Multiplex Immunofluorescence Studies on T cell subsets in Oral Squamous Cell Carcinoma of the Tongue.

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University of Bergen, Norway

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Philosophy in Global Health at the University of Bergen.

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Abstract

Background

Cancer of the oral cavity is a common malignancy, notably in low-middle income countries (LMIC). Almost half of the cases of oral cancer appear in the tongue (41.5%). Oral Squamous cell carcinoma (OSCC) is the major histology, and the most common etiological factors are excessive tobacco and alcohol usage. Even after successful intervention and treatment, almost 50% of OSCC patients pass away within 5 years. Globally, there was an incidence of 377,713 new cases in 2020, most notable in South Asian populations.

The immune system performs a critical function in eliminating cancers. Tumor-infiltrating T cells have been demonstrated to relate to overall survival and reaction to the treatment of OSCC. In particular, memory-resident cytotoxic T cells are reported as crucial to a successful immune response against tumors, spontaneously but also in the context of immunotherapy

Objectives

The main objective of the present study was to assess the prognostic value of tumor-infiltrating cytotoxic and memory T cells in OSCC of the tongue and establish a protocol for sequential staining of markers defining this T cell subset in OSCCT tissue samples and determine the amount and prognostic value of these cells in OSCC of the tongue when analyzed against patient clinical and pathological data.

Methods

TMAs obtained from 123 cases of tongue OSCC part of the NOROC cohort were tested in this study. A protocol for multiplexing immunofluorescence was devised. The TMAs were tested for CD8+, CD103+, CD45RO+, CD3+, KI67+, Granzyme B+, markers for T cells, and Pan Cytokeratin+ to identify malignant epithelia. The targeted antigens were visualized using an Olympus VS120 S6 Slide scanner. QuPath software was used for image analysis, and we started the study with the quantification of CD8+, CD103+, and CD8+CD103+ cells. Statistical analysis of the data was achieved using IBM SPSS 27 statistical analysis software.

Results

We were able to devise a successful protocol for multiplexed staining. We were able to quantify and analyze the TILs we wanted to visualize. Most of the patients were above 50 years of age, and overall survival for all stages was 56.8%. Almost half of the patients were diagnosed with TNM stage III. There was recurrency regionally in 22.6% of patients, and it was distant in 6.4% of patients. There was high

survival with patients having early TNM stage (stage I and II), no perineural thickness in tumors less < than 8 mm, depth of invasion < 5 mm, and no recurrent metastasis. A high number of CD8+ cells was associated with tumors that had poor differentiation, low keratinization but low nuclear polymorphism, and low local recurrence. A high number of CD103+ cells were associated with high keratinization, lower tumor stage, and no local recurrence. Double positive CD8+CD103+ cells were higher in number in low nuclear polymorphism and low regional recurrence. The amount of CD8+, CD103+, and CD8+CD103+ frequency had no impact on overall survival.

Conclusion

The protocol for multiplex staining we devised is suitable for sequential staining of TMAs from cancer tissues. This is useful since it permits staining the same cells for several markers maintaining the tissue morphology and with minimal bleeding of the signal. Even if we analyzed only T cell markers so far, results show that the expression of CD8 and CD103 in tumor infiltrates could be of prognostic value in OSCCT. This study can be a significant contribution to the knowledge of the significance of immune response in OSCCT and we look forward to better understanding the significance of the T cell subsets and exploring further the meaning and expression of CD103 in tumor infiltrates.

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Acronyms and Abbreviations

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
APCs	Antigen-presenting cells
BSA	Bovine serum albumin
CTLs	Cytotoxic T Lymphocytes
DAMPs	Damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
EBV	Epstein-Barr Virus
GST	Glutathione-S-transferase
HPV	Human Papilloma Virus
HSV	Herpes Simplex Virus
IF	Immunofluorescence
IFNs	Type I Interferons
IHC	Immunohistochemistry
IMC	Imaging Mass Cytometry
MHC	Major Histocompatibility Complex
MIBI	Molecular Ion Beam Imaging
MIC	Molecular Imaging Centre
NK cells	Natural Killer cells
NNK	4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
NOROC	Norwegian Oral Cancer
OSCC	Oral Squamous Cell Carcinoma
OSCCT	Oral Squamous Cell Carcinoma of the Tongue
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD-1	Programmed cell death 1
PD-L1	Programmed death-ligand 1
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species

SD	Standard Deviation
Tc cells	Cytotoxic T cells
TCRs	T cells receptors
TMA	Tissue Microarray
TME	Tissue Microenvironment
TNF	Tumor necrosis factor
TNM	T describes the size of the primary tumor, N describes regional lymph nodes, M describes distant metastasis
TSNs	Tobacco-specific nitrosamines
WHO	World Health Organization

Acknowledgements

I would like to dedicate this thesis to my late father, Professor Emeritus Dr. Mohammad Tariq Jan.
My idol, my inspiration, and my closest ally.

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1. Introduction

1.1. Oral Squamous Cell Carcinoma (OSCC)

Cancers arising in the oral cavity are one of the widest spreading diseases from a global health perspective. It has great health and economic consequences and reduces the quality of life immensely (Peres et al., 2019). Among oral cancers, oral squamous cell carcinoma (OSCC) is the most widespread histological subtype, constituting more than 90% of all oral cancers (Coletta et al., 2020). OSCC is epithelial in origin while other oral cancers may surface from other tissues in the oral cavity such as the connective tissue, minor salivary glands, lymphoid tissue, and melanocytes, or represent metastasis from malignancies in other sites (Montero & Patel, 2015). According to a review by Weatherspoon et al, almost one-half of all oral cancers appear in the tongue (41.5%), and 90% of those are OSCC (Weatherspoon et al., 2015).

The disease is often diagnosed in advanced clinical stages (clinical TNM stage III and IV, see also below) which requires extensive treatment, disfigurement, and poor quality of life. Surgical resection is the preferred treatment whenever possible, and can be followed by postoperative treatment i.e. chemotherapy and radiotherapy (Ow & Myers, 2011). More recently, immunotherapy has also been introduced as a treatment option for OSCC (Almangush et al., 2021).

In their study on a cohort from England, Rogers et al found that, regardless of advances in diagnosis and management, overall survival was only 56% after 5 years with 10% and 21% of the patients developing local or loco-regional recurrence respectively; in addition, 7 % of the individuals developed a second primary OSCC (Rogers et al., 2009).

The TNM system (T as in size and depth of invasion of a tumor, N as regional lymph node involvement, and M as in distant metastasis) is the main prognostic stratification in most malignancies (Brierley et al., 2017). The latest TNM classification for oral carcinomas, the 8th edition, is reported in, Table 1.1.

T – Primary tumor
TX Primary tumor cannot be assessed.
T0 No evidence of primary tumor
Tis Carcinoma in situ
T1 Tumor ≤ 2 cm in greatest dimension and ≤ 5 mm depth of invasion

T2 Tumor > 2 cm but ≤ 4 cm in greatest dimension and ≤ 10 mm depth of invasion T3 Tumor > 4 cm in greatest dimension > 10 mm depth of invasion T4a (lip) Tumor invades through cortical bone, inferior alveolar nerve, floor of the mouth, or skin (of chin or nose) T4a (oral cavity) Tumor invades through cortical bone of the mandible or maxillary sinus, or invades the skin of the face T4b (lip and oral cavity) Tumor invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery
N – Regional lymph nodes
NX Regional lymph nodes cannot be assessed. N0 No regional lymph node metastasis N1 Metastasis in a single ipsilateral lymph node, ≤ 3 cm in greatest dimension N2 Metastasis as specified in N2a, N2b, or N2c below N2a Metastasis in a single ipsilateral lymph node, > 3 cm but ≤ 6 cm in greatest dimension N2b Metastasis in multiple ipsilateral lymph nodes, all ≤ 6 cm in greatest dimension N2c Metastasis in bilateral or contralateral lymph nodes, all ≤ 6 cm in greatest dimension N3a Metastasis in a lymph node > 6 cm in greatest dimension N3b Metastasis in a single or multiple lymph nodes with clinical extra nodal extension
M – Distant metastasis
M0 No distant metastasis M1 Distant metastasis
Stage grouping
Stage 0 Tis N0 M0 Stage I T1 N0 M0 Stage II T2 N0 M0 Stage III T1-3 N1 M0 T3 N0 M0 Stage IVA T1-4a N2 M0 T4a N0-1 M0 Stage IVB Any T N3 M0 T4b Any N M0 Stage IVC Any T Any N M1

Table 1.1. TNM classification of carcinomas of the lip and oral cavity

1.1.1. Global Distribution

In the GLOBOCAN 2020 report, Oral cancer was reported to be the 16th most reported malignancy in the world, with 377,713 new cases reported in 2020 (https://gco.iarc.fr/today/).

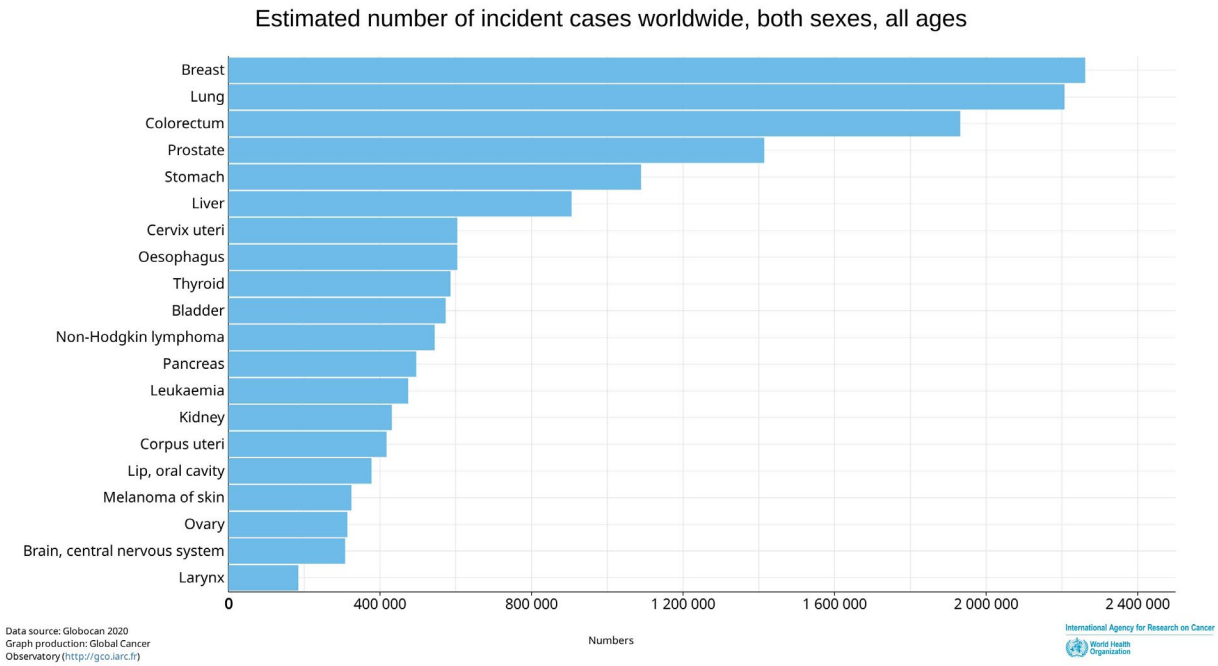


Fig 1.1. Incidence of Cancer Worldwide. Source; GLOBOCAN 2020 (https://gco.iarc.fr/today/)

Ferlay et al reported the highest incidence of oral cancer worldwide in Papua New Guinea, but its incidence was also very high in South Asian countries, especially in Pakistan, India, and Sri Lanka. Oral cancer represented the primary cause of cancer mortality amongst males in India according to the report (Ferlay et al., 2021). Sung et also report high rates of oral cancer in Eastern Europe as well as Australia and New Zealand (Sung et al., 2021).

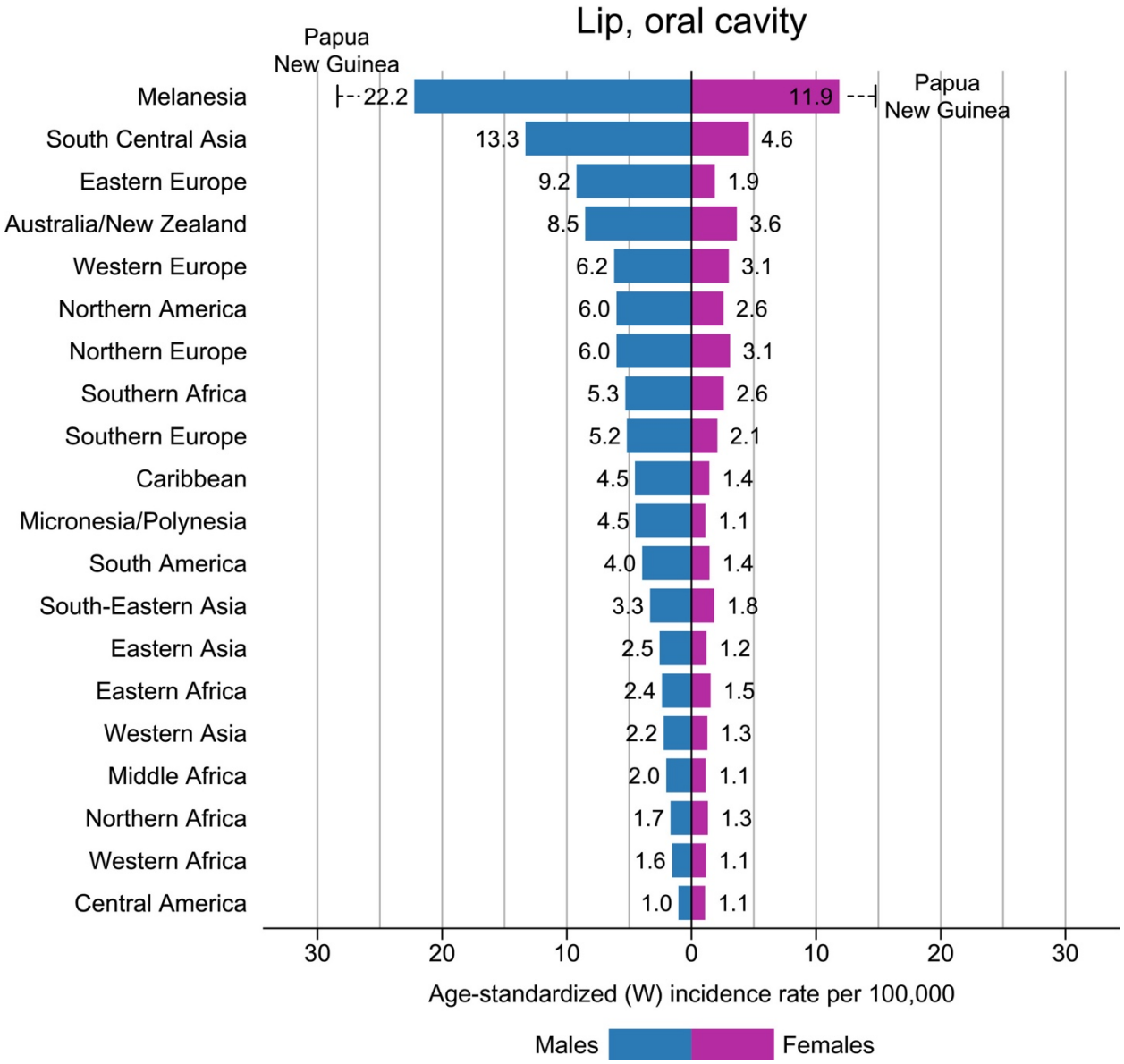


Fig 1.2. Region-Specific Incidence Age-Standardized Rates by Sex for Cancers of the Oral Cavity in 2020. Source: GLOBOCAN 2020. (https://gco.iarc.fr/today/)

1.1.2. Etiology of OSSC

The most common etiological factors of OSCC are tobacco, alcohol, viruses, genetic predisposition, immunosuppression, dental factors, and occupational risks (Markopoulos, 2012), with tobacco and alcohol consumption being the key etiological factors in OSCC (Bugshan & Farooq, 2020).

Warnakulasuriya et al. report in their analysis of the effect and treatment of tobacco dependence, that the risk for developing OSCC was seven to ten times higher in smokers compared to non-smokers (Warnakulasuriya et al., 2005).

The primary carcinogens from smoking tobacco are aromatic hydrocarbon benz-pyrene and tobacco-specific nitrosamines (TSNs) specifically, 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). Various analyses show that NNK and NNN present in tobacco are important etiological factors in the development of OSCC since these metabolites covalently bind with deoxyribonucleic acid (DNA) of keratinocyte stem cells developing DNA adducts (Yershova et al., 2016). Importantly the metabolic processes of such carcinogens include oxygenation by P450 enzymes in cytochromes and conjugation by glutathione-S-transferase (GST). A crucial part of the genetic predisposition to tobacco-induced OSCC may be due to the genetic polymorphisms in the genes coding for these enzymes (Scully et al., 2000).

Chewing of betel nut and tobacco chewing are also habits very prevalent in Asian populations (Salian et al., 2016), and a specific contribution of these substances to carcinogenesis has been suggested since betel quid components and metabolic intermediates can act as oxidating and methylating agents causing DNA damage, cytotoxicity, and cell proliferation (Kumar et al., 2016).

Alcohol abuse has also been recognized as a main etiological factor for OSCC (Tenore et al., 2020). N-nitroso compounds, mycotoxins, urethane, and inorganic arsenic are among the components of alcohol beverages that in addition to alcohol metabolites can be cytotoxic. The main metabolite of alcohol is acetaldehyde, a product of alcohol dehydrogenase (ADH) metabolism which is itself oxidized to acetate by aldehyde dehydrogenase (ALDH). Acetaldehyde may affect the DNA synthesis and repair and provoke sister chromatid exchanges and specific gene mutations (Zakhari, 2006).

There is as well evidence of a synergistic effect between alcohol and tobacco in the OSCC etiology (Mello et al., 2019). According to Rodriguez et al., those having heavy smoking and drinking habits have up to forty-eight times higher risk of developing OSCC than non-smoking and non-drinking adults (Rodriguez et al., 2004).

Viruses have also been associated with the development of OSCC. Viral genes and gene products may disturb cell growth and proliferation when proto-oncogenes integrate into the host’s DNA acting as oncogenes, alternating the regulation of the cell cycle, and contributing to malignant transformation.

Viruses that have been associated with OSCC are human herpes virus (mainly Epstein–Barr virus (EBV)), human papillomavirus (HPV), and herpes simplex virus (Metgud et al., 2012). Chronic inflammation is also reported as a possible mechanism that favors malignant transformation of the oral mucosa (Feller et al., 2013).

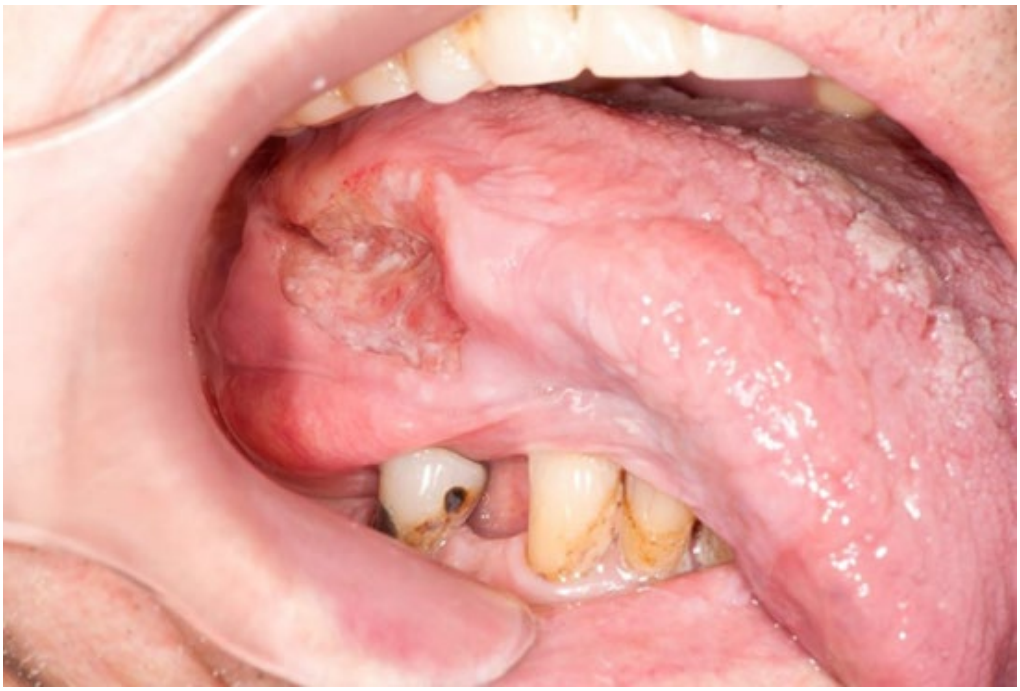


Fig 1.3. Oral Squamous Cell Carcinoma of the tongue (Wong & Wiesenfeld, 2018).

1.2. NOROC

The Norwegian Oral Cancer (NOROC) study is a retrospective study that has enrolled a large cohort (535) of all individuals diagnosed with Oral Squamous Cell Carcinoma in Norway in the period between January 1st, 2005, and December 31st, 2009, with at least 5 years follow up after the end of their treatment. Cases were pooled from all four health regions of Norway, and descriptive clinicopathological characteristics and five-year survival outcomes were collected in a database. 273 patients (45%) from the NOROC cohort had OSCC of the tongue (OSCCT) (Bjerkli et al., 2019), but only patients undergoing primary surgery with curative intent were included in the investigation in this Master thesis.

NOROC was established with the aim of providing a retrospective large cohort with well documented clinicopathological data to better characterize OSCC according to the site, to further knowledge of the biological behavior of OSCCs, and provide putative prognostic markers that would stratify tumors in TNM or in addition to this (Bjerkli, Jetlund, et al., 2020a). NOROC studies have identified tumor budding

and lymphocytic infiltrates combined with tumor differentiation as prognostic markers in OSCC (Bjerkli, Jetlund, et al., 2020b).

1.3. The Immune System

The role of the immune system is to eliminate infections and damaged tissues, among these also malignant cells. The immune system also performs a critical function in the healing of wounded tissues (Larouche et al., 2018). A deficient immune system would cause an enhanced vulnerability to infections, potentially higher risk of cancer, and damaged or incomplete repair of tissues (Haas, 2019). The immune system protects our body from harm by eliciting an immune response or reaction to microbes, as well as other molecules that are identified as foreign or damaged, and sometimes it can damage the surrounding tissue even though they are healthy (Chaplin, 2010).

In the latter case, the immune system can cause harm to the host when an inappropriate or too exuberant inflammatory response leads to otherwise physiological pathways activation or cell injury. This results in allergic, autoimmune, or inflammatory diseases. The immune system may also be responsible for rejection and damage to tissue grafts and transplants (Chen et al., 2017).

The immune system is conventionally separated into two parts: innate immune system and adaptive immune system (Medzhitov & Janeway, 2000).

1.3.1. Innate Immunity

Innate immunity, also known as natural immunity or native immunity, is the first line of defense. This mechanism first evolved in invertebrates and then persisted in all higher vertebrates. The innate immune cells are ready to recognize and eliminate foreign/damaged/dead cells upon activation (Medzhitov & Janeway, 2000), but also other structures and molecules are incorporated into the concept of innate immunity such as epithelial barriers of the skin and mucosal surface and by cells and natural antibiotics present in epithelia. These block the entry of microbes and can destroy them (Aristizábal & González, 2013).

If the microbes breach the epithelia, other components of the innate immune system, namely phagocytes, granulocytes, certain types of lymphoid cells such as NK cells, and plasma proteins of the complement system, will elicit a response. As the adaptive immune response requires time to be stimulated and then undergo proliferation and differentiation, the innate immune response provides protection during the

activation of the adaptive immune response. Importantly, the innate immune system also instructs the adaptive immune response through the presentation of antigens and the production of cytokines that recruit adaptive lymphocytes and regulate and control their cell division (Justiz Vaillant et al., 2022).

Because of its role in the clearance of dead and damaged tissues and the initiation of repair after tissue damage the innate immune response is key to the maintenance of homeostasis and the regeneration (Turvey & Broide, 2010).

The innate immune response can be both intracellular and extracellular. Innate immune defense against intracellular and extracellular viruses is mediated by Natural Killer (NK) cells, which kill virus-infected cells, and by cytokines called type I interferons (IFNs), which block viral replication within host cells (Beutler, 2004).

The innate immune system recognizes structures that are shared by various classes of pathogens and are not present in normal host cells. These microbial molecules stimulate innate immunity and are termed pathogen-associated molecular patterns (PAMPs). The receptors of the innate immune system that recognize these structures are called pattern recognition receptors (PRRs). Innate immune receptors are specific to structures of microbes that are often essential for the survival and infectivity of these microbes (Medzhitov & Janeway, 1997). The molecules that are released from damaged or necrotic host cells and recognized by PRRs on the innate immune system also are called damage-associated molecular patterns (DAMPs) (Rubartelli & Lotze, 2007).

It is important to mention that the innate immune response can not only be extracellular – as by activated leukocytes that can destroy and/or ingest microbes and damaged cells, the immune cells – host cells also display an intracellular immune response as that elicited by the cytokines type I interferons (IFNs), which block viral replication within host cells (Beutler, 2004) (Rauch et al., 2013).

1.3.2. Adaptive Immunity

Adaptive immunity is the hallmark of the mammalian immune system. This response consists of antigen-specific reactions through T lymphocytes (CD3+) and B lymphocytes (CD20+). The most important characteristic of the adaptive response is the generation of memory so that new exposures to the same antigen lead to a more robust and faster adaptive reaction (Borghesi & Milcarek, 2007).

T and B lymphocytes express different receptors that recognize cognate antigens. T lymphocytes have T cell receptors (TCRs) which are not secreted, while B lymphocytes have membrane-bound antibodies, also called immunoglobulins, that can also be secreted and target microbes and microbial toxins and eliminate them by activating various effector mechanisms (Moser & Leo, 2010).

The structure of an antibody consists of two identical heavy chains and two identical light chains, forming a disulfide-linked complex. Each chain contains a variable region that recognizes antigens and a constant region which provides stability and structure to its (Wang et al., 2007).

The adaptive lymphocytes T and B cells display a targeted effector response in two stages. First, the cognate antigen is presented by an antigen-presenting cell (APC) and recognized by the antigen-specific T or B cell leading to cell priming, activation, and differentiation, which occurs within the specialized environment of lymphoid tissue and in the periphery. Second, the effector response takes place, either due to the activation and proliferation of central or peripheral T cells or due to the release of antibodies from activated B cells (plasma cells) into blood and tissue fluids (Arstila et al., 1999).

As briefly mentioned previously this second phase also generates memory clones of the activated adaptive T cell and B cells (Parkin & Cohen, 2001).

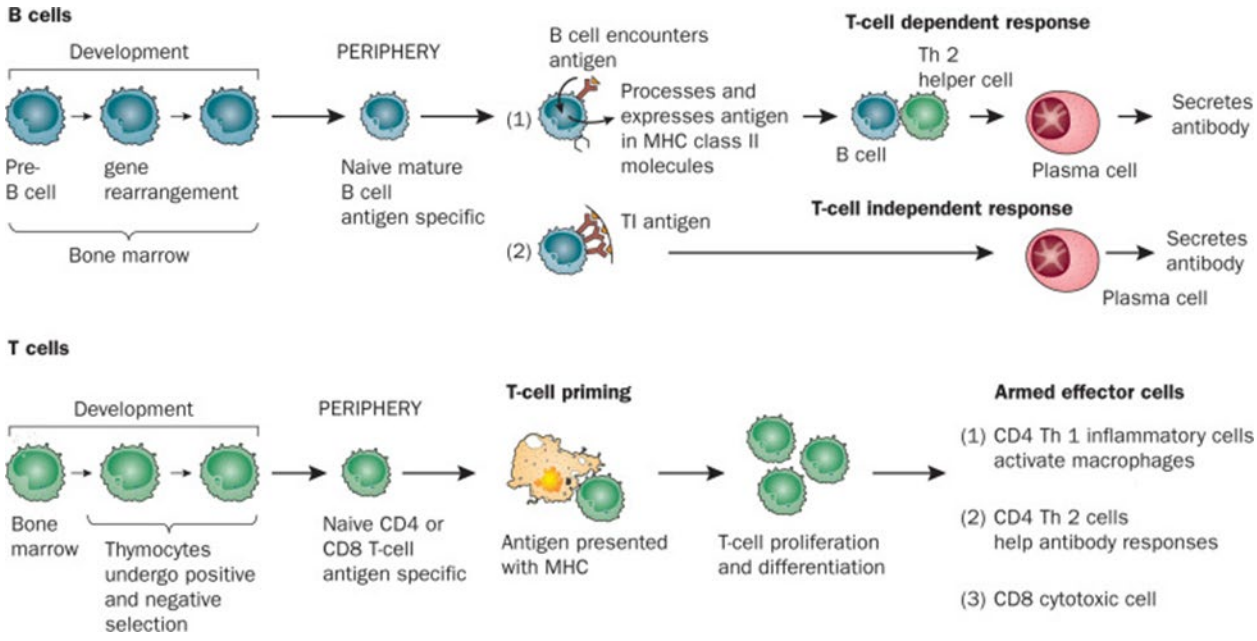


Fig 1.4. Adaptive T and B lymphocytes effectors (Parkin & Cohen, 2001).

1.3.3. T Lymphocytes

Because of their centrality in our study, we will focus now on a more detailed description of the role and functions of T lymphocytes. T lymphocytes have subsets mainly responsible for cell-mediated immunity (mainly CD8+) and others with activating and regulatory roles (mainly CD4+). Upon activation, naïve T cells initiate proliferation of the activated clones and their differentiation into effector and memory cells (Fabbri et al., 2003) Memory T cells can effectively mount an effector response in the wake of reinfection/reencounter with antigen and can be identified by the expression of the RO form of CD45 (Kaech & Cui, 2012). The adaptive response is self-limiting, in fact, persistent antigen exposure in the wake of chronic disease and/or cancer can compromise T cell function, rendering its effector response capability restricted with reduced proliferative capacity and expression of inhibitory receptors as PD-1 (Schietinger et al., 2016) (Wherry et al., 2007) (Wherry & Kurachi, 2015).

CD4+ and CD8+ T lymphocytes recognize cognate antigens when these are presented by APCs (as macrophages, dendritic cells, etc.) associated with specialized surface molecules on APCs, the Major Histocompatibility Complex (MHC) molecules. They are highly polymorphic and here we focus on the two main classes, class I and class II. A detailed description of the structure of MHC I and MHC II is beyond the scope of this introduction, suffice to say that they are protein complexes with a peptide-binding cleft, that binds the antigens peptides (Parkin & Cohen, 2001).

Class I MHC molecules are expressed on all nucleated cells and are mostly recognized by the TCRs of CD8+ T cells. Thus, any such cell infected by an intracellular pathogen or mutated hence generating tumor-associated antigens (TAAs) or autoantigens can be a target of CD8 T cells. Class II MHC molecules are expressed mainly on APCs, but can also be upregulated on other nucleated cells, and when they present peptidic antigens they activate TCRs of CD4 lymphocytes (von Andrian & Mackay, 2000). We will present more in detail CD8+ T cells that have been the object of this study.

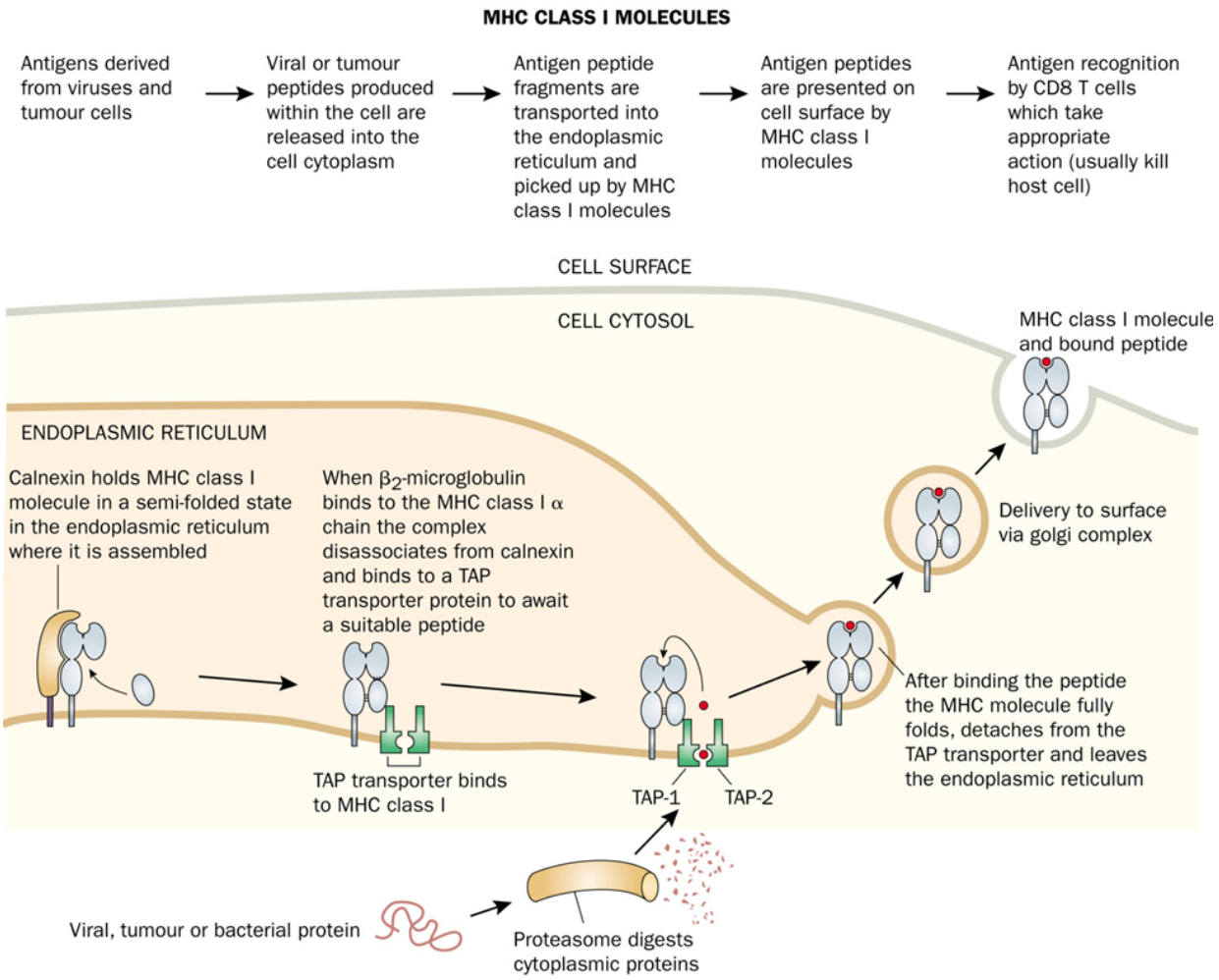


Fig 1.5. The pathway of endogenous antigen delivery to class I MHC molecules (Parkin & Cohen, 2001).

1.3.4. CD8+ T cells and Cytotoxic activity

CD8 T cells are at the forefront of host immunity against cancer especially in their cytotoxic role because they can kill malignant cells controlling and possibly eradicating malignancies (Tanaka et al., 1999). Upon activation, CD8+ T lymphocytes can differentiate into Cytotoxic T Lymphocytes (CTLs) that kill target cells through an intracellular enzymatic cascade (caspase cascade) resulting in apoptosis of the target cell. CTLs act either through 1) granzymes and perforins and 2) through the surface protein Fas-ligand. Perforins disrupt the integrity of the target cell plasma membrane and endosomal membranes, thereby facilitating the delivery of granzymes into the cytosol caspase cleavage.

Fas ligand binds a death-inducing receptor called Fas (CD95), present on the surface of the target cells. The interaction with Fas activates caspases and induces programmed cell death of the target cells (Mond et al., 1995) (Rosenberg & Huang, 2018).

1.4. T cells and cancer

Immune cells are a prominent part of the tumor microenvironment (TME) and have represented a very successful therapeutic target (Canning et al., 2019) (Hinshaw & Shevde, 2019), and together with others of its components has utmost importance the biological behavior and the metastatic potential of malignancies (Hanahan & Weinberg, 2011).

Because of the key role of cytotoxicity in immunity against cancer, most cancer immunotherapy efforts are dedicated to eliciting cellular immune responses against the tumors (Rosenberg et al., 2004). Three conditions are needed for the immunologic elimination of tumors: (i) adequate numbers of immune cells that recognize cognate antigens must be generated (ii) these cells must circulate to and penetrate the tumor, and (iii) the immune cells must be stimulated at the tumor site to proliferate and generate effector and memory clones (Overwijk et al., 2003).

This last point is particularly important since cancer had previously been considered a non-immunogenic disease (Stutman, 1975) (Pardoll, 2003) (Balkwill & Mantovani, 2001) (Karin et al., 2002). The evidence of the immunogenicity of malignant tissues and the description of immunosuppression by tumor cells and TMA components has paved the way for the development of immunotherapies and cancer vaccines. The last decade has witnessed the advent of immune checkpoint antibodies and programmed death-ligand (PD-1) treatment options (Luke & Ott, 2015). It is also clear nowadays that the tumor microenvironment can suppress the immune system through different mechanisms, and resistance to immune checkpoint treatment has been explained by experimental findings (Sharma et al., 2017).

A subset of CD8+ Memory cells that express the integrin CD103 has recently emerged as an important role player in cytotoxic response to cancer since this integrin gives confers T cells the capability to be retained in the tissues and also to better interact with their epithelial targets (Corgnac et al., 2018).

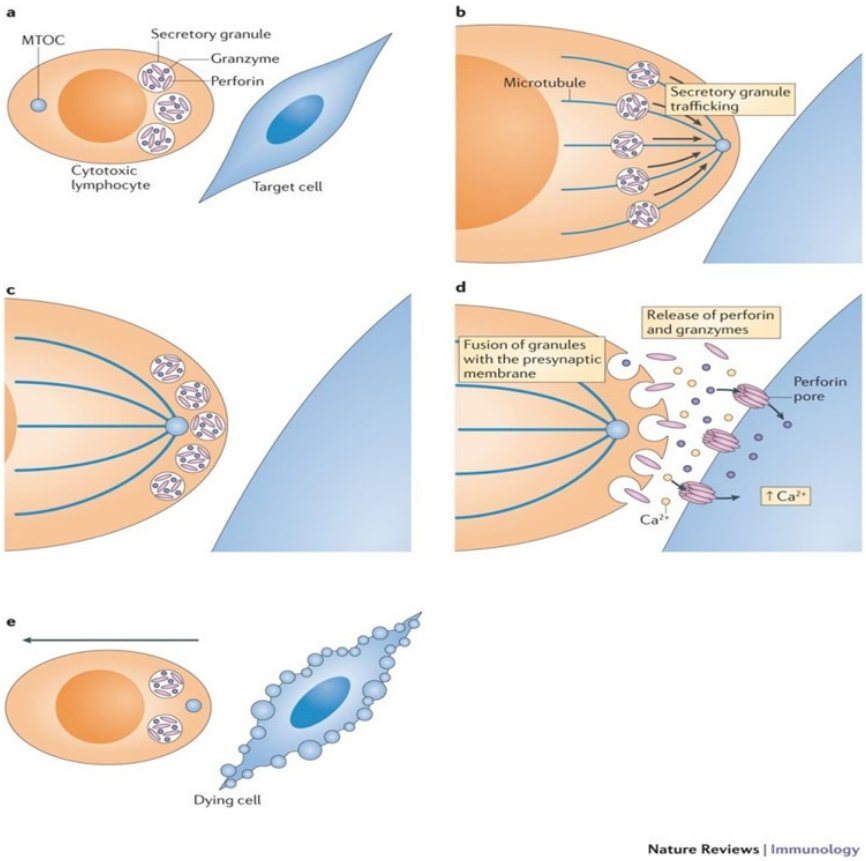


Fig 1.6. Interaction of a cytotoxic lymphocyte with a target cell (Voskoboinik et al., 2015).

1.5. The immune system and OSCC

As summarized by Feller et al, and discussed above, a chronic immune response can favor malignant transformation of the oral squamous lining (Feller et al., 2013). In the last decades though, a large body of research has explored and described the multiple roles immune cells can have in the TME and their importance in the biological behavior and the clinical progression of malignancies. This is true for OSCC as well as for other types of cancer as we can observe in the literature most relevant to our study summarized below.

OSCC of the tongue with a heavier immune infiltrate and moderate/high histological differentiation presented with a better diagnosis both in the Norwegian NOROC cohort (Bjerkli, Hadler-Olsen, et al., 2020), that is the same cohort we investigate in the present study.

High CD8s: CD4s ratio has been identified as an independent prognostic factor for Disease-Specific Survival (DSS) in a Head and Neck SSC cohort (Russell et al., 2013). In another study on a cohort of

Head and Neck SCC, Lechner et al, found that tumor-infiltrating lymphocytes were mostly of effector memory populations, and they expressed PD-1, PD-L1, and CTL-4, especially in smaller tumors (Lechner et al., 2017).

Boxberg et al, describe a high number of CD4+FOXP3+ lymphocytes as a good prognostic factor, defining tumors, the authors define as FOXP3 inflamed, and interestingly CD8+ lymphocytes hold no prognostic value in their investigation (Boxberg et al., 2019).

A recent meta-analysis reported that high infiltration of CD163+ macrophages and CD57 lymphocytes positively impacted overall survival in individuals affected by OSCC (Hadler-Olsen & Wirsing, 2019), while a meta-analysis by Huang et al, stated that a high number of tumors infiltrating CD8+ T cells and CD45RO+ T cells are also positive predictors of survival in OSCC patients (Huang et al., 2019).

This summary of published literature is by any means not exhaustive, but it helps to give a background for our study.

Due to its importance in the interaction between tumor and cells and immune cells we were interested in particular about CD103 positive subsets in OSCC, and recent work on a smaller cohort pointed to a positive prognostic association of CD103 expression in populations of CD8+ T cells and dendritic cells (Xiao et al., 2019).

2. Aims

2.1. General Aim of the Study

Here we focus on the description of residential T memory cells in the OSCCs of the tongue in NOROC (CD3+CD8+CD45RO). In particular, we wonder how many cells in this population are proliferating (KI67+) and how many do express granzyme B, thus exhibiting a cytotoxic potential. Since there could be differences between the intraepithelial and stromal compartments of the tumor tissues, we also stained for Pan Cytokeratin to identify the malignant epithelial islands.

To collect this data, we devised a protocol for multiplex immunofluorescence of the TMAs from the cohort that could be digitally imaged through scanning epifluorescence and analyzed for quantification.

2.2. Specific Aims

- Develop a protocol for sequential IHC staining (multiple immunofluorescence) of the NOROC OSCC of the tongue TMA cohort for CD3, CD8, CD45RO, Ki67, granzyme B, CD103, and Pan Cytokeratin.
- Determine the number of CD8+, CD103+, and CD8+CD103+ cells infiltrating the tissue.
- Determine the prognostic value of these cells in the OSCC of the tongue.
- Determine the number of CD8+CD103+CD3+ cells infiltrating the tissues by image overlapping.
- Determine the prognostic value of these cells in the OSCC of the tongue.
- Determine the number of CD8+CD103+CD3+ expresses also granzyme B by image overlapping.
- Determine the prognostic value of these markers in the OSCC of the tongue.
- Determine the number of CD8+CD103+CD3+ express also Ki67 by image overlapping.
- Determine the prognostic value of these markers in the OSCC of the tongue.
- Determine the number of CD8+CD103+CD3+granzyme B+Ki67 infiltrating the tissues by image overlapping.
- Determine the prognostic value of these markers in the OSCC of the tongue.

Aims a. to c. have been successfully achieved (due to time constraints) and are presented in the Results section.

3. Materials and Methods

3.1. NOROC

TMAs obtained from 123 cases of tongue OSCC part of the NOROC cohort were tested in this study. The cases with tissues and descriptive clinicopathological characteristics and five-year survival outcomes were diagnosed between the period of January 1st, 2005, and December 31st, 2009, and the last day of follow-up was June 15th, 2015. All patients had been followed up for at least 5 years after the end of their treatment (Bjerkli et al., 2019). Three patients whose cores were larger resulting in annotated area > 1.8 mm^2 were excluded from our statistical analyses, see also results.

3.2. Immunofluorescence

A new protocol modified from Bernstock et al, for multiplexing immunofluorescence was devised changing the stripping method according to Osman et al, (Bernstock et al., 2019) (Osman et al., 2013). Initially, each antibody was optimized singularly in chromogenic IHC according to the manufacturer's recommendations, and then antibodies were coupled for double staining according to requirements for antigen retrieval method and host so that one mouse primary antibody was coupled with a rabbit primary antibody. Human tonsil tissue was used as a positive and when the primary was omitted as a negative control.

The TMAs were tested for **CD8**, **CD103**, **CD45RO**, **CD3**, **KI67**, **Granzyme B,** and **Pan Cytokeratin**, and the sequential double stainings were CD8 + CD103, followed by CD45RO + CD3, then Ki67 + Granzyme, and lastly Pan Cytokeratin was stained singularly. The protocol is represented in flowchart 3.1 and explained more in detail in the results section (table 4.1.).

Briefly, the TMA sections were deparaffinized by immersion in xylene for 10 minutes and rehydrated by sequential immersion in 100% ethanol for 6 minutes, 96% ethanol for 3 minutes, and 70% ethanol for 3 minutes, and then dipped in distilled water.

For retrieval of antigen, the sections were submerged in pH 6.0 or pH 9.0 retrieval solution and heated at 950W for 8 minutes and then at 350W for 17 minutes in rounds 1-3, while they were incubated with Proteinase-K for 5 minutes at a dilution of 1:2 for the last round. Before incubation with the primary antibodies, the sections were incubated with 0.1M Glycine for 30 minutes and then blocked with 0.2% BSA/PBS for 20 minutes before applying primary antibodies in each round. After each round, a

stripping/denaturation of antibodies was performed by submerging the sections in the respective retrieval solution and heating at 950W for 2 minutes and then at 350W for 5.5 minutes.

For the first round of primary antibodies, the sections were stained with mouse monoclonal anti-CD8 (1:100/blocking solution) mixed with rabbit monoclonal anti-CD103 (1:50/blocking solution) after antigen retrieval in pH 6.0 antigen retrieval solution. Subsequently, they were stained with Mouse monoclonal anti-CD45RO (1:2000/blocking solution) and Rabbit monoclonal anti-CD3 in a (1:150/blocking solution) with antigen retrieval at pH 6.0; Mouse monoclonal anti-Ki67 (1:150/blocking solution) and Rabbit monoclonal anti-Granzyme B (1:250/blocking solution) with antigen retrieval at pH 9.0 and Mouse monoclonal anti-Pancytokeratin (1:200/blocking solution) with Protease-K retrieval.

For the staining with fluorescent secondary antibodies, slides were incubated after washing with a mix of Goat Anti-Rabbit IgG Alexa Fluor 594, and Goat Anti-Mouse IgG Alexa Fluor 488 in a dilution of 1:400 each in blocking solution at the end of each round, followed by counterstaining with DAPI in a dilution of 1:5000 for 5 minutes.

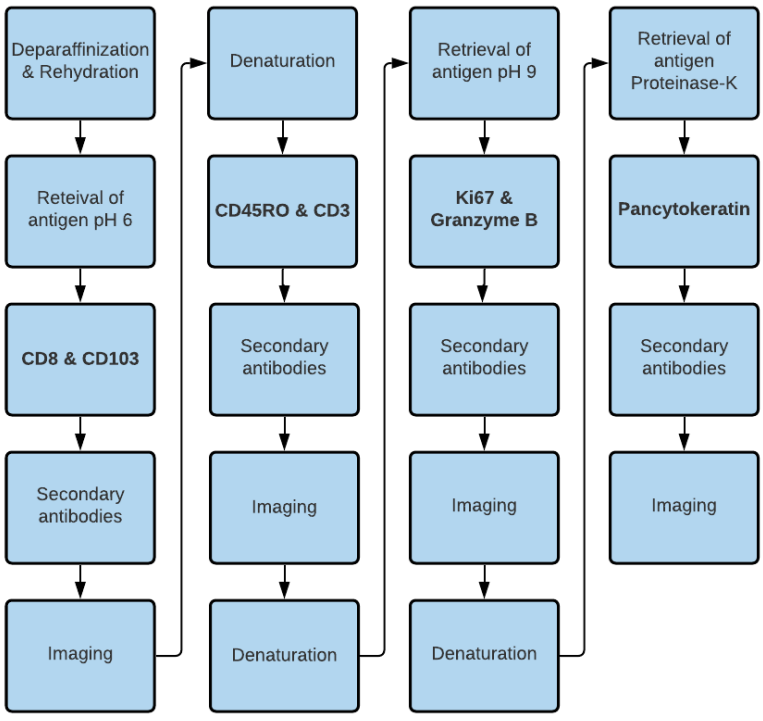


Fig 3.1. Flowchart for Immunofluorescence protocol

Antibody	Host	Clone	Supplier	Epitope retrieval	Dilution
CD8	Mouse	C8/144B	Dako	MW 25 min, pH 6	1:100
CD103	Rabbit	ITGAE	Sigma	MW 25 min, pH 6	1:50
CD45RO	Mouse	UCH-L1	Abcam	MW 25 min, pH 6	1:2000
CD3	Rabbit	SP162	Abcam	MW 25 min, pH 6	1:150
Ki67	Mouse	MIB-1	Dako	MW 25 min, pH 9	1:150
Granzyme B	Rabbit	EPR20129-217	Abcam	MW 25 min, pH 9	1:250
Pan Cytokeratin	Mouse	MNF116	Dako	Proteinase-K incubation 1:2	1:100

Table 3.1. Primary Antibodies

Antibody	Host	Conjugation	Supplier	Dilution
Anti-mouse IgG	Goat	Alexa Fluor 488	Abcam	1:400
Anti-rabbit IgG	Goat	Alexa Fluor 594	Abcam	1:400

Table 3.2. Secondary Antibodies

3.3. Imaging

Imaging of the stained TMAs was achieved at the Molecular Imaging Centre (MIC), Department of Biomedicine, The University of Bergen, using an Olympus VS120 S6 Slide scanner. The microscope can fit up to 6 slides simultaneously. It can provide magnification of 2x, 4x, 10x, 20x, and 40x and our slides were scanned at 20x magnification with a UPlanSApo 0,75 objective (pixel size 0,33 um). The Fluorescence filters available are blue, green, red, and far-red (see table 3.3 below), we used DAPI, FITC, and TRITC channels. The exposure time for each channel was set at 5ms, 150ms, and 100ms, respectively.

Filter	*Excitation*	*Emission*	*Filter type*
DAPI	340-380	430-480	Quad filter
FITC	480-500	507-543	
TRITC	543-568	579-631	
CY5	630-660	669 -741	
CFP	418-442	460-500	Single filter

Table 3.3. Available fluorescence filters on Olympus VS120 S6 (https://www.uib.no/en/rg/mic/120747/olympus-vs120-s6-slide-scanner)

3.4. Image Analysis and Quantification

The software QuPath was used for image analysis and quantification of CD8+ and CD103+ cells. QuPath is free software that opens raw format image files acquired from the Olympus VS120 S6 slide scanner and permits quantification (Bankhead et al., 2017).

The TMA dearrayer was used to detect TMA cores whenever possible. The cells were detected and measured by cell analysis via positive cell detection after setting classifiers.

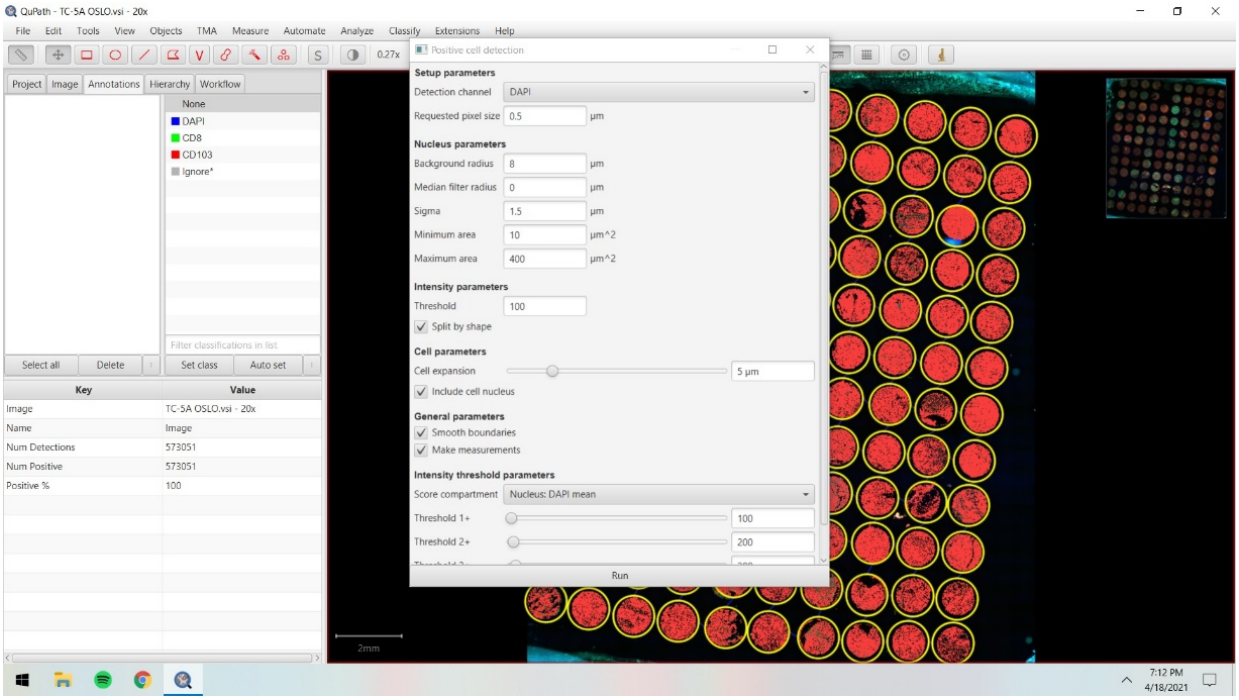


Fig 3.2. Detecting and measuring cells (screenshot of QuPath software).

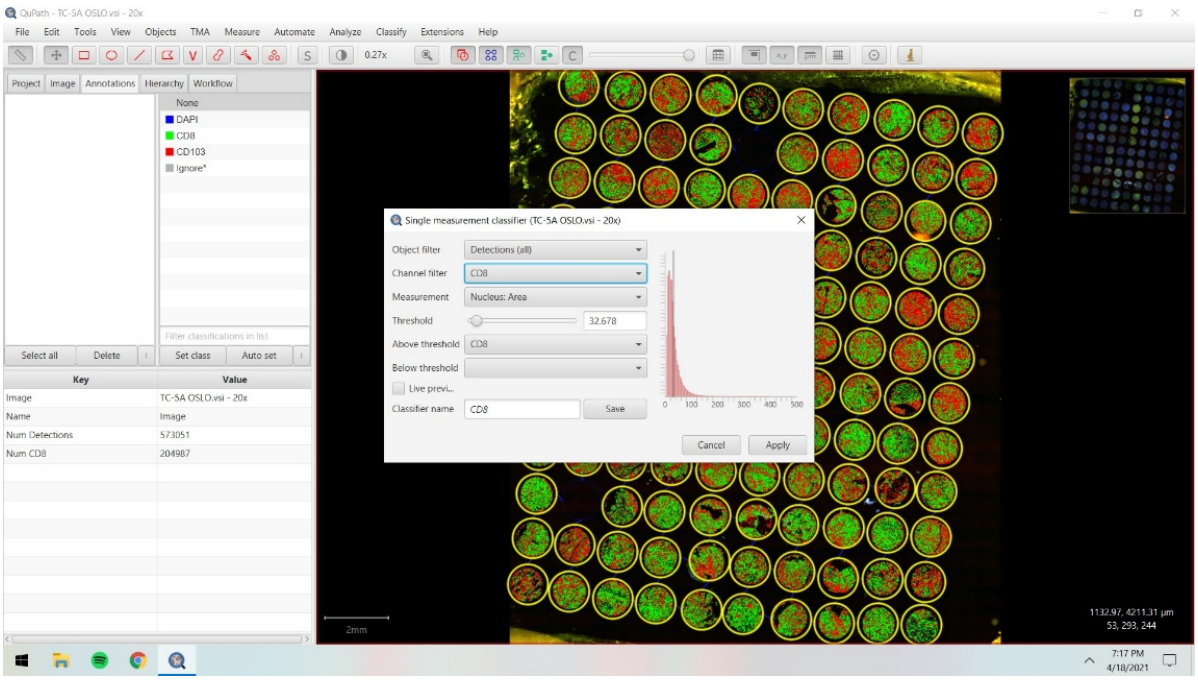


Fig 3.3. Measurement classification (screenshot of QuPath software)

3.5. Statistical Methods

After quantification, data from each core were exported in Excel sheets identified with the anonymized patient ID. Statistical analysis of the data, along with the patient's clinical and pathological data was achieved using IBM SPSS 27 statistical analysis software.

Initially, descriptive analysis was performed on the data to give us information regarding the patient cohort. This data was then used to form pie charts to describe the information using Microsoft Excel. As the data was skewed, non-parametric analysis was performed, to compare CD8+ and CD103+ cells between groups based on clinical and pathological data, using the Kruskal-Wallis test for comparison of more than two independent groups, while the Mann-Whitney test was used to compare two independent groups. Survival analysis was also performed on the clinical and pathological data using the Kaplan-Meier Survival test.

4. Results

4.1. Successful Multiplex Staining of Seven Markers on the Same Tissue

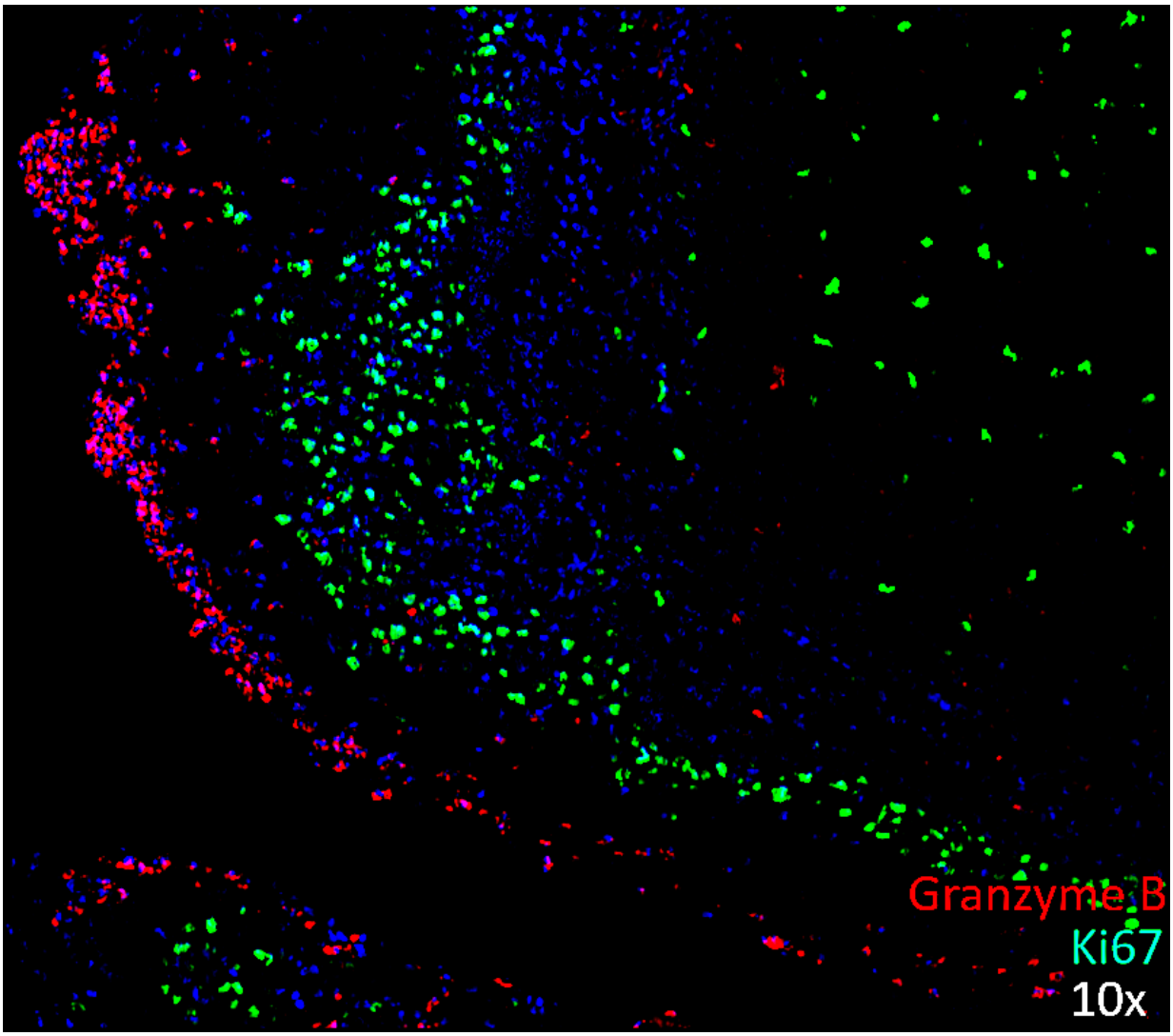


Fig 4.1. Human Tonsil Tissue stained with Granzyme B and Ki67

Initially, the antibodies, both primary and secondary antibodies, were titrated for different dilutions to choose the best dilution. This was particularly in the case of the primary antibody of **CD3**. The dilution of 1:150 showed the best results on the human tonsil tissue and thus that dilution was selected for the staining of the TMA slides. Also, for fluorescence, the secondary antibody in the dilution of 1:400 showed the best results, therefore that was the selected dilution for the main round of staining and imaging.

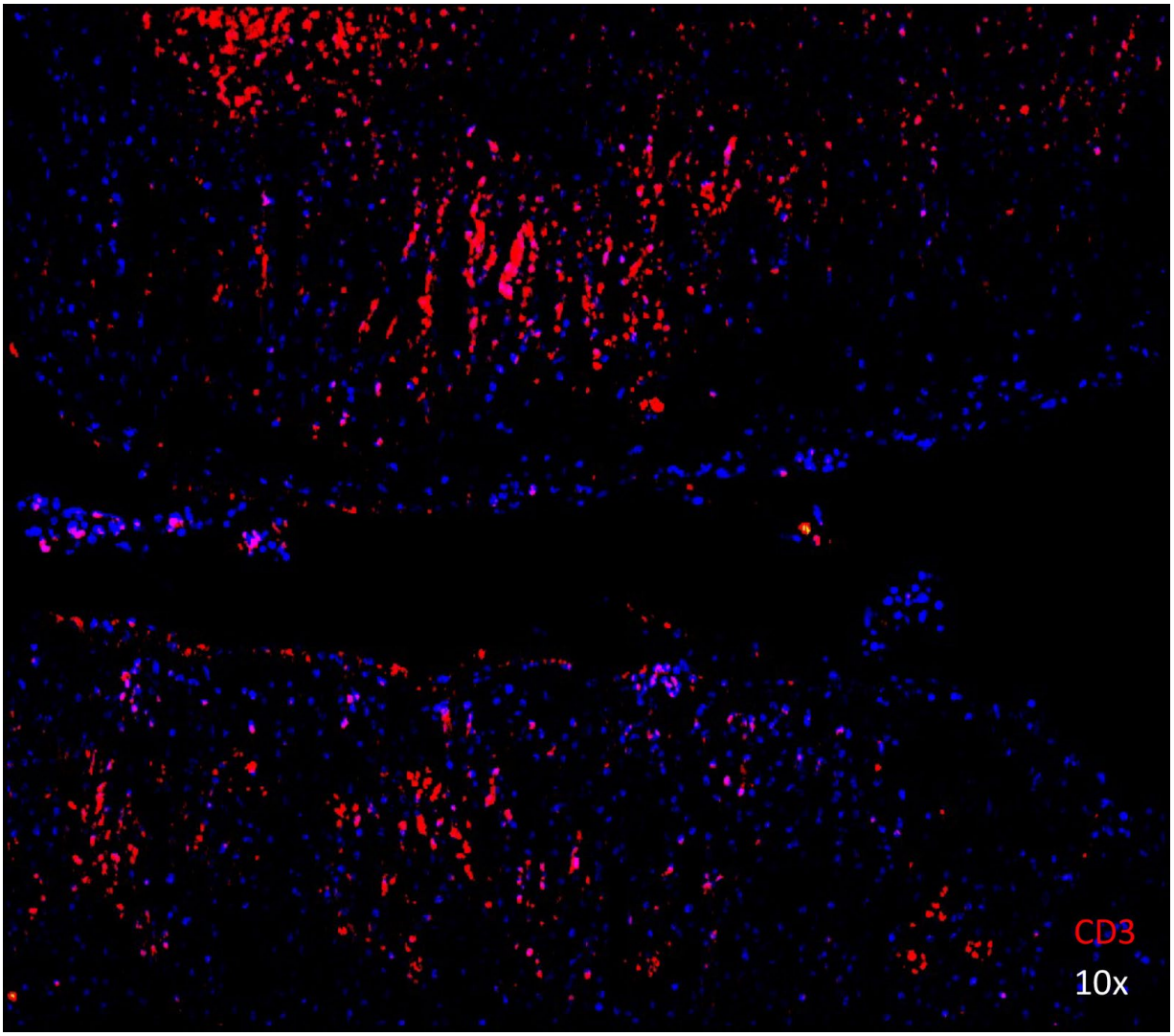


Fig 4.2. Human Tonsil Tissue stained with Primary antibody CD3

Another area where the protocol had to be modified from company recommendations to obtain optimum results was in the case of Pan Cytokeratin. For retrieval of antigen, instead of heat retrieval by placing the tissue in pH 9 buffer solution and heating in the microwave for 25 minutes, it was established that better results could be achieved by using a non-heat method. This was achieved by incubating the tissue with Proteinase-K for 5 minutes at a dilution of 1:2.

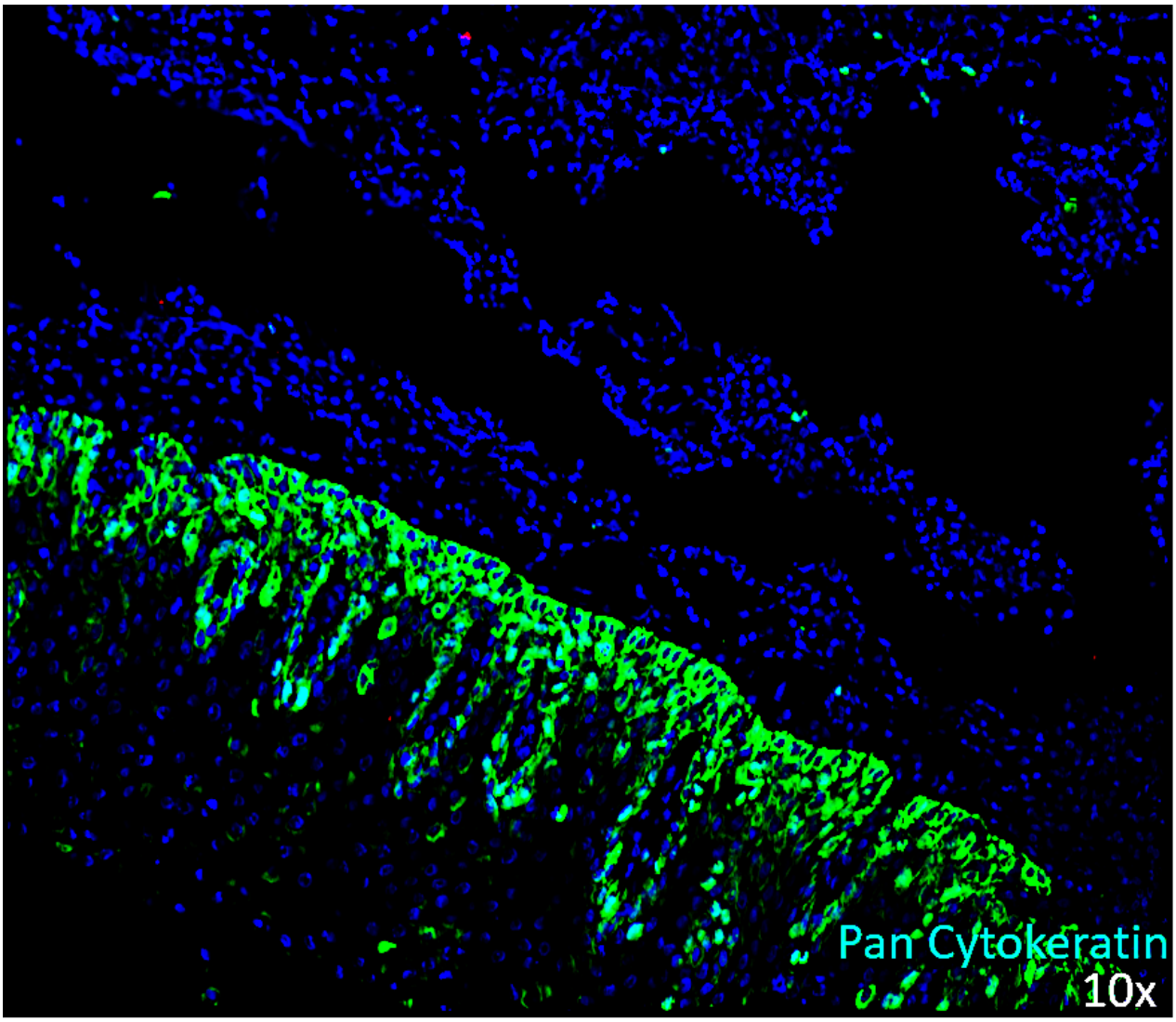


Fig 4.3. Human Tonsil Tissue stained with primary antibody Pan Cytokeratin.

Protocol		
Xylene	In ventilation	2 x 5 min
Ethanol	Absolut 100%	2 x 3 min
Ethanol	96%	3 min
Ethanol	70%	3 min
Distilled water		Rinse
Retrieval of the antigen	Buffer pH 6 Microwave 950 W 8 min, 350 W 17 min	25 min total
Cooling	Leave on the bench for cooling	15-20 min

Wash	Slightly pouring tap water	Until room temperature
Put sections in wash buffer, take out one or two slides at a time, wipe around the tissue and draw around the tissue sections with Hydrophobic Pen (in ventilation). Put the slides back in the wash buffer after drawing with the pen.		
Incubate	0.1M Glycine	30 min
Shake off most of the liquid		
Blocking	0.2 % BSA in PBS solution	20 min
Shake off most of the serum		
Primary antibody	CD8 1:100 and CD103 1:50 in blocking solution	30 min
Wash	TBST (wash buffer)	10 min (shaking)
Secondary antibody	Anti-mouse and anti-rabbit in blocking solution	60 min
Wash	TBST (wash buffer)	10 min (shaking)
DAPI	1:5000	5 min
Mount sections with cover Overnight in the fridge before imaging		
Denaturation (stripping)	Buffer pH 6 Microwave 950 W 2 min, 350 w 5.5 min	7.5 min total
Cooling	Let it be on bench for cooling	10 min
Wash	TBST	10 min (Shaking)
Put sections in wash buffer, take out one or two slides at the time, wipe around the tissue and draw around the tissue sections with Hydrophobic Pen (in ventilation). Put the slides back in wash buffer after drawing with the pen.		
Incubate	0.1M Glycine	30 min
Shake off most of the liquid		
Blocking	0.2 % BSA in PBS solution	20 min
Shake off most of the serum		
Primary antibody	CD45RO 1:2000 and CD3 1:150 in blocking solution	30 min
Wash	TBST (wash buffer)	10 min (shaking)

Secondary antibody	Anti-mouse and anti-rabbit in blocking solution	60 min
Wash	TBST (wash buffer)	10 min (shaking)
DAPI	1:5000	5 min
Mount sections with cover Overnight in the fridge before imaging		
Denaturation (stripping)	Buffer pH 6 Microwave 950 W 2 min, 350 w 5.5 min	7.5 min total
Cooling	Let it be on bench for cooling	10 min
Wash	TBST	10 min (Shaking)
Retrieval of the antigen	Buffer pH 9 Microwave 950 W 8 min, 350 W 17 min	25 min total
Cooling	Let it be on the bench for cooling	15-20 min
Wash	Slightly pouring tap water	Until room temperature
Put sections in wash buffer, take out one or two slides at the time, wipe around the tissue and draw around the tissue sections with Hydrophobic Pen (in ventilation). Put the slides back in wash buffer after drawing with the pen.		
Incubate	0.1M Glycine	30 min
Shake off most of the liquid		
Blocking	0.2 % BSA in PBS solution	20 min
Shake off most of the serum		
Primary antibody	Ki67 1:150 and Granzyme B 1:250 in blocking solution	30 min
Wash	TBST (wash buffer)	10 min (shaking)
Secondary antibody	Anti-mouse and anti-rabbit in blocking solution	60 min
Wash	TBST (wash buffer)	10 min (shaking)
DAPI	1:5000	5 min
Mount sections with cover Overnight in fridge before imaging		
Denaturation (stripping)	Buffer pH 9 Microwave 950 W 2 min, 350 w 5.5 min	7.5 min total
Cooling	Let it be on bench for cooling	10 min

Wash	TBST	10 min (Shaking)
Retrieval of the antigen	Proteinase-K incubation 1:2 (Ready to use) in blocking solution	5 min
Put sections in wash buffer, take out one or two slides at the time, wipe around the tissue and draw around the tissue sections with Hydrophobic Pen (in ventilation). Put the slides back in wash buffer after drawing with the pen.		
Incubate	0.1M Glycine	30 min
Shake off most of the liquid		
Blocking	0.2 % BSA in PBS solution	20 min
Shake off most of the serum		
Primary antibody	Pancytokeratin 1:100 in blocking solution	30 min
Wash	TBST (wash buffer)	10 min (shaking)
Secondary antibody	Anti-mouse in blocking solution	60 min
Wash	TBST (wash buffer)	10 min (shaking)
DAPI	1:5000	5 min
Mount sections with cover Overnight in the fridge before imaging		

Table 4.1. Complete Protocol for Immunofluorescence of TMA slides.

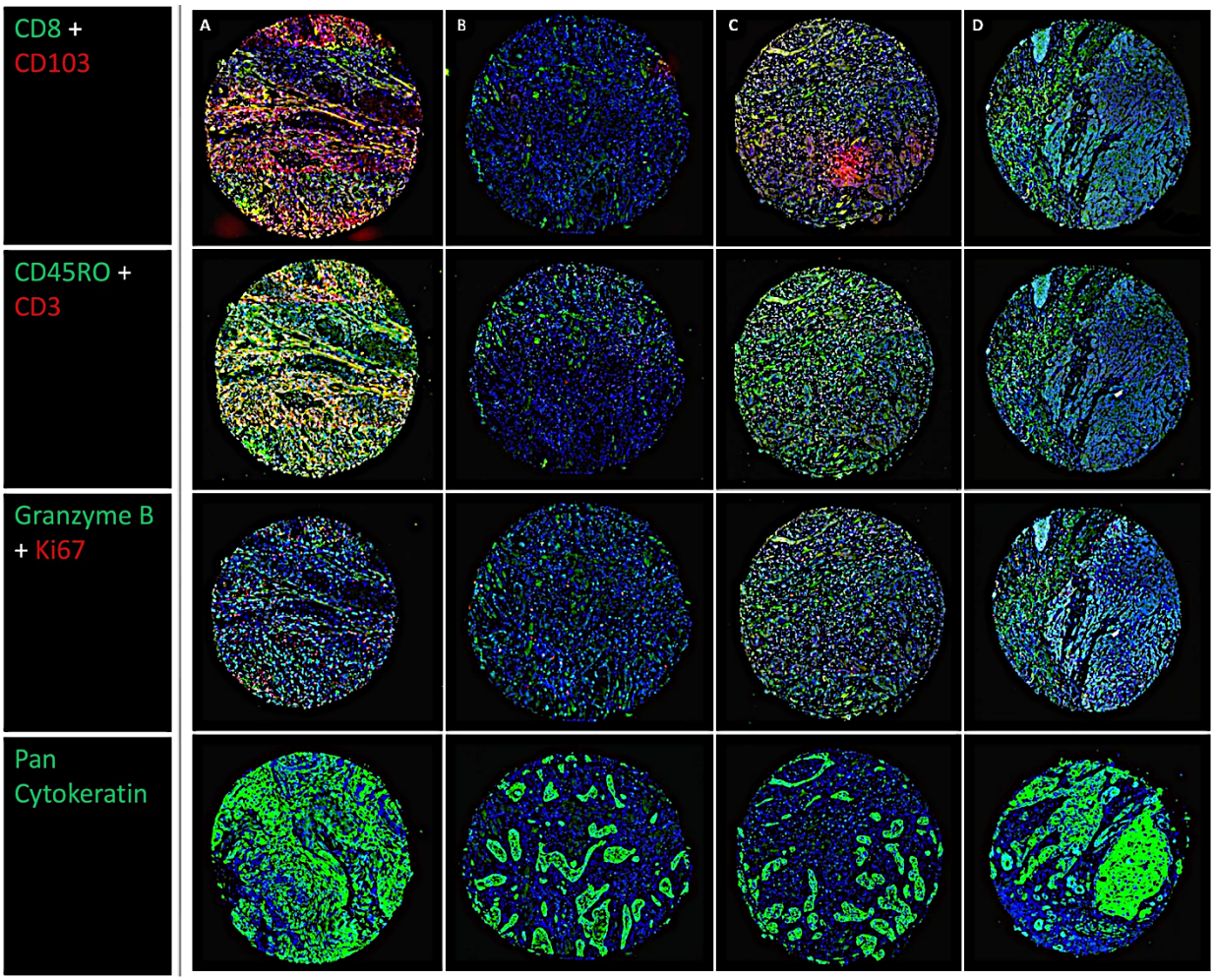


Fig 4.4. TMA Cores with multiplex staining. A. TMA i Centre, B. TMA i Front, C. TMA ii Centre, D. TMA ii Front

4.2. Criteria for Analysis

Two important questions arose while attempting an analysis of the data: 1) Was standardization needed? and 2) How to choose which of the cores would be included since each patient could have up to four cores and most patients had cores from tumor center areas and tumor front areas.

The TMA dearrayer function in the QuPath software was used to identify and annotate the TMA cores, unfortunately, we experienced the software was unable to identify the TMA cores in one slide where contamination caused a strong fluorescent object. Hence the cores had to be marked individually, and a few had a larger annotation area. These were excluded from subsequent analysis since the used annotation

area was a form of standardization of results between all core samples. In particular, descriptive statistics based on the area of cores, and cores larger than 1.8mm^2 were excluded in one TMA slide.

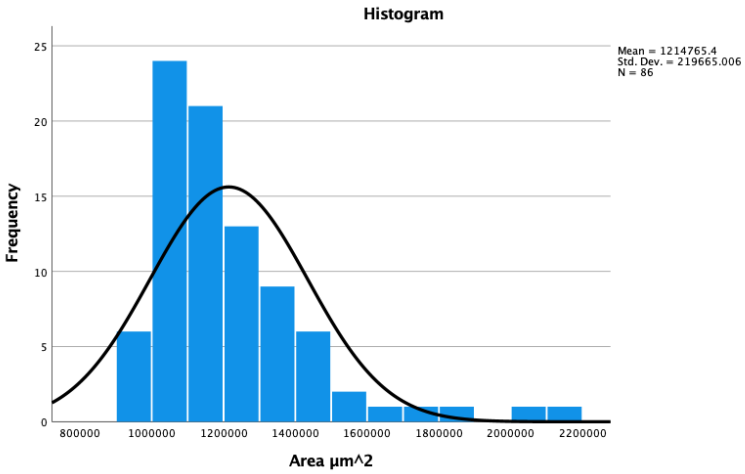


Fig 4.5. Frequency of TMA cores based on the area (µm^2)

To decide which tumor cores should be included for analysis, statistical analysis was performed on cores from both the tumor center and tumor front of the same patient, based on the number of detections of positive cells. After analysis of part of the cohort, it was observed that there was no significant difference between the number of detections of positive cells in the cores from both locations of the same tumors. Therefore, the average mean of detections was acquired from a combination of all tumor cores of each patient, and this was decided to be the data that would be used for further analysis against the clinical and pathological data of the patient.

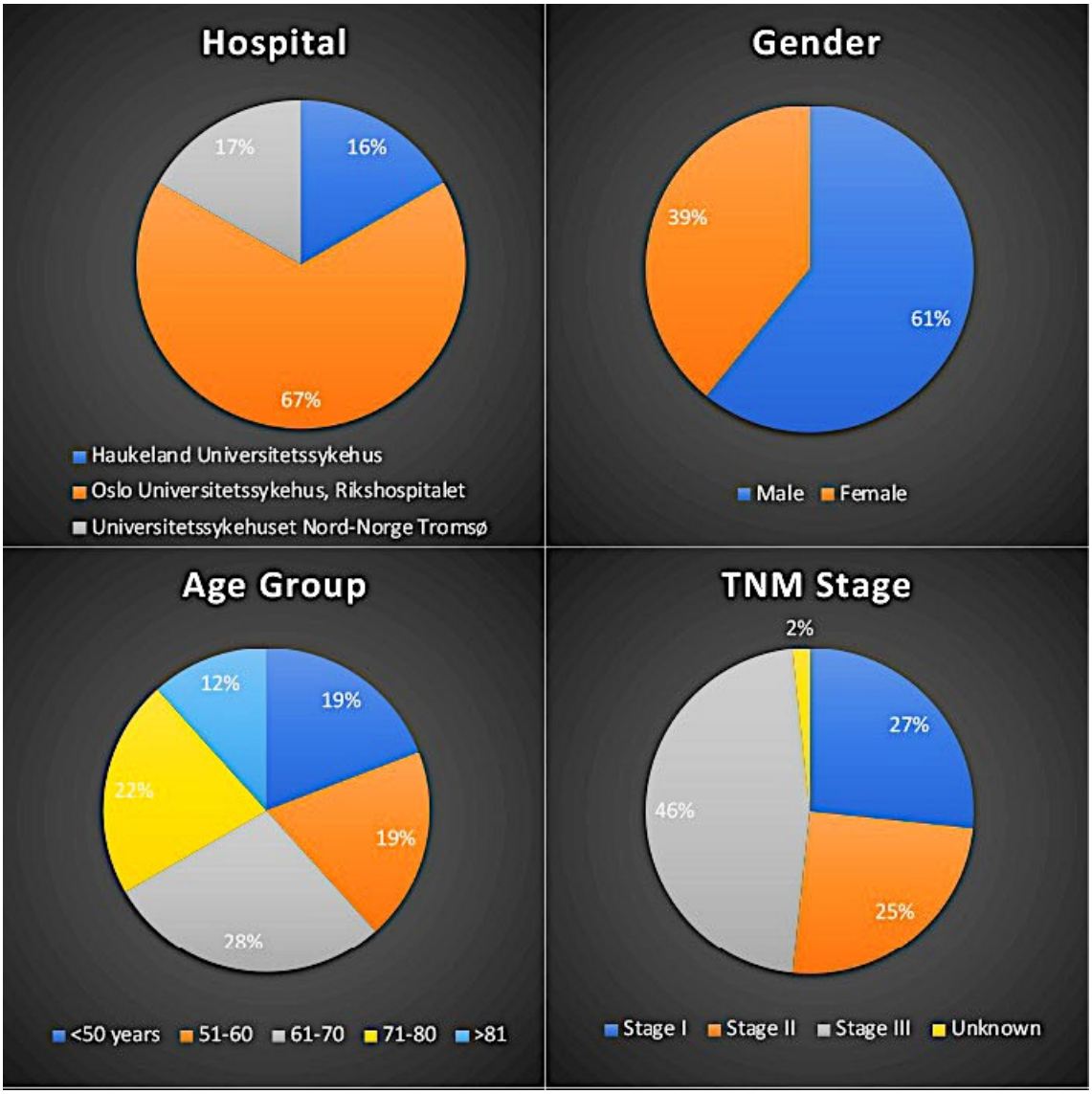
4.3. Characteristics of Norwegian OSCCT Patients

This research was based on tissue samples from 120 oral cancer patients from Norway. These tissue samples were acquired from the NOROC study. All of these tissue samples were from patients diagnosed with OSCC of the tongue.

The majority of the samples were from Oslo University Hospital, Rikshospitalet (66.7%). The male to female ratio was found to be 1: 0.64. Most of the patients were above 50 years of age at the time of diagnosis (80.8%). Analysis of survival data showed that 56.8% of the patients survived 5 years after diagnosis. Almost half of the patients (47%) were diagnosed with TNM Stage III, none of the patients included in this study had TNM Stage IV, and only 26.3% of them had already lymph node metastasis at

the diagnosis time. However, 22.6% of the patients had recurrent regional metastasis during the follow-up period, and 6.4% had recurrent distant metastasis.

The available data indicated that only 25.2% of the patients had never smoked and only 10% had never consumed alcohol. It should be noted that there was a significant amount of missing data on alcohol consumption (40.8%). An overview of the cohort characteristics is presented in figure 4.3.



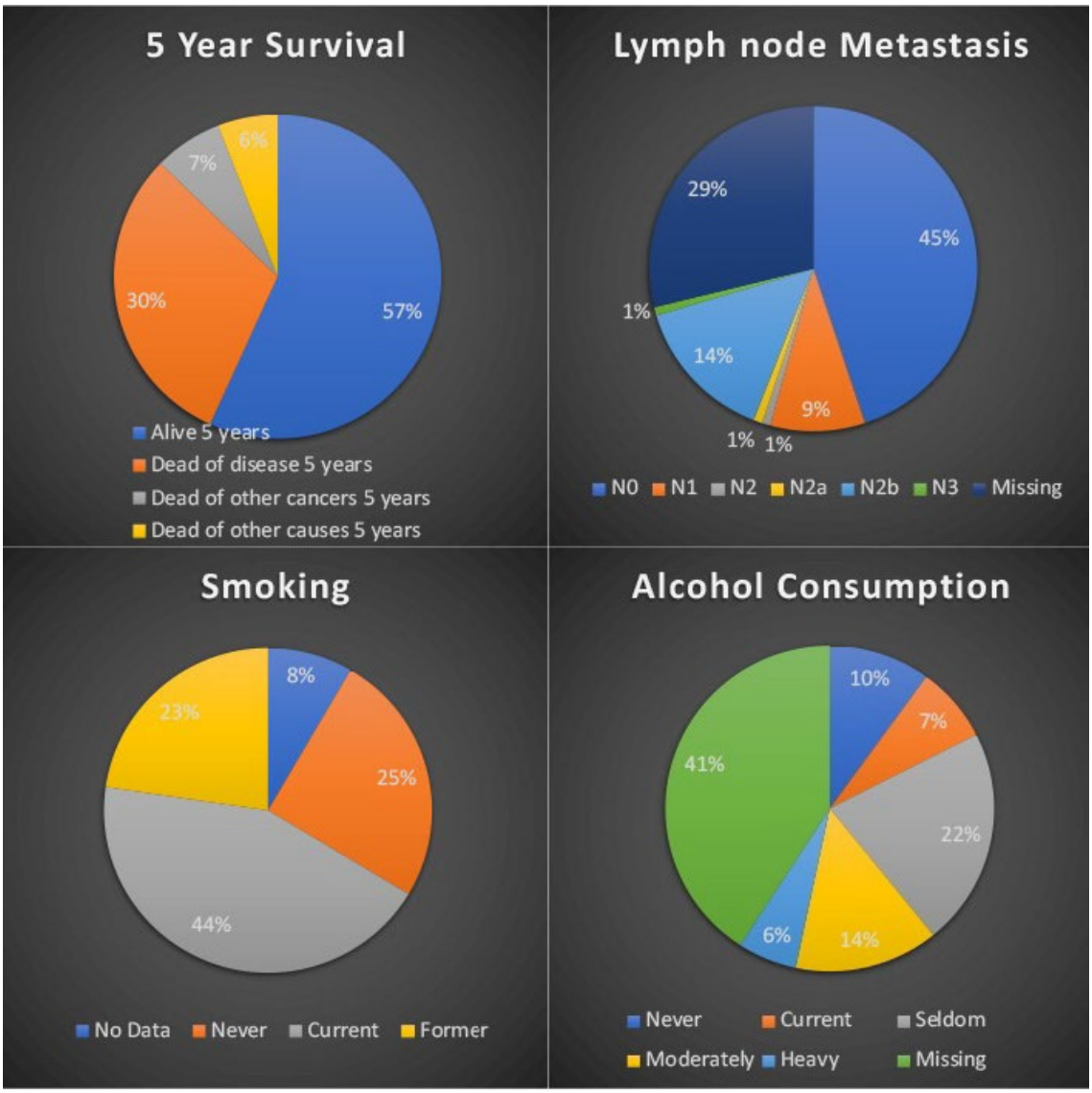


Fig 4.6. Cohort Description of Norwegian OSCCT patients.

4.4. Frequency of CD8+ and CD103+ cells infiltration in OCT

Although CD8 and CD103 are not only expressed by T cells, CD8+ cells and double-positive cells will be referred to as T cells in this study.

CD8+ and CD103+ cells were detected in the majority (84.2% and 70.8% respectively) of the samples included in this study. Positive cells were counted based on the expression of either CD8+ and CD103+ individually, or as a combination of them. Nineteen samples (15.8%) showed no expression of CD8+, while thirty-five (29.2%) samples had no CD103+ signal. Interestingly, only two cases were lacking expression of both markers, while cells that co-expressed both CD8+ and CD103+ were detected in the

majority of the samples (90.8%). The mean and standard deviations of the numbers of cells expressing the markers under investigation are presented in Table 4.2.

	N	Minimum	Maximum	Mean	Std. Deviation
AverageCD8	120	0	708	71.28	111.088
AverageCD103	120	0	4018	282.52	582.463
AverageCD8CD103	120	0	2492	365.64	584.296

Table 4.2. Expression of cells of CD8, CD103, and CD8+CD103.

4.5. Prognostic Value of CD8+ and CD103+ cells

4.5.1. Abundance of CD8+ and CD103+ cells Subpopulation is Associated with Specific pathological OSCC Phenotype

Differentiation of Whole Tumor

Data analysis indicated that the numbers of CD8+ T cells were different between tumors based on the WHO classification of differentiation of tumors (Kruskal-Wallis, p-value = 0.025) figure 4.7.

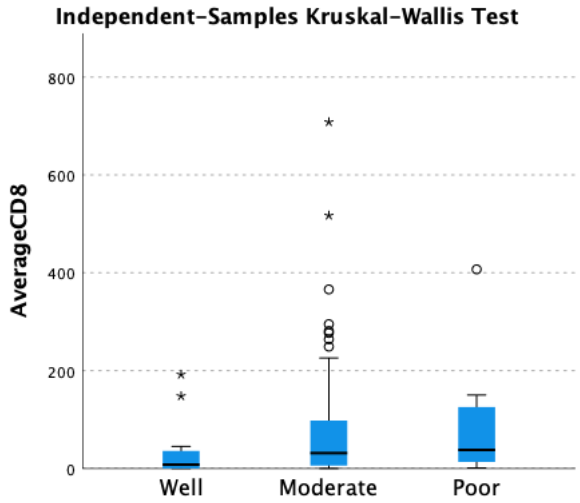


Fig 4.7. Expression of CD8+ cells according to Differentiation of the whole tumor.

Differentiation, WHO classification, whole tumor		N	Minimum	Maximum	Mean	Std. Deviation
Well	AverageCD8	21	0	192	27.95	50.072
Moderate	AverageCD8	86	0	708	78.05	119.223
Poor	AverageCD8	11	1	407	92.09	118.673

Table 4.3. Comparison of CD8+ cells according to Differentiation of the whole tumor.

Further analysis indicated that several CD8+ cells were found to be significantly higher in patients that had poor differentiation in the whole tumor according to WHO classification, as compared to patients who had well-differentiated tumors (Mann-Whitney, p-value = 0.034) table 4.4, but not in comparison to moderately differentiated ones (not shown).

Test Statistics[a]

	AverageCD8
Mann-Whitney U	62.500
Wilcoxon W	293.500
Z	-2.120
Asymp. Sig. (2-tailed)	.034
Exact Sig. [2*(1-tailed Sig.)]	.034[b]

a. Grouping Variable: 1Differentiation, WHO classification, whole tumor

b. Not corrected for ties.

Table 4.4. Comparison of CD8+ cells in two groups using the Mann-Whitney test.

Degree of Keratinization

Analysis showed a variation in distribution of CD8+ and CD103+ cells between tumors with variable degree of keratinization (Kruskal-Wallis, p-value of CD8+ = 0.003, p-value of CD103+ = 0.017) figure 4.8.

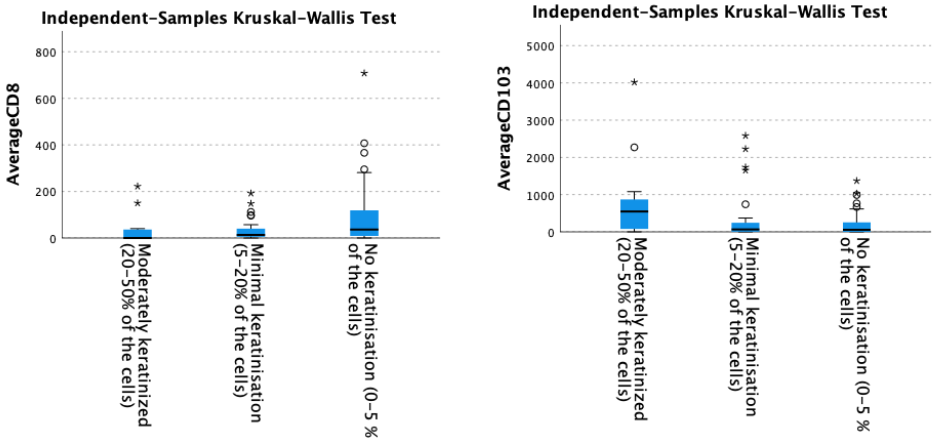


Fig 4.8. Distribution of T-cells based on Degree of Keratinization.

Keratinization is associated with transformation and increased malignant potential of oral squamous epithelial cells, the presence of CD8+ cells was much higher in tumor tissue with no keratinization (mean = 85.64, SD = 121.388), compared to tumor tissue with keratinization (Mann-Whitney, p-value = 0.007) table 4.6.

Descriptive Statistics

Degree Of Keratinization		N	Minimum	Maximum	Mean	Std. Deviation
Moderately keratinized (20–50% of the cells)	AverageCD8	13	0	222	35.46	69.808
	Valid N (listwise)	13				
Minimal keratinisation (5–20% of the cells)	AverageCD8	31	0	192	33.13	47.600
	Valid N (listwise)	31				
No keratinisation (0–5 % of the cells)	AverageCD8	70	0	708	85.64	121.388
	Valid N (listwise)	70				

Table 4.5. Comparison of CD8+ cells according to Degree of Keratinization.

Test Statistics[a]

	AverageCD8
Mann–Whitney U	239.500
Wilcoxon W	330.500
Z	-2.706
Asymp. Sig. (2–tailed)	.007

a. Grouping Variable: Degree Of Keratinization

Table 4.6. Comparison of CD8+ cells in two groups using the Mann-Whitney test.

Keratinization may also be used as a diagnostic tool and prognostic marker. There was an increased presence of CD103+ cells in moderately keratinized cells in the tumor tissue (mean = 807.38, SD = 1149.775), compared to tumor tissue with no keratinization (Mann-Whitney, p-value = 0.004) table 4.8.

Descriptive Statistics

Degree Of Keratinization		N	Minimum	Maximum	Mean	Std. Deviation
Moderately keratinized (20–50% of the cells)	AverageCD103	13	0	4018	807.38	1149.775
	Valid N (listwise)	13				
Minimal keratinisation (5–20% of the cells)	AverageCD103	31	0	2582	359.42	692.291
	Valid N (listwise)	31				
No keratinisation (0–5 % of the cells)	AverageCD103	70	0	1371	169.99	271.948
	Valid N (listwise)	70				

Table 4.7. Comparison of CD103+ cells according to Degree of Keratinization

Test Statistics[a]

	AverageCD103
Mann-Whitney U	231.000
Wilcoxon W	2716.000
Z	-2.846
Asymp. Sig. (2-tailed)	.004

a. Grouping Variable: Degree Of Keratinization

Table 4.8. Comparison of CD103+ cells in two groups using Mann-Whitney test.

Nuclear Polymorphism of Whole Tumor

Analysis showed a variation in distribution of CD103+, and combination of CD8+ and CD103+ cells between tumors with variable nuclear polymorphism of whole tumor (Kruskal-Wallis, p-value of CD103+ = <0.001, p-value of CD8CD103+ = 0.018) figure 4.9.

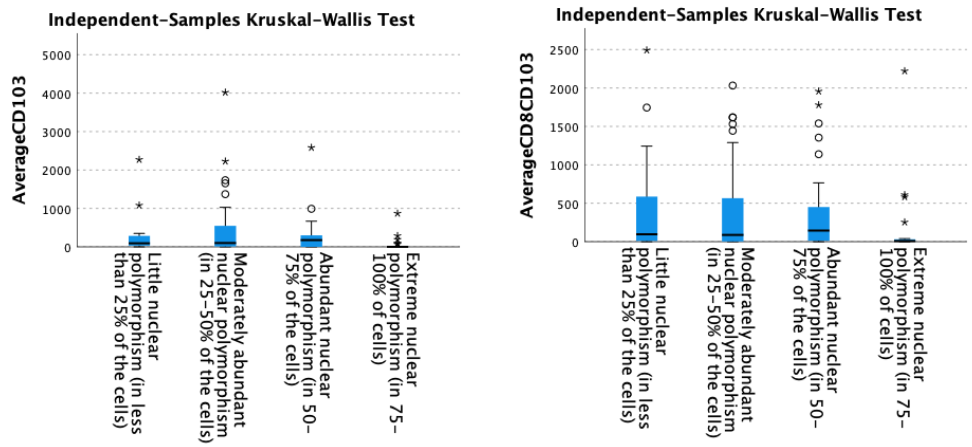


Fig 4.9. Expression of CD103+ and CD8CD103+ cells according to Nuclear Polymorphism.

Nuclear polymorphism is a common change in the genetic code in DNA. Polymorphisms can have a harmful effect and increase the risk of cancer. There was a much higher presence of CD103+ cells in tumor tissue having little nuclear polymorphism (mean = 280.42, SD = 541.692), compared to tumor tissue having extreme nuclear polymorphism, regarding the whole tumor (Mann-Whitney, p-value = 0.002) table 4.10.

Descriptive Statistics

5Nuclear polymorphism, whole tumor:		N	Minimum	Maximum	Mean	Std. Deviation
Little nuclear polymorphism (in less than 25% of the cells)	AverageCD103	19	0	2269	280.42	541.692
	Valid N (listwise)	19				
Moderately abundant nuclear polymorphism (in 25-50% of the cells)	AverageCD103	42	0	4018	436.67	776.249
	Valid N (listwise)	42				
Abundant nuclear polymorphism (in 50-75% of the cells)	AverageCD103	29	0	2582	285.38	503.665
	Valid N (listwise)	29				
Extreme nuclear polymorphism (in 75-100% of cells)	AverageCD103	26	0	869	61.46	178.196
	Valid N (listwise)	26				

Table 4.9. Comparison of CD103+ cells according to Nuclear Polymorphism of the whole tumor.

Test Statistics[a]

	AverageCD103
Mann-Whitney U	119.000
Wilcoxon W	470.000
Z	-3.059
Asymp. Sig. (2-tailed)	.002

a. Grouping Variable: 5Nuclear polymorphism, whole tumor:

Table 4.10. Comparison of CD103+ cells in two groups using Mann-Whitney test.

Similarly, there was a much higher presence of a combination of CD8+ and CD103+ cells in tumor tissue having little nuclear polymorphism (mean = 462.26, SD = 709.619), compared to tumor tissue having extreme nuclear polymorphism, regarding the whole tumor (Mann-Whitney, p-value = 0.013) table 4.12. This might indicate that a tumor environment with an abundant combination of CD8+ and CD103+ cells could lead to reduced nuclear polymorphism in OSCC as well.

Descriptive Statistics

5Nuclear polymorphism, whole tumor:		N	Minimum	Maximum	Mean	Std. Deviation
Little nuclear polymorphism (in less than 25% of the cells)	AverageCD8CD103	19	0	2492	462.26	709.619
	Valid N (listwise)	19				
Moderately abundant nuclear polymorphism (in 25-50% of the cells)	AverageCD8CD103	42	0	2032	413.55	598.279
	Valid N (listwise)	42				
Abundant nuclear polymorphism (in 50-75% of the cells)	AverageCD8CD103	29	0	1957	421.97	577.198
	Valid N (listwise)	29				
Extreme nuclear polymorphism (in 75-100% of cells)	AverageCD8CD103	26	0	2221	149.88	452.905
	Valid N (listwise)	26				

Table 4.11. Comparison of CD8CD103+ cells according to Nuclear Polymorphism of the whole tumor.

Test Statistics[a]

	AverageCD8 CD103
Mann-Whitney U	139.500
Wilcoxon W	490.500
Z	-2.474
Asymp. Sig. (2-tailed)	.013

a. Grouping Variable: 5Nuclear polymorphism, whole tumor:

Table 4.12. Comparison of CD8CD103+ cells in two groups using the Mann-Whitney test.

Nuclear Polymorphism of most invasive layers

Data analysis indicated that the numbers of CD103+ T cells were different between tumors with variable Nuclear Polymorphism of most invasive layers (Kruskal-Wallis, p-value = <0.001) figure 4.10.

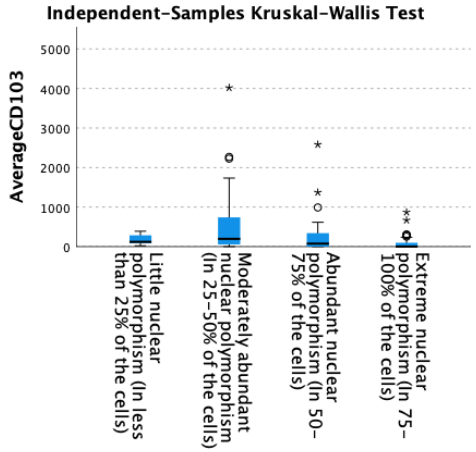


Fig 4.10. Expression of CD103+ cells according to Nuclear Polymorphism.

There was a much higher presence of CD103+ cells in tumor tissue having moderate nuclear polymorphism (mean = 587.64, SD = 888.974), compared to tumor tissue having extreme nuclear polymorphism, regarding nuclear polymorphism within the lowest differentiated parts of the most invasive 2-3 cell layers at the advancing front of tumors (Bryne et al 1998) (Mann-Whitney, p-value = <0.001) table 4.14. This might indicate that a tumor environment with abundant CD103+ cells could be beneficial in patient prognosis.

Descriptive Statistics

6Nuclear polymorphism, within the lowest differentiated parts of the most invasive 2-3 cell layers at the advancing front of tumors (Bryne et al 1998):		N	Minimum	Maximum	Mean	Std. Deviation
Little nuclear polymorphism (In less than 25% of the cells)	AverageCD103	11	19	389	181.18	123.835
	Valid N (listwise)	11				
Moderately abundant nuclear polymorphism (In 25-50% of the cells)	AverageCD103	33	0	4018	587.64	888.974
	Valid N (listwise)	33				
Abundant nuclear polymorphism (In 50-75% of the cells)	AverageCD103	26	0	2582	315.42	577.306
	Valid N (listwise)	26				
Extreme nuclear polymorphism (In 75-100% of the cells)	AverageCD103	44	0	869	89.80	178.758
	Valid N (listwise)	44				
Not evaluable	AverageCD103	4	0	37	11.25	17.576
	Valid N (listwise)	4				

Table 4.13. Comparison of CD103+ cells according to Nuclear Polymorphism in the most invasive layers.

Test Statistics[a]

	AverageCD103
Mann-Whitney U	291.500
Wilcoxon W	1281.500
Z	-4.542
Asymp. Sig. (2-tailed)	<.001

a. Grouping Variable: 6Nuclear polymorphism, within the lowest differentiated parts of the most invasive 2-3 cell layers at the advancing front of tumors (Bryne et al 1998):

Table 4.14. Comparison of CD103+ cells in two groups using Mann-Whitney test.

Taken together, these results indicate that high numbers of CD8+ cells are associated with tumors that had poor differentiation and less keratinization. On the other hand, a high number of CD103+ cells were associated with the OSCC phenotype characterized by high keratinization and lower degree of nuclear polymorphism in the whole tumor, as well as in the most invasive layers. A combination of CD8CD103+ cells also indicated less degree of nuclear polymorphism.

Comparison of numbers of T cells between groups of patients based on differentiation according to the worst pattern, perineural infiltration, lymphocytic infiltrate, depth of invasion, and worst pattern of invasion revealed no significant difference (p-value greater than 0.05, Kruskal-Wallis).

4.5.2. Effect of the abundance of CD8+ and CD103+ cells on Clinical variables of patients

Tumor Stage

Analysis indicated that the numbers of CD103+ T cells were different between patients with variable Tumor stages (Kruskal-Wallis, p-value = 0.041) figure 4.11.

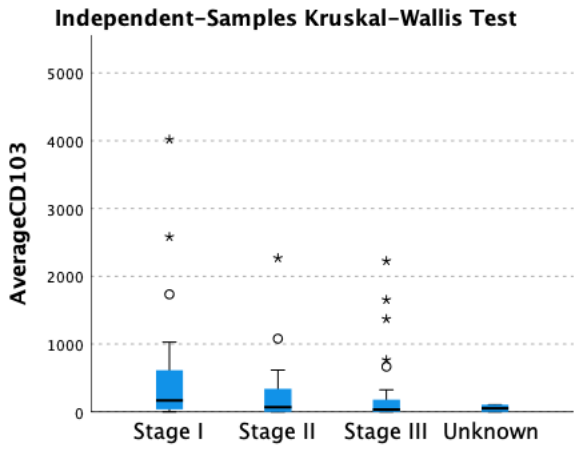


Fig 4.11. Expression of CD103+ cells according to TNM Staging.

There was a much higher presence of CD103+ cells in tumor tissue having Stage I OSCC (mean = 497.56, SD = 854.724), compared to tumor tissue having Stage III OSCC (Mann-Whitney, p-value = 0.005) table 4.16.

Descriptive Statistics

Stage revised 8 ed		N	Minimum	Maximum	Mean	Std. Deviation
Stage I	AverageCD103	32	0	4018	497.56	854.724
	Valid N (listwise)	32				
Stage II	AverageCD103	30	0	2269	251.53	459.998
	Valid N (listwise)	30				
Stage III	AverageCD103	56	0	2226	184.45	414.077
	Valid N (listwise)	56				

Table 4.15. Comparison of CD103+ cells according to TNM Staging.

Test Statistics[a]

	AverageCD103
Mann-Whitney U	576.500
Wilcoxon W	2172.500
Z	-2.800
Asymp. Sig. (2-tailed)	.005

a. Grouping Variable: Stage revised 8 ed

Table 4.16. Comparison of CD103+ cells in two groups using Mann-Whitney test.

Recurrent Regional Metastasis

Analysis indicated that the numbers of CD8+, and combination of CD8CD103+ T cells was different between patients with variable recurrent regional metastasis (Mann-Whitney, p-value of CD8+ cells = 0.032, p-value of CD8CD103+ cells = 0.010) figure 4.12.

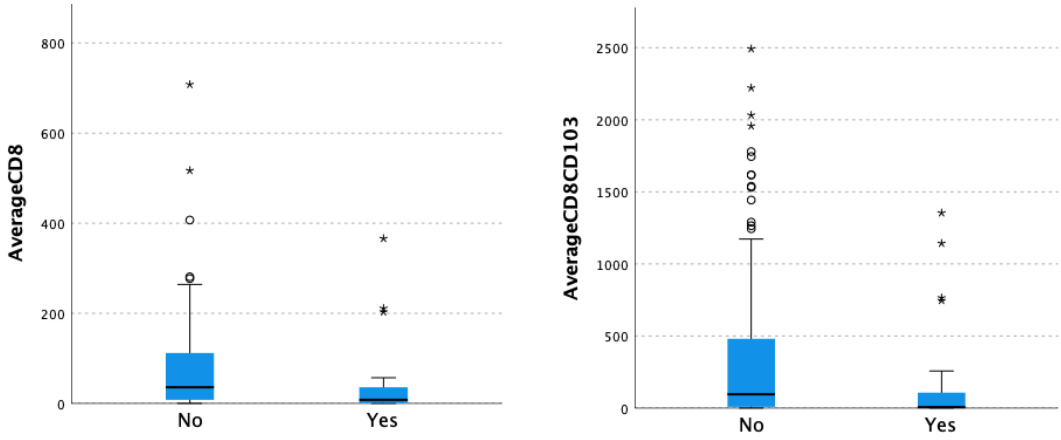


Fig 4.12. Expression of CD8+ and CD8CD103+ cells according to recurrent regional metastasis.

Patients with no recurrent regional metastasis had higher numbers of CD8+ cells (mean = 79.80, SD = 116.391), and combination of CD8+ and CD103+ cells (mean = 423.04, SD = 628.238) table 4.17.

Descriptive Statistics

Recidive regional metastases		N	Minimum	Maximum	Mean	Std. Deviation
No	AverageCD8	89	0	708	79.80	116.391
	AverageCD8CD103	89	0	2492	423.04	628.238
	Valid N (listwise)	89				
Yes	AverageCD8	26	0	366	42.77	85.728
	AverageCD8CD103	26	0	1354	186.12	374.428
	Valid N (listwise)	26				

Table 4.17. Comparison of CD8+ cells according to recurrent regional metastasis.

Alcohol Consumption

Analysis indicated that the numbers of CD103+ T cells were different between patients with variable alcohol consumption (Kruskal-Wallis, p-value = 0.015) figure 4.13.

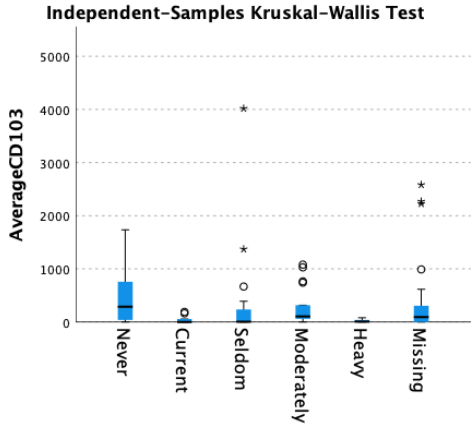


Fig 4.13. Expression of CD103+ cells according to Alcohol Consumption.

Results indicated that those patients who had never consumed alcohol had a higher presence of CD103+ cells (mean = 527.08, SD = 612.216) compared to those who had been heavy drinkers (Mann-Whitney, p-value = 0.017) table 4.19.

Alcohol consumption		N	Minimum	Maximum	Mean	Std. Deviation
Never	AverageCD103	12	0	1734	527.08	612.216
	Valid N (listwise)	12				
Current	AverageCD103	9	0	190	48.44	75.343
	Valid N (listwise)	9				
Seldom	AverageCD103	26	0	4018	294.65	815.646
	Valid N (listwise)	26				
Moderately	AverageCD103	17	0	1080	288.65	369.873
	Valid N (listwise)	17				
Heavy	AverageCD103	7	0	79	23.57	29.921
	Valid N (listwise)	7				
Missing	AverageCD103	49	0	2582	294.04	571.668
	Valid N (listwise)	49				

Table 4.18. Comparison of CD103+ cells according to Alcohol Consumption.

Test Statistics[a]

	AverageCD103
Mann-Whitney U	14.500
Wilcoxon W	42.500
Z	-2.329
Asymp. Sig. (2-tailed)	.020
Exact Sig. [2*(1-tailed Sig.)]	.017[b]

a. Grouping Variable: Alcohol consumption

b. Not corrected for ties.

Table 4.19. Comparison of CD103+ cells in two groups using Mann-Whitney test.

Taken together, these results indicate that high numbers of CD8+ T cells are associated with patients that had no recurrent regional metastasis. Similarly, a high number of CD103+ cells was associated with patients that had no recurrent regional metastasis, as well as, lower Tumor stage, and never consumed alcohol.

Comparison of numbers of T cells between groups of patients based on Overall 5-year survival, Disease-specific 5-year survival, Primary tumor according to TNM 8th edition, Clinical lymph node metastasis, Metastasis of OSCC, and Recurrent distant metastasis, revealed no significant difference (p-value greater than 0.05, Kruskal-Wallis).

4.6. Survival Analysis

Kaplan-Meier survival analysis indicated that patients with early TNM stage, no perineural infiltration, less tumor thickness, less depth of invasion, and no recurrent regional or distant metastasis had significantly higher survival probability (figure 4.14.).

Survival analysis was performed using the Kaplan-Meier test, 5-year survival calculated in months was used for a time, while survival of patient was used as status.

Similar survival analysis indicated no effect of the number of CD8+ and CD103+ T cells on patient overall survival.

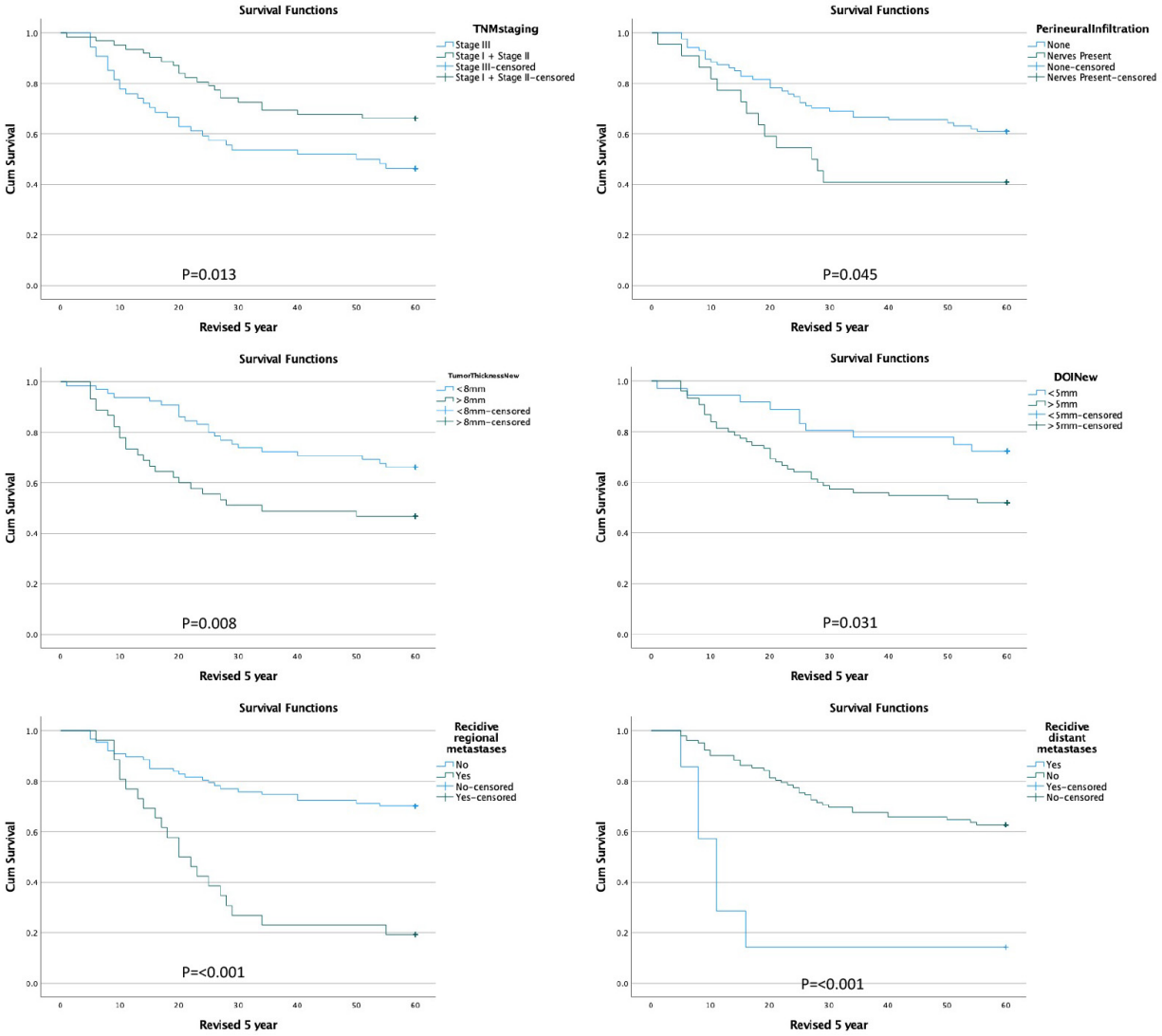


Fig 4.14. Kaplan-Meier Survival Analysis Tests.

5. Discussion

5.1 Multiplex Staining of Tumor TMA Cores

For this study, we successfully performed multiplex staining of tumor TMA cores using the immunofluorescence (IF) method with 7 different markers CD8, CD103, CD45RO, CD3, KI67, Granzyme B, and Pan Cytokeratin.

IF was chosen instead of using the standard chromogenic immunohistochemistry method to be able to reuse the same tissue for multiple staining to observe the expression of different markers on the same cells, thus giving us a much clearer picture of the tumor microenvironment, and also a result, reduce the amount of tissue that was used (Tan et al., 2020). Also, IF has more sensitivity than standard chromogenic IHC, and better visualizes subcellular localization permitting the multiple staining of antigens from the same subcellular localization at the same time. Unfortunately IF does not a give a permanent signal and has to be assessed with a dedicated microscope to detect each emission signal (Warnke et al., 1978).

Both chromogenic IHC and IF employ a primary antibody against the desired molecular target and either use a secondary antibody conjugated to an enzyme or a fluorophore or use a conjugated primary antibody. (Mori & Cardiff, 2016). The benefit of IHC over other protein detection techniques (for example, Flow cytometry or Western Blot) is its capability to connect the presence of an antigen with its position in a tissue. (Taylor CR, 2013). The technique is not only used in scientific research, but it has wide applications in histopathology and diagnostic cytology and has been even dubbed as ‘brown revolution’ (Bodey, 2002) (Leong & Wright, 1987) (Taylor, 2000). Therefore, the advent and progress of immunohistochemistry have benefited patients assuring increasingly diagnostic accuracy in a relatively low-cost (Raab, 2000).

Potential drawbacks of multiplex staining using immunofluorescence could be that there could be incomplete stripping of antibodies after each round of staining, thus we would observe bleeding of the signal of previous antibodies during the image of analysis of the next round (Taube et al., 2020). However, after implementing our protocol for multiplex staining on the tumor TMA cores, we observed appropriate antibody signal during each round of staining and weak levels of signal from previous rounds of staining (the staining is visible in different rounds). The tumor TMA cores also remained intact even after multiple rounds of staining and stripping of antibodies, and this is notable because those repeated cycles of retrieval and stripping could have damaged the epitopes as well as the morphology of the tissue.

Also, this protocol allowed us to stain for multiple antigens although commercially available antibodies are produced in a limited number of species (mostly mouse and rabbit) while reducing the number of staining rounds by combining antibodies for double staining. Both Xiao et al (Xiao et al 2019) and Bernstock et al stained for one antibody for each round, and our approach can reduce the time consume for the execution of the experiment as well as potentially reduce tissue damage. The use of secondary antibodies can amplify the signal, but unconjugated antibodies are also often less expensive the conjugated ones.

The immunohistochemistry interpretation is usually made in qualitative terms, however, the technique's quantification potential in terms of antigen concentration has been explored for a long time (True, 1988) (Polak et al., 1975). Firstly, visual scoring was provided for semi-quantitative measurements (Biesterfeld et al., 1996) (Walker, 2006) and although this is still the method preferred in diagnostic routine, computer-assisted analysis of images with potentially better accuracy was considered quite an early (Bacus et al., 1988; Zhu, 1989).

Here we used the software QuPath, user-friendly and open source it represented in our experience an optimal tool for quantification. We plan to use it also for the overlay and quantification including the rest of the markers in this study (Bankhead et al., 2017).

Other methods such as Imaging Mass Cytometry (IMC), and Multiplexed Ion Beam Imaging (MIBI) could be used to achieve the same results, these methods need dedicated equipment and reagents and are very costly. IMC has lower sensitivity compared to IF, therefore it may be difficult to perform subcellular analysis using IMC method (Giesen et al., 2014). In the case of MIBI, it is a new alternative method and is quite costly compared to IF, as well as requires a longer acquisition time (Parra et al., 2019).

This leads us to the conclusion that the multiplex staining protocol devised for this study using the immunofluorescence method is a viable method to observe the expression of multiple markers on the same tissue sample. This was used to observe the expression of different cell markers in combination with each other Especially, when subsets require multiple markers to be identified, as is the case for T cells subtypes that we wanted to analyze in this study.

5.2. Characteristics of patients in the NOROC OSCCT cohort

More than 80% of the patients in this cohort were older than 50 years of age at the time of diagnosis but age did not show any effect on survival, in contrast to other studies (Carreras-Torras & Gay-Escoda, 2015) (Seoane-Romero et al., 2012). More than half of the patients survived after 5 years, and all of the patients included in the were diagnosed with a stage III or under at the time of diagnosis.

This can be viewed as a much better survival rate compared to studies performed in other countries such as Pakistan and India, where the survival rate is much lower (Khan et al., 2012) (Dikshit et al., 2012). This could be due to different reasons, it may be due to better education and health system in Norway compared to these countries, as well as a higher incidence of pre-existing conditions and cancer-inducing habits in countries like Pakistan and India compared to Norway (Khan et al., 2012) (Singh et al., 2016), though it is possible that more tumors would be diagnosed at higher stages in the latter countries due to social and political factors.

NOROC OSCCT patients having Stage I or Stage II at the time of diagnosis had a much better survival prognosis compared to patients having Stage III OSCCT, something that is in line with previous research (Omar, 2013) (Almangush et al., 2020). This observation further shows the importance that patients are diagnosed at earlier stages to detect OSCC in earlier stages for improved treatment and prognosis.

In addition, patients whose tumors displayed no perineural infiltration were more likely to survive compared to patients having perineural infiltration in the nerves at the invasive front and tumor center. Perineural infiltration is the growth of tumor cells infiltrating into the nerve bundles in the surrounding stroma. Our results fall in line with recent literature (Binmadi & Basile, 2011) (Varsha et al., 2015) (Jardim et al., 2015).

Patients having tumor thickness less than 8mm and depth of invasion less than 5mm had a much better chance of survival. According to relevant literature, increased tumor thickness and depth of invasion can lead to infiltration of tumor cells into increased blood vessels and lymphatic tissue, therefore increasing the chances of metastasis (Dirven et al., 2017). Also, there is a much higher chance for the tumor emboli to form in the wider lymphatic vessels, compared to the small caliber lymphatics of superficial areas (Pentenero et al., 2005).

Also, as expected from previous literature, patients with no recurrent regional and distant metastasis had a considerably improved chance of survival. Postoperative tumor recurrences can lead not only to a poor prognosis but also to poor quality of life (Wang et al., 2013) (Sklenicka et al., 2010).

5.3 Expression of CD8 and CD103 and correlation with clinicopathological features

A high number of CD103+ cells were associated with the OSCC phenotype characterized by a lower tumor stage. This could lead to an indication that the presence of CD103+ cells in the tumor microenvironment may lead to slower progression of cancer according to TNM staging.

High numbers of CD103+ and a combination of CD8CD103+ cells were associated with tumors that had no recurrent regional metastasis. According to previous results, a higher number of CD103+ were associated with a lower stage of OSCC, and lower stage tumors can have less potential for recurrency, therefore a high number of CD103+ cells could be a feature of smaller tumors, and not predictive of recurrence.

According to a systematic review and meta-analysis by Jin et al, a high number of CD8+ T cells indicated a better overall survival and better prognosis for cancer patients. Our study did not show any significant results in that regard (Jin et al., 2021). Similarly, a study by Lenouvel et al indicated a favorable prognosis for OSCC patients with an abundance of CD8+CD103+ cells, but our study showed no significant results in that regard (Lenouvel et al., 2020).

A high number of CD103+ cells were associated with the OSCC phenotype characterized by patients that never consumed alcohol. As indicated from previous results, CD103+ cells may be beneficial to reduce the spread of the tumor, therefore we could surmise that restriction of alcohol may be beneficial to the prognosis of cancer patients.

We also observed that 40.8% of cases were missing data on alcohol consumption, though this can be relevant for the biology of the immune response since we find that a high number of CD103 was seen in individuals who report no alcohol consumption. According to research, this may be due to patients hiding their smoking and alcohol habits due to the stigma or embarrassment of admitting their unhealthy habits to a healthcare worker (Fainzang, 2002) (Frick et al., 2021). Better and more sensitive methods for collecting patient data should be devised instead of the normally used basic questionnaire that is to be filled by the patient when it comes to factors that the patient may not feel comfortable disclosing easily.

We found that high numbers of CD8+ cells were associated with tumors that had poor differentiation degree in the whole tumor. We also observed a high number of CD8+ cells associated with lower degrees of nuclear polymorphism but only when expressed in a combination with CD103+ cells. While high numbers of CD103+ cells were associated with a low degree of nuclear polymorphism both in the whole tumor and also in the most invasive layers. Interestingly, the degree of differentiation combined with the amount of lymphocytic tumoral infiltrate was shown as a prognostic marker in a recent study on the NOROC cohort (Bjerkli, Hadler-Olsen, et al., 2020).

High numbers of CD8+ T cells were associated with tumors that had less keratinization in the whole tumor. In contrast, a high number of CD103+ cells were associated with the OSCC phenotype characterized by high keratinization in the whole tumor. We could not find relevant literature researching the association of keratinization with TILs, but in our opinion, this is a very relevant biological question.

Current studies are concentrating on the identification of inflammatory and immunological processes that may provide prospective therapeutic targets in OSCC, especially after recent successes of checkpoint inhibitors for specific human cancers (Lechner et al., 2017), but we can see that the mechanisms that regulate an efficient response to immune checkpoint targeting are still incompletely clear, due to the complexity of the interactions among TME compartments and those and tumor cells.

5.4. Limitations of Study

Unfortunately, all aims could not be achieved, and we hope to complete them. Although this cohort includes cases with valuable and largely complete clinicopathological datasets, we found a very high standard deviation in the data resulting from our IHC experiments and we wonder if a larger study cohort may change the normality of distribution permitting us to run parametric tests instead of non-parametric tests and we ask if the results would be different.

As the tumor microenvironment is extremely complex with several cell types, exploring the relationship of the subset we study here with other components of the tumor microenvironment, as well as each other, may provide us with invaluable information on the immune response in OSCCT and possibly allow us to discover more prognostic and/or predictive tools in OSCCT.

6. Conclusions and future perspective

a. The protocol for multiplex IHC staining we devised is suitable for sequential staining of TMAs from cancer tissues, maintaining the tissue morphology and with minimal bleeding of the signal.
b. In a preliminary analysis of just two markers results show that the expression of CD8 and CD103 in tumor infiltrates could be of prognostic value OSCCT.

We conclude that this study can be a significant contribution to the knowledge of the significance of immune response in OSCCT and we look forward to better understanding the significance of the T cell subset object of this study completing the rest of the aims listed above and exploring further the meaning and expression of CD103 in tumor infiltrates.

We also hope that findings in this work can suggest which pathways of cell-cell interaction in the immune infiltrates and with malignant epithelia could be explored in terms of treatment targeting and predictivity.

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