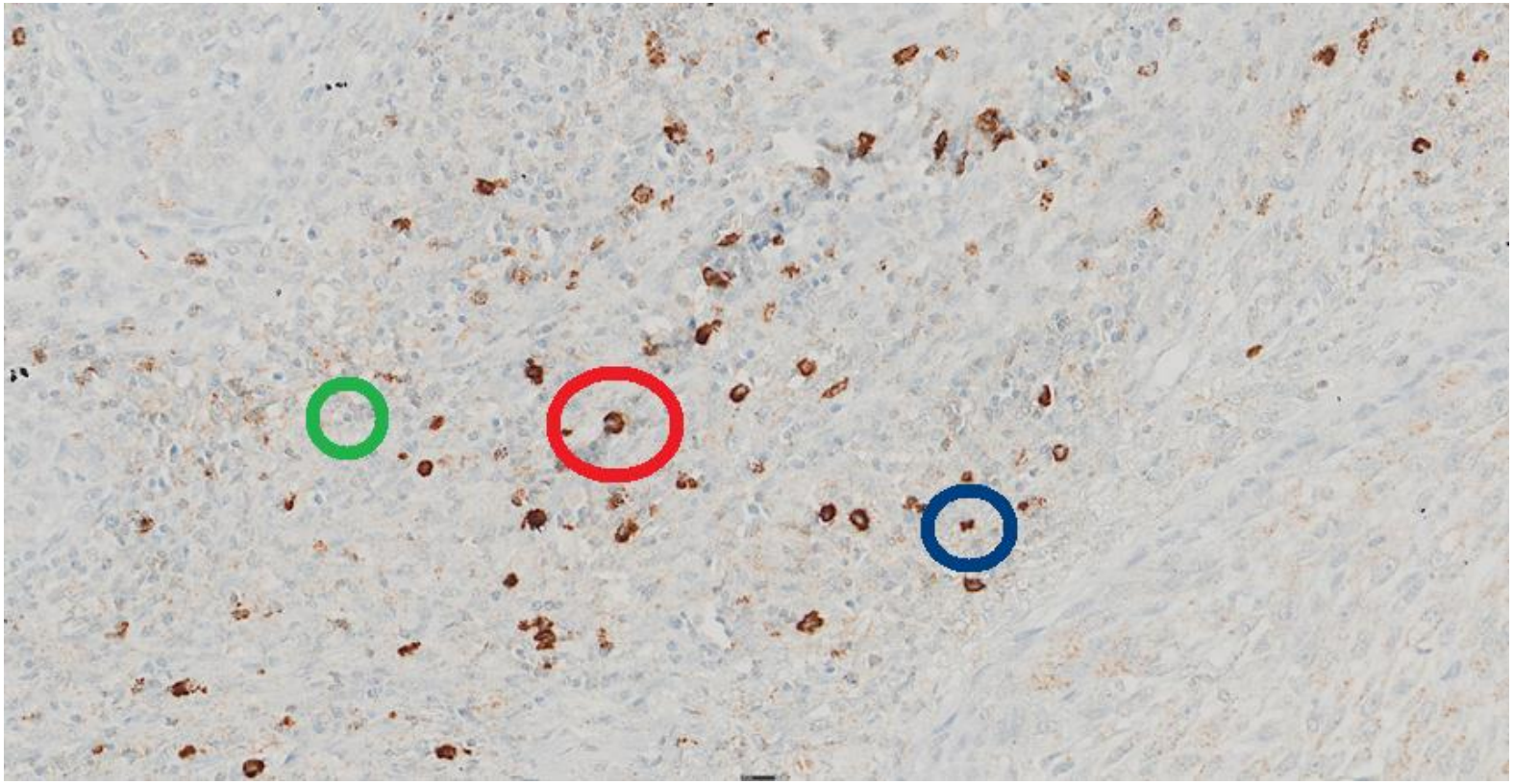


Image 3: example of immunohistochemical staining of a nerve and unspecific staining in the tumor tissue itself. These structures were not included in the scoring of the CD57 marker.

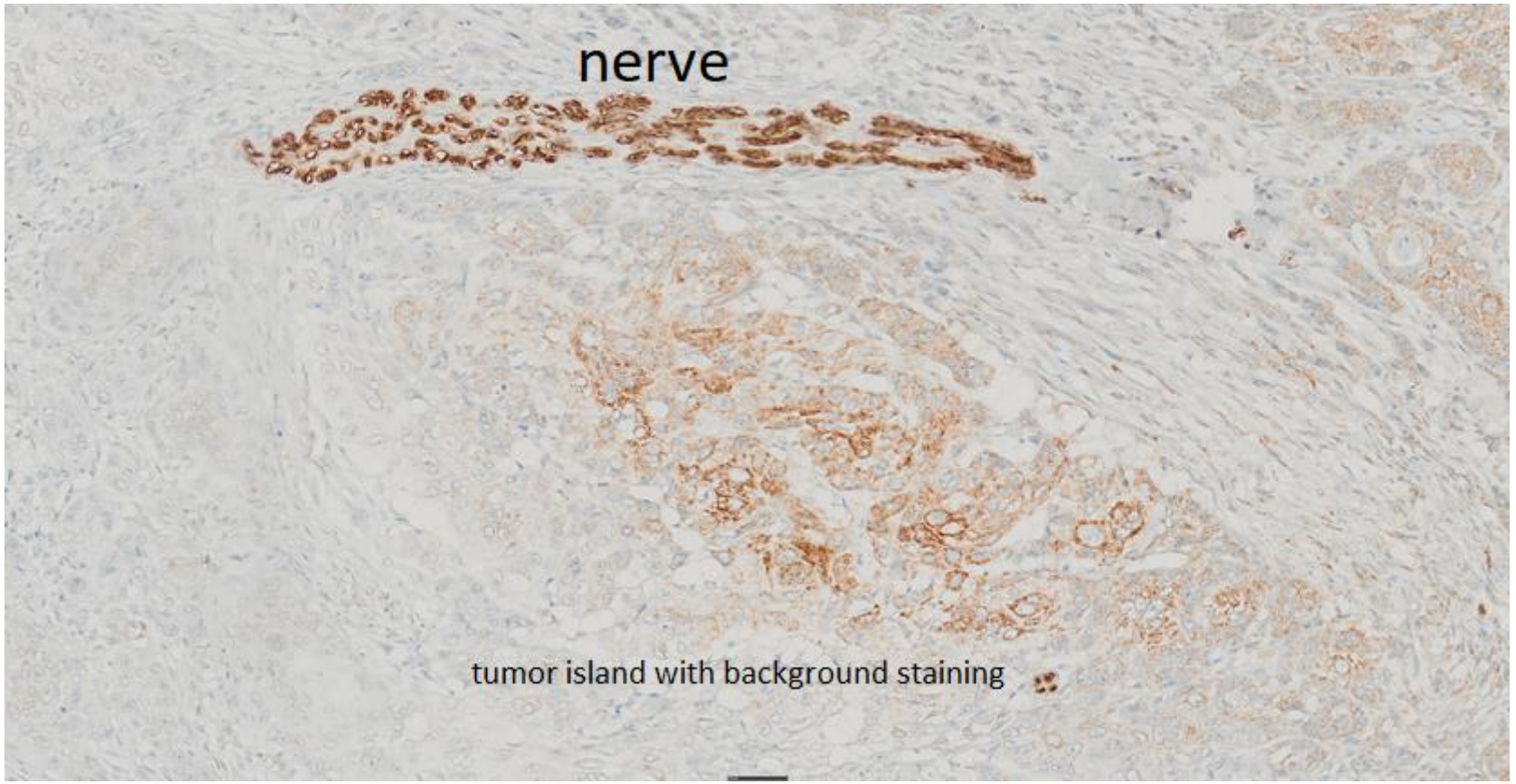


Table 1 TNM-stage, eight edition for OSCC (1, 10)

TNM-status	*Code*	*Description*
T-Primary tumor	TI	Tumor ≤ 2 cm or less in greatest dimension, and ≤ 0,5 cm depth of invasion
	T2	Tumor ≤ 2 cm or less in greatest dimension, and ≥ 0,5 -1,0 cm ≤ depth of invasion.
	T3	>2 cm ≤ 4 cm in greatest dimension, and ≤ 1,0 cm depth of invasion
	T4	T4 >4 cm or any tumor with > 1,0 cm depth of invasion T4a Tumor invades through the cortical bone of the mandible or maxillary sinus, or invades the skin of the face T4b Tumor invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery
N-regional lymph nodes	N0	No regional lymph node metastasis
	N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension without extranodal extension
	N2	N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension without extranodal extension N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension, without extranodal extension N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension, without extranodal extension
	N3	N3a Metastasis in a lymph node more than 6 cm in greatest dimension without extranodal extension N3b Metastasis in a single or multiple lymph nodes with clinical extranodal extension
M-Metastasis	M0	No distant metastasis
	M1	Distant metastasis

Table 2 Stages of OSCC, with corresponding TNM-status(10)

Stage	*Tumor size*	*Node affection*	*Metastasis -/+*
Stage 0	Tis (Carcinoma in situ)	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
Stage IVA	T4a T1, T2, T3, T4a	N1	M0
Stage IVB	Any T	N0, N1	M0
Stage IVC	Any T	Any N	M1

Table 3: Freuency statistics for the patient cohort in this thesis, 5-year disease spesific survival, and if there is significant differences between the groups

	N (%)	*5-year DSS (%)*	*p*
Gender			
Men	80 (61,1)	43 (62,3)	0,623
Women	51 (38,9)	30 (68,2)	
Age at diagnosis			
≤50	24 (18,3)	20 (83,3)	0,007
51-60	26 (19,8)	15 (60,0)	
61-70	40 (30,5)	24 (66,7)	
71-80	27 (20,6)	12 (57,1)	
≥81	14 (10,7)	2 (28,6)	
Smoking			
Never	34 (26,0)	21 (70,0)	0,659
Current	53 (40,5)	26 (60,5)	
Former	32 (24,4)	18 (60,0)	
Missing	12 (9,2)	8 (80,0)	
Differentiation			
Well	24 (18,6)	15 (88,2)	>0,001
Moderate	92 (71,3)	55 (67,1)	
Poor	13 (10,1)	2 (16,7)	
Missing	2 (1,5)		
T-status			
T1	42 (33,6)	30 (85,7)	0,014
T2	50 (40,0)	24 (54,5)	
T3	33 (26,4)	17 (60,7)	
Missing	6 (4,6)		
N-status			
N0	92 (70,2)	61 (79,2)	>0,001
N+	39 (29,8)	12 (33,3)	
Stage			
I	37 (28,2)	28 (93,3)	0,001
II	31 (23,7)	17 (65,4)	
III	60 (2,3)	26 (48,1)	
IV	0	0	
Missing	3 (2,3)	2 (66,7)	
CD57 25 percentile			
Low	13 (11,7)	5 (45,5)	0,095
High	98 (88,3)	58 (68,2)	
CD57 50 percentile			
Low	46 (41,4)	26 (66,7)	0,934
High	65 (58,6)	37 (64,9)	
CD57 75 percentile			
Low	80 (72,1)	45 (65,2)	0,880
High	31 (27,9)	18 (66,7)	

7 Appendix: Collection of quality-controlled studies, GRADE

<table>
<tr><td colspan="3">Reference:

R Agarwal, M Chaudary, S Bora, S Bajaj
Evaluation of natural killer cell (CD57) as a prognostic marker in oral squamous cell carcinoma: An immunohistochemistry study</td><td>Design: Prospective cohort study
Quality of evidence — Low
Recommendation — None</td></tr>
<tr><td>Aim</td><td>Material and methods</td><td>Results</td><td>Discussion/comments</td></tr>
<tr><td>to assess the expression of CD57 in oral squamous cell carcinoma (OSCC) and to correlate the expression of CD57 with 3 years survival in patients with OSCC.
conclusion
CD57 could be a good prognostic marker for OSCC patients.
country
USA
Year of data collection
2007-2010</td><td>Data material:
About 100 histopathologically diagnosed cases of OSCC of various grades were divided into two groups, i.e., Group I (dead patients) and Group II (live patients) from the archives of Department of Oral Pathology and Microbiology. CD57 was detected in these tissues by immunohistochemistry.

Exclusions:
No exclusion criteria mentioned

Statistical methods:
Spearman's correlation coefficient and students unpaired t-test.</td><td>3 years after surgery, 56 of 100 (56%) patients were alive and disease free, 5 (5%) patients were alive with recurrence of disease and 39 (39%) patients died of the disease.

CD57 LI: CD57 was expressed in OSCC in decreasing order from well differentiated to poorly differentiated SCC

CD57 expressed predominantly in mononuclear inflammatory cells around the tumor islands of OSCC. However, few CD57 positive cells were scattered even away from the tumor island. CD57 was significantly higher in patients who were alive than those who were dead.

The mean CD57 labeling index in Group II was significantly higher than that found in Group I (P = 0.000). There was a significant correlation (P = 0.00) in the mean CD57 levels between Groups I and II and prognosis of patient.</td><td>▪ Is the aim of the study clearly formulated? yes
▪ Were the groups recruited from the same population? yes
▪ Were the groups comparable with the respect to underlying factors? No mention of underlying factors other than presence of OSCC of various stages, so unsure.
▪ Were the exposed individuals representative for a defined population not mentioned if the 100 samples were ALL cancers diagnosed in the time period 2007-2010 or if it is a selection of cases
▪ Were exposures and outcomes measured equally and in a reliable manner in all groups? No mention of exposures (i.e known risk factors), outcome measured by calling relatives, which seems like an unreliable method compared to registration of deaths by more formal means
▪ Were those who evaluated outcomes blinded? Not mentioned
▪ Was the study prospective? yes
▪ Were a sufficient amount of participants followed up? All patients were followed up
▪ Was the follow-up period sufficient to measure significant results? 3 years is short for cancer, seeing as 5-year survival is usually used to measure survival. But they had survival rates in accordance with former knowledge.
▪ Were confounding factors adjusted for? Not mentioned
▪ Can these results be transferred to the general population to some degree? There are international differences in OSCC based on exposure to risk factors, but tumor microenvironment results should not be affected by this
▪ Are these results supported by prior literature? yes
▪ Do these results have any clinical implication? Yes, the results indicate that CD57 cells in the tumor tissue could be a prognostic marker for OSCC.

▪ Limitations:
No systematic representation of information of the patients other than outcome at the end of the follow up (disease free, recurrence of disease or dead). Different stages is the only other factor mentioned, but no further information on the division of the sample in cancer stages is given. The follow up time is low compared to the norm in cancer research.</td></tr>
</table>

Reference:
IH Bjerkli, O Jetlund, G Karevold, Á Karlsdóttir, E Jaatun, L Uhlin-Hansen et al.
Characteristics and prognosis of primary treatment-naïve oral cavity squamous cell carcinoma in Norway, a descriptive retrospective study

Design: retrospective cohort study

Quality of evidence	**Moderate**
Recommendation	**None**

Aim	**Material and methods**	**Results**	**Discussion/comments**
To present a large cohort of primary oral cavity squamous cell carcinoma from all four health regions of Norway, with descriptive clinicopathological characteristics and five-year survival outcomes. **conclusion** Five-year disease-specific survival was 52%, and patients eligible for curative treatment had a five-year disease-specific survival up to 62%. **country** Norway **Year of data collection** 2005-2009	**Data material:** Patients diagnosed with primary treatment-naïve oral cavity squamous cell carcinomas at all four university hospitals in Norway between 2005–2009 were retrospectively included in this study. Clinicopathological data from the electronic health records were compared to survival data. **Exclusions:** ICD-10 codes C05.1 and C05.2 which are regarded as oropharyngeal sites, and cancer of the external upper or lower lip (vermilion), because these almost exclusively arise in the lower lip and are more likely to act as skin cancer. Tumors with different histopathology than SCC were also excluded, as well as patients with HN second primaries or previous cancer treatment. Approximately 27% of the patients had been incorrectly coded; the majority of these had oropharyngeal cancer and were excluded. **Statistical methods:** The correlation between gender and different variables was evaluated using Spearman bivariate correlation (2-tailed) and bootstrapping at 95%confidence interval (CI). For evaluating survival, Cox regression allowed us to report significance, hazard ratio (HR), and 95% CI after bootstrapping. Results were considered to be significant at $p<0.05$. For survival the variables significant in univariate analysis were analyzed for multicollinearity (VIF), applying linear regression, testing independent variables against a dependent variable. VIF values <2 were regarded to indicate no multicollinearity. The variables with limited data (few in number), were excluded from calculations because of risk of sparse-data bias. Kaplan-Meier (Log Rank) was used to construct survival analyses plot. For survival analysis the definitions used were overall survival (OS) and disease specific survival (DSS); the latter was equivalent to cause-specific survival	The study identified 646 patients with cancer in the oral cavity, of which 111 were excluded. Giving a final cohort of 535 patients diagnosed with primary treatment-naïve OCSCC. **Table 1. Clinicopathological characteristics of 535 primary oral cavity squamous cell carcinomas 2005–2009.** |	Male n (%)	Female n (%)	(r_s)	(CI 95%)	p
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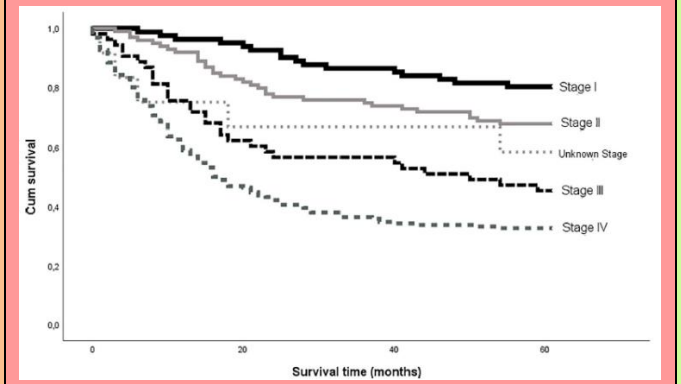
Variable	294 (55)	241 (45)			
Age, median (range)	64 (25–101)	72 (24–96)			
Age groups					
≤50	31 (10.5)	19 (7.9)			
51–60	72 (24.5)	36 (14.9)			
61–70	108 (36.7)	55 (22.8)	0.245	(0.162–0.325)	<0.001
71–80	54 (18.4)	66 (27.4)			
>80	29 (9.9)	66 (27.0)			
Primary site					
Mobile tongue	142 (48.3)	98 (40.7)			
Gingival/alveolar	46 (15.6)	61 (25.3)			
Floor of mouth	69 (23.5)	33 (13.7)	0.062	(-0.026–0.147)	0.154
Cheek/buca/retromolar	35 (11.9)	44 (18.3)			
Hard palate	2 (0.7)	5 (2.1)			
Tumor status					
T1	65 (22.1)	46 (19.1)			
T2	100 (34.0)	73 (30.3)			
T3	29 (9.9)	29 (12.0)	0.059	(-0.030–0.141)	0.180
T4	92 (31.3)	85 (35.3)			
Unknown*	8 (2.7)	8 (3.3)			
Lymph node status					
N0	186 (63.3)	143 (59.3)			
N1	34 (11.6)	23 (9.5)			
N2	53 (18.1)	56 (23.2)	0.050	(-0.039–0.136)	0.266
N3*	4 (1.4)	1 (0.4)			
Unknown*	17 (5.8)	18 (7.5)			
Stage of disease					
Stage I	61 (20.7)	40 (16.6)			
Stage II	75 (25.5)	51 (21.2)			
Stage III	35 (11.9)	28 (11.6)	0.089	(-0.004–0.172)	0.043
Stage IV	112 (38.1)	111 (46.1)			
Unknown*	11 (3.7)	11 (4.6)				 * Unknown data were not included in the calculations. **Not included in calculations because of risk of sparse-data bias. r_s = Spearman rank correlation, rho.  	▪ Is the aim of the study clearly formulated? **yes** ▪ Were the groups recruited from the same population? **yes** ▪ Were the groups comparable with the respect to underlying factors? **yes** ▪ Were the exposed individuals representative for a defined population **yes** ▪ Were exposures and outcomes measured equally and in a reliable manner in all groups? **yes** ▪ Were those who evaluated outcomes blinded? **yes** ▪ Was the study prospective? **No, retrospective** ▪ Were a sufficient amount of participants followed up? **yes** ▪ Was the follow-up period sufficient to measure significant results? **yes** ▪ Were confounding factors adjusted for? **yes** ▪ Can these results be transferred to the general population **yes** ▪ Are these results supported by prior literature? **yes** ▪ Do these results have any clinical implication? **yes** ▪ **Limitations:** There are some limitations in clinical information on the patients, as it as retrieved from medical journals, where not all relevant information was documented (for example information on smoking was missing for a few of the patients).

<table>
<tr><td colspan="3">Reference:

S lopez-Vergès, JM Miluish, S Pandey, VA York, J Arakawa-Hoyt, H Pircher et al.
CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16^{+} NK-cell subset</td><td colspan="2">Design: in-vitro research
Quality of evidence | Low
Recommendation | None</td></tr>
<tr><td>Aim</td><td>Material and methods</td><td>Results</td><td colspan="2">Discussion/comments</td></tr>
<tr><td>define the transcriptional signature, distinctive phenotype, and functional properties of CD57^{+} NK cells
conclusion
CD57 defines a functionally distinct population of mature NK cells in the human CD56^{dim} CD16^{+} NK-cell subset
country
USA
Year of data collection
Not mentioned</td><td>Data material:
PBMCs were obtained from leukocyte reduction Pall filters (Blood Centers of the Pacific) and from healthy adult volunteers after density gradient centrifugation over Ficoll-Paque (GE Healthcare)

Exclusions: not mentioned

Statistical methods:
2-tailed paired t test was used in the phenotypic studies to compare CD57^{+} and CD57^{-} NK cells. The nonparametric Wilcoxon matched pairs test was used in the functional analysis of CD57^{+} and CD57^{-} NK cells. The statistical significance threshold was set at P less than .05.</td><td>CD57 is expressed on 30% to 60% of CD56dimCD16^{+} cells, which represent mature NK cells, whereas less than 1% of CD56bright NK cells, which are considered immature, express CD57 (Figure 1A; supplemental Figure 1), consistent with prior observations. Indeed, more than 98% of CD57^{+} NK cells were CD56dimCD16^{+} NK cells. CD56bright NK cells have higher surface density of NKp46, NKp30, NKG2A, and NKG2D compared with CD56dim NK cells.1,29 During maturation, the cell-surface density of CD56 and natural cytotoxic receptors decreases, whereas CD56dim NK cells acquire expression of CD16, KIRs, 2B4, and LIR-1. We hypothesized that within the CD56dimCD16^{+} NK-cell subset CD57 marks more mature NK cells, indicating a final stage of maturation. The differentiation of NK cells involves maturation from CD56bright to CD56dim NK cells and we propose, from CD57^{-} CD56dim to CD57^{+} CD56dim cells.

CD57^{-}CD56^{dim} CD16^{+} NK cells have a greater proliferative capacity than CD57^{+} NK cells when stimulated with cytokines and target cells, suggesting that CD57+ NK cells, like CD57^{+} CD8^{+} T cells, have a proliferation defect in vitro.</td><td colspan="2">▪ Is the aim of the study clearly formulated? Yes
▪ Were the groups recruited from the same population? no, there were to different ways they collected blood samples
▪ Were the groups comparable with the respect to underlying factors? not known
▪ Were the exposed individuals representative for a defined population not relevant
▪ Were exposures and outcomes measured equally and in a reliable manner in all groups? yes
▪ Were those who evaluated outcomes blinded? Not relevant
▪ Was the study prospective? no
▪ Were a sufficient amount of participants followed up? Not relevant
▪ Was the follow-up period sufficient to measure significant results? Not relevant
▪ Were confounding factors adjusted for? Not relevant
▪ Can these results be transferred to the general population maybe
▪ Are these results supported by prior literature? yes
▪ Do these results have any clinical implication? yes, in the long run

▪ Limitations:
The research is done in vitro, and it is uncertain if the results can be transferred to in vivo cases.</td></tr>
</table>

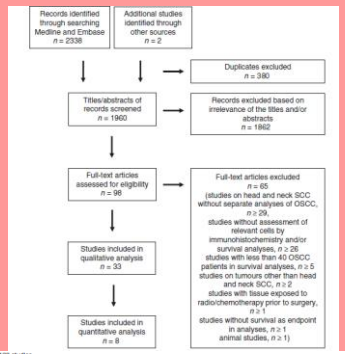
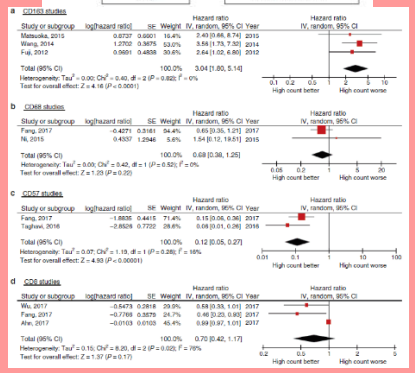
Reference:

N Tagavi, S Bagheri, A Akbarzadeh
Prognostic implication of CD57, CD16, and TGF-β expression in oral squamous cell carcinoma

Design: retrospective cohort study

Quality of evidence	**Low**
Recommendation	**None**

Aim	**Material and methods**	**Results**	**Discussion/comments**
to evaluate the prognostic significance of CD57+ and CD16+ cells and TGF-b expression in samples of oral squamous cell carcinoma (OSCC). **conclusion** findings suggest that CD57 expression and mode of invasion are independent prognostic factors of survival in OSCC patients **country** Iran **Year of data collection**	**Data material:** samples were collected from 57 patients with primary OSCC diagnosed at the Department of Oral and Maxillofacial Pathology, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Clinicopathologic information on each case including age, sex, tumor location, mode of invasion, and survival rate obtained from medical records and reviewing slides. **Exclusions:** Patients without complete data and insufficient paraffin embedded tumor material were excluded from the study **Statistical methods:** Chi-square test, t-test, and Fisher's exact test were used to assess association between CD57, CD16, and TGF-b expression and clinicopathologic variables. Multivariate analysis was performed according to Cox's regression model to estimate hazard ratios (HR). Analysis of expression correlation between CD57, CD16, and TGF-b was conducted using Spearman correlation coefficient. The significant level of all tests was set as 0.05.	Fifty-seven patients with OSCC (27 males and 30 females) were studied. Follow-up was available for all of the cases, and the average follow-up was 29 months ranging from 10 to 85 months. The mean and median overall survival (OS) was 59.9 and 84 months, respectively. Twenty patients died of cancer, and 37 patients survived No significant correlation between CD16, TGF-b, and CD57 expression with mode of invasion was Seen Multivariate Cox regression analysis showed that CD57 expression [HR 17.34 (95% CI 3.815–78.830); P < 0.001] and mode of invasion [HR 0.362 (95% CI 0.138–0.947); P = 0.038] are significantly correlated with OS (Table 2) and can be considered as independent prognostic factors in OSCC patients higher expression of CD57 increases OS 17 times **Table 2** Multivariate analysis of prognostic factors of survival using Cox's proportional hazard model *Parameters* / *Coefficient (SE)* / *P* / *Hazard ratio* / *95% CI*[a] Age: 0.13 (0.018) / 0.482 / 1.013 / 0.997–1.050 Sex[b]: 0.523 (0.513) / 0.309 / 1.69 / 0.617–4.613 Tumor location: – / 0.504 / – / – Mode of invasion: −1.017 (0.491) / 0.038* / 0.362 / 0.138–0.947 CD57 expression: 2.853 (0.773) / <0.001* / 17.34 / 3.815–78.830 CD16 expression: 0.397 (0.341) / 0.243 / 1.48 / 0.763–2.900 TGF-β expression: 0.394 (0.348) / 0.257 / 1.483 / 0.750–2.931 *P < 0.05. [a]95% CI = 95% confidence interval for hazard ratio. [b]Reference category: male.	▪ Is the aim of the study clearly formulated? **yes** ▪ Were the groups recruited from the same population? **yes** ▪ Were the groups comparable with the respect to underlying factors? **yes** ▪ Were the exposed individuals representative for a defined population **yes** ▪ Were exposures and outcomes measured equally and in a reliable manner in all groups? **yes** ▪ Were those who evaluated outcomes blinded? **not mentioned** ▪ Was the study prospective? **no** ▪ Were a sufficient amount of participants followed up? **all patients were followed up, but time varied** ▪ Was the follow-up period sufficient to measure significant results? **unsure, the time of follow-up ranged from 10-85 months among the patients, while 20 were counted as dead with no survival time given.** ▪ Were confounding factors adjusted for? **yes** ▪ Can these results be transferred to the general population **possibly** ▪ Are these results supported by prior literature? **yes** ▪ Do these results have any clinical implication? **yes** ▪ **Limitations:** Inadequate data on patients, they do multivariate analysis on some important parameters, but should have had parameters like TNM-Status and stage as well.

Reference: E Hadler-Olsen, AM Wirsing **Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma: a systematic review and meta-analysis**			**Design: systematic review and meta-analysis of 33 cohort studies** **Quality of evidence** — **Moderate** **Recommendation** — **None**
Aim	**Material and methods**	**Results**	**Discussion/comments**
conclusion Deficiencies in the reporting of study design and conduct make it difficult to draw reliable conclusions about the suggested markers. The prognostic value of CD163+ M2 macrophages and CD57+ natural killer cells should be validated in large, standardised studies. **country** Norway **Year of data collection** Not mentioned, but before May 2018	**Data material:** 1960 records in our search, of which 33 articles were eligible for this systematic review **Inclusions:** Included in the review were original articles that fulfilled all the following criteria: ● were written in English, ● presented data from patients with SCC in the oral cavity proper ● analysed tissue that had not been previously exposed to radiotherapy and/or chemotherapy, ● used immunohistochemistry on tumour tissue sections to recognise the immune cells of interest, ● addressed the prognostic value of tumour associated macrophages, DC, NK cells, mast cells, T cells and/or B cells by univariate and/or multivariate survival analyses of at least 40 OSCC patients, and ● employed some kind of survival as endpoint in the survival analyses **Statistical methods:** random-effect meta-analysis of overall survival to estimate the summary HR and the associated 95% CI for immune cell markers that had been reported in at least two studies with necessary statistical data. Based on multivariate estimates of HR	Of the 1960 articles identified, 33 were eligible for this systematic review and 8 were included in the meta-analysis. CD163+ M2 macrophages and CD57+ natural killer cells were the most promising predictors of survival in oral cancer patients. Many studies lacked important information on their design and conduct. CD163+ macrophages and CD57+ NK cells have prognostic Potential.  	▪ Is the aim of the study clearly formulated? **yes** ▪ Were the groups recruited from the same population? **yes** ▪ Were the groups comparable with the respect to underlying factors? **yes** ▪ Were the exposed individuals representative for a defined population **yes** ▪ Were exposures and outcomes measured equally and in a reliable manner in all groups? **yes** ▪ Were those who evaluated outcomes blinded? **Not relevant** ▪ Was the study prospective? **no** ▪ Were a sufficient amount of participants followed up? **not relevant** ▪ Was the follow-up period sufficient to measure significant results? **Not relevant** ▪ Were confounding factors adjusted for? **not relevant** ▪ Can these results be transferred to the general population **most likely yes** ▪ Are these results supported by prior literature? **yes** ▪ Do these results have any clinical implication? **yes** ▪ **Limitations:** Important clinical information or reporting of immunohistochemical staining and scoring protocols as well as statistical data were often missing in the papers reviewed in the study, decreasing the usefulness of results based on them.