UiT
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Faculty of Health Sciences
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Characterization and prognostic markers

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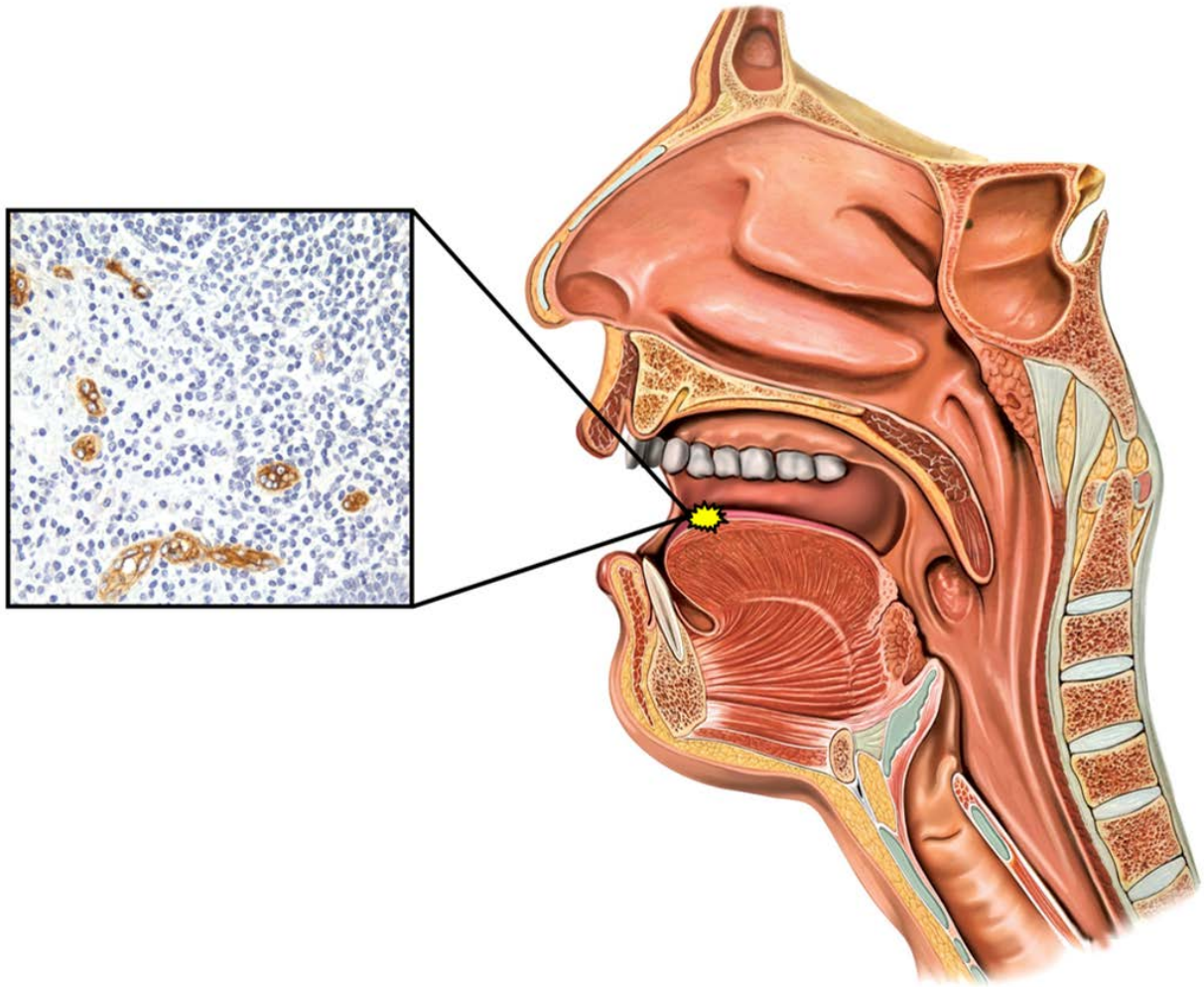
Anna Maria Wirsing

A dissertation for the degree of Philosophiae Doctor, September 2018



UNIVERSITY OF TROMSØ

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Title picture

Microscopy image of immunohistochemically stained (brown) high-endothelial venules in the tumor microenvironment of a tissue section from a patient with oral squamous cell carcinoma (left).

Sagittal section of the oral cavity and the pharynx, reprinted and adapted from ‘Asklepios Atlas of the Human Anatomy’/ Science Photo Library / NTB Scanpix, license purchased (right).

Abstract

Oral squamous cell carcinomas (OSSCs) are aggressive tumors often associated with a low survival rate. Today, OSCCs of the same size and extent usually receive the same treatment although they may differ in aggressiveness and response to therapy. Thus, new prognostic markers that reflect the biological diversity of these tumors are requested to better tailor the treatment to the patient's need. The immune system influences the development of OSCCs, and the immune infiltrate surrounding the tumor is an indicator of the host's anti-tumor response. The main goal of this thesis was to describe how the tumor microenvironment in OSCC is organized, and to determine whether different subsets of the immune infiltrate can be used to predict patient outcome. In tissue from 80 OSCC patients, we used immunohistochemistry to detect distinct cellular components of the immune infiltrate, and real-time quantitative polymerase chain reaction to analyze the gene expression of a number of inflammation-related cytokines and chemokines. We correlated the presence of the various components to each other as well as to clinicopathological data and patient survival. A systematic review of the literature was conducted to identify promising prognostic factors in OSCC and to evaluate the quality of the published studies. In our review, we found that CD163+ M2 macrophages and CD57+ mature natural killer cells were the most promising prognostic markers. In our Norwegian OSCC patient cohort, the presence of specialized blood vessels called high-endothelial venules (HEVs) was found to be an easy-to-use, independent prognostic marker for improved survival and had the most promising potential of the factors analyzed. However, larger studies adhering to best-practice guidelines are needed to draw reliable conclusions about the prognostic value of the markers identified in our Norwegian OSCC cohort and our literature review. HEVs are known to be gateways for lymphocyte entry to the tumor site, and we found these vessels to be associated with a potentially tumor-suppressive immune microenvironment. Therefore, we hypothesize that HEVs may be master regulators of a favorable immune reaction and potential targets to enhance anti-tumor responses in OSCC patients.

Contents

List of Papers

This thesis is based on the following papers, which are referred to as **Papers I-IV** in the text.

Paper I

Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma.

Wirsing AM, Rikardsen OG, Steigen SE, Uhlin-Hansen L, Hadler-Olsen E.

BMC Clinical Pathology. 2014 Aug 23; 14:38. doi: 10.1186/1472-6890-14-38.

Paper II

Presence of tumour high-endothelial venules is an independent positive prognostic factor and stratifies patients with advanced-stage oral squamous cell carcinoma.

Wirsing AM, Rikardsen OG, Steigen SE, Uhlin-Hansen L, Hadler-Olsen E.

Tumour Biology. 2016 Feb; 37(2):2449-59. doi: 10.1007/s13277-015-4036-4.

Paper III

Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma.

Wirsing AM, Ervik IK, Seppola M, Uhlin-Hansen L, Steigen SE, Hadler-Olsen E.

Modern Pathology. 2018 Feb 7. doi: 10.1038/s41379-018-0019-5.

Paper IV

Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma – a systematic review.

Hadler-Olsen E, Wirsing AM. Manuscript submitted for publication.

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I have greatly benefited from my work as Vice-president of Tromsø Doctoral Students (TODOS), the local interest organization for PhD candidates at UiT. I am equally grateful for the experiences as Board Member of the Association of Doctoral

Organizations in Norway (SiN), which aims at securing a good doctoral program at a national level.

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This work has been part of The Norwegian Oral Cancer study, a national multicenter study aiming at optimizing the treatment of oral cancer patients, and I am grateful for fruitful discussions with the collaborators as well as for financial support. Being part of this big project is inspiring, and I believe that combining the energy, knowledge, and skills of this motivated group of people can lead to great achievements in the development of new biomarkers for oral cancer patients.

My dear Maarten, thank you for your patience, love and support throughout these years. I feel privileged that I can discuss both science and the banalities of everyday life with you. Last but not least, I thank my son Simon for making me smile every single day.

Tromsø, June 2018

Anna Wirsing

Abbreviations

CCL	CC-chemokine ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CXCL	CXC-chemokine ligand
FDC	Follicular dendritic cell
FFPE	Formalin-fixed, paraffin-embedded
HEV	High-endothelial venule
HPV	Human papillomavirus
IHC	Immunohistochemistry
IFN	Interferon
LT	Lymphotoxin
NK	Natural killer
PD-L1	Programmed-death ligand 1
PD-1	Programmed-death 1
PNAd	Peripheral node addressin
PRISMA	Preferred reporting items for systematic review and meta-analysis
qPCR	Real-time quantitative polymerase chain reaction
REK	The regional committee for medical health research ethics
REMARK	Reporting recommendations for tumor marker prognostic studies
RQI	RNA quality indicator
SCC	Squamous cell carcinoma
Th	T helper
TLS	Tertiary lymphoid structure
UiT	University of Tromsø – The Arctic University of Norway
UNN	University Hospital of North Norway

Introduction

1. Oral squamous cell carcinoma

Oral (O) squamous cell carcinomas (SCCs) originate within the epithelium lining of the oral cavity and include tumors located in the mobile part of the tongue (anterior two thirds), floor of the mouth, buccal mucosa, gingiva and the hard palate (Figure 1). OSCCs belong to a group termed head and neck SCCs. This group comprises amongst others cancers arising in the oral cavity, oropharynx, larynx, hypopharynx, paranasal sinuses, nasal cavity, and the salivary glands, each with distinct, subsite-specific patterns of presentation and behavior [1, 2]. OSCCs are the most common oral malignancy, and are particularly aggressive tumors with early tendency to metastasize to the regional lymph nodes [2].

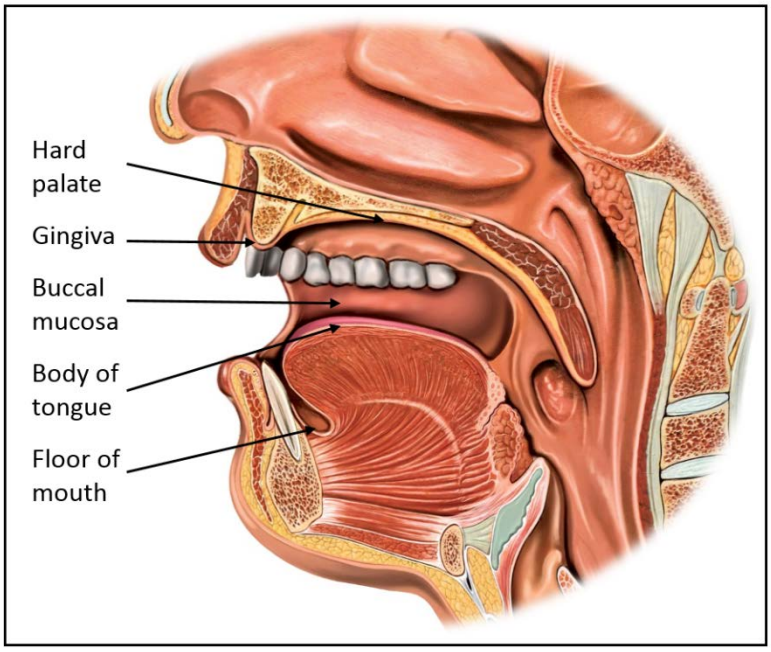

Figure 1. Anatomical location of oral squamous cell carcinomas. Sagittal section of the oral cavity, reprinted and adapted from 'Asklepios Atlas of the Human Anatomy'/ Science Photo Library / NTB Scanpix, license purchased.

1.1 Epidemiology, risk factors, and survival

Oral cancer (with the oropharyngeal sites included) is the sixth most common cancer worldwide [3]. Globally, oral cancer accounted for an estimated 202.000 new cases in 2012 [4]. In Norway, oral cancer was in 2015 responsible for 234 new cases and 83 cancer deaths [5]. Oral cancer is most frequent in the fifth and sixth decades of life, with higher incidence in males compared to females [2]. There is a marked global variation in the distribution of oral cancer, which can be mostly attributed to differences in exposure to risk factors. In Europe, oral cancer incidence is highest in eastern and central parts (e.g. Hungary, Slovakia, Slovenia, and France). In the rest of the world, oral cancer incidence is particularly high in southern Asia, where these cancers account for about 25% of all new cancer cases [3]. Overall, oral cancers seem to have stabilized or declined in many countries, which is consistent with the decreasing use of tobacco and betel quid [4, 6].

Tobacco and alcohol consumption are the most important risk factors for oral cancers and, if used together, strongly increase the risk for oral cancer [7, 8]. The habit of chewing betel quid (i.e. a mixture of areca nut and/or tobacco with other substances such as slaked lime) is widely prevalent in many Asian countries, and is another strong etiological factor for oral cancer [9]. Other more speculative risk factors include amongst others poor oral health and low consumption of fruit and vegetables [10, 11]. Human papillomavirus (HPV) has been identified as a cause of oropharyngeal cancer [12, 13], but may only play a minor role in tumors arising in the oral cavity [14].

In many parts of the world, survival rates have not improved significantly for decades [2]. In Europe, five-year survival rates for oral and oropharyngeal cancers are approximately 50% [15]. In Norway, preliminary data from a multicenter study including more than 500 OSCC patients showed that the overall 5-year survival rate of this patient group is approximately 45% [16].

1.2 Clinical features and treatment

OSCCs gradually develop from normal epithelium, which through multiple genetic alterations transforms into dysplasia and invasive cancer [17]. Clinical presentations that carry a risk of oral cancer development are painless white (leukoplakia) or red (erythroplakia) plaques of the oral mucosa, but only a minority of these lesions will undergo malignant change [18]. Early disease is generally subtle and asymptomatic, which is why small lesions often remain undetected. Depending on the anatomical location of the tumor, patients with more advanced disease may suffer from ulcers, pain, bleeding, and difficulties with chewing, swallowing and speaking as well as weight loss, and neck swelling. Preliminary data from a Norwegian multicenter study revealed that about 40% of patients had metastatic spread to the lymph nodes, and 10% had distant metastasis at the time of diagnosis [16]. Patient assessment is managed by a multidisciplinary team of health care providers, if available, and includes amongst others thorough clinical examination of the primary site and the neck, pathological confirmation of the diagnosis by biopsy, and employment of imaging techniques to evaluate the extent of the disease [2].

The choice of treatment largely depends on the localization and anatomical extent of the primary tumor as well as the patient's health status and individual desire. For oral cavity cancers, surgery with or without adjuvant radiotherapy are the gold standards for treatment of advanced and early disease, respectively [18]. Large tumors sometimes receive radiotherapy prior to surgery, and palliative radiochemotherapy is often the treatment of choice for the most advanced cancers. Complications related to radiotherapy include amongst others mucositis (inflammation and ulceration of the oral mucosa), xerostomia (dry mouth), osteoradionecrosis (irreversible, progressive devitalization of irradiated bone), and local infections [19]. This, again, causes secondary effects such as problems with eating and speech, taste dysfunction, pain, and deformation, which may lead to social exclusion and impair the quality of life of these patients [20].

About 12% of head and neck cancer patients develop recurrent disease within five years after the treatment [21]. Genetically altered epithelial cells in close proximity to the primary tumor, a phenomenon called 'field cancerization', is believed to be an important factor in the recurrence or persistence of oral cancer after therapy [22]. Of those who survive the disease, about 4% per year develop independent, new tumors in the upper aerodigestive tract, so-called second primary tumors [23].

1.3 Classification and histopathological factors

The main tool for staging oral cancers is the TNM system, which classifies tumors according to the size and extent of the primary tumor (T), the absence or presence and extent of regional lymph node metastasis (N), and the absence or presence of distant metastasis (M). The latest, eighth edition of the TNM system of the American Joint Committee on Cancer and Union for International Cancer Control includes substantial revisions for the classification of head and neck SCCs [24] and accounts both for the better treatment response and survival of patients with HPV-induced oropharyngeal carcinomas [25, 26] as well as for the importance of depth of invasion (tumor thickness) [27] and extranodal extension (extracapsular spread in cervical lymph node metastases) [28] in oral cavity tumors. Table 1 gives an overview over the TNM staging of OSCCs according to the current [24] and the fifth edition [29]. The latter was used to classify the tumors included in **Papers I-III** of this thesis. The individual TNM categories form the base for stage grouping of oral cancer patients (Table 1), where advanced tumor stage and especially metastatic spread to regional lymph nodes are strong prognosticators for poor patient outcome [21]. Distant metastasis is far less common compared to nodal involvement [30], but reduces survival drastically [31].

Table 1. TNM classification and stage grouping for oral cavity tumors. The table is adapted from the 5th [29] and 8th edition [24] of the TNM classification of Malignant Tumors, and includes the TNM categories and stages relevant for this thesis. The 8th edition includes substantial changes for the classification of oral cavity tumors. The clinical and pathological TNM classifications largely correspond to each other.

	Fifth edition TNM	Eighth edition TNM
Size and extent of primary tumor (T category)		
T1	Size ≤ 2 cm	and depth of invasion (doi) ≤ 5mm
T2	Size >2-4 cm	Size ≤ 2 cm and doi 5-10 mm, or size >2-4 cm, and doi ≤ 10 mm
T3	Size > 4 cm	or depth of invasion > 10 mm
T4	Tumor invades adjacent structures	sub-classified as T4a and T4b
Regional lymph node metastasis (N category)		
N0	No regional lymph node involvement	
N1	Ipsilateral, single ≤ 3 cm	and no extranodal extension*
N2	(a) Ipsilateral, single >3-6 cm, (b) Ipsilateral, multiple ≤ 6 cm (c) Bilateral, contralateral ≤ 6 cm	(a) and no extranodal extension (b) and no extranodal extension (c) and no extranodal extension
N3	> 6 cm	(a) and no extranodal extension (b) Any N with extranodal extension
Distant metastasis (M category)		
M0	No distant metastasis	
M1	Distant metastasis	
Stage grouping of individual TNM categories		
Stage I	T1, N0, M0	
Stage II	T2, N0, M0	
Stage III	T3, N0, M0 or T1/T2/T3, N1, M0	
Stage IV	Any T4 lesion. Any N2 or N3. Any M1. (sub-classified as IVA, IVB, IVC)	

* differences in definition and classification of clinical and pathologic extranodal extension apply

OSCCs are further graded as well, moderately or poorly differentiated based on the degree of keratinization, cellular and molecular pleomorphism, and mitotic activity [2]. Most OSCCs have either a well or moderate degree of differentiation. The degree of differentiation often varies within a tumor and is determined based on the area of the tumor with the lowest degree of differentiation. However, defining precise histological criteria to differentiate between low- and high-risk tumors can be

challenging and often results in poor reproducibility between the observers [32]. This is part of the reasons why classification based on histologic grading alone is considered to be of limited clinical use, and only poorly correlates with outcome [33]. During the last decades, a number of multifactorial grading systems addressing histologic parameters of the tumor-host relationship have been proposed to give a more accurate estimate of the patient's prognosis [34-37]. These parameters include amongst others the pattern of invasion and the degree of the lymphocytic infiltrate, with special emphasis on the invasive margins of the tumor. In 2005, Brandwein-Gensler et al. proposed a histological risk-assessment score for OSCCs based on perineural invasion, lymphocytic infiltrate and worst pattern of infiltration, which has shown independent effects on patient survival in a large cohort [33, 38]. However, the histological risk assessment score was found not to have prognostic significance in two recent studies [39, 40]. Tumor budding, that is the presence of single cells or small-cell clusters in the stroma ahead of the invasive tumor front, has recently shown promising prognostic value in patients with tongue cancer [40, 41].

At present, the TNM classification system, and to a lesser extent the degree of differentiation, are used to predict outcome and make treatment decisions for OSCC patients. However, diagnostic assessment based on these factors has its limitations for personalized therapeutic approaches as patients with similar stages of oral cancer may have diverse clinical courses and responses to treatment [42]. This calls for the development of new prognostic markers that reflect the unique biology of an individual tumor.

1.4 Biological markers

From the definition of the Biomarker Definition Working Group [43], a biological marker (biomarker) can be understood as an objectively measured and evaluated indicator of diagnosis or prognosis of disease, or as a sensitive and specific tool for risk stratification. The great potential of biomarkers for personalized medicine, together with the emerge of molecular techniques, has resulted in an enormous and

growing amount of investments and academic outputs within this field [44]. Likewise, during the last decades, an increasing number of biomarkers have been launched as potentially useful prognosticators for oral cancer patients, and in 2017, 182 publications on oral cancer biomarkers were indexed in Medline (Figure 2). In recent years, the analyzation of the saliva and blood of patients have been promising approaches for biomarker discovery in this cancer type [45-47]. Several proteins as well as mutated tumor suppressor gene tp53 have in the latest edition of the TNM classification system been acknowledged as new and promising tumor-related prognosticators for oral cavity tumors [24]. However, none of these or the numerous other suggested biomarkers are currently being utilized in routine clinical management of this patient group. Poor data and lack of clinical usefulness are among the most important reasons why biomarkers fail clinical implementation [48].

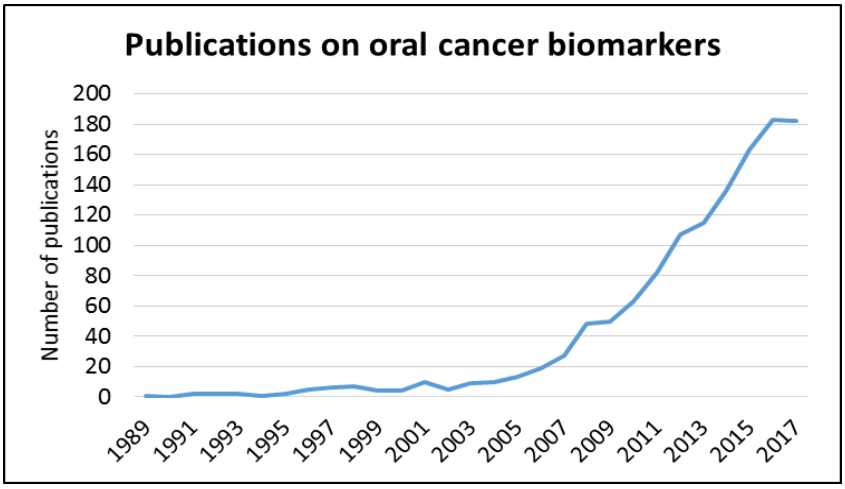

Figure 2. Publications on oral cancer biomarkers. Number of publications containing the term 'biomarker' in the tile, keyword or abstract, as derived from Medline using the search strategy for the entry terms 'prognostic value' and 'oral cancer' as described in **Paper IV**. The first publication was indexed in 1989 (Ovid Medline was searched from 1967 to 2017 and accessed 23rd May 2018).

2. The immune microenvironment

The immune system is an important mediator of tumor initiation and progression. Cells of the innate and adaptive immune system work together to maintain tissue homeostasis by clearing the host from pathogenic antigens, and help to eliminate developing tumors long before they become clinically apparent [49]. However, inefficient antigen clearance due to persistent, excessive exposure to pathogens or other irritating agents can result in chronic inflammatory processes, which may increase the risk of cancer development [50]. Since Rudolf Virchow in 1863 described a link between infiltrating leukocytes and neoplastic tissue, inflammatory processes have been shown to precede a variety of human malignancies including OSCCs [51, 52] and are considered one of the hallmarks of cancer [53]. For head and neck cancers, it is recognized that the immune system plays a key role during all stages of tumorigenesis, from precancerous lesions to the establishment, progression and metastatic spread of the primary tumor [54]. Due to the complexity of the tumor microenvironment and host-tumor interactions during tumorigenesis, only a selection of factors relevant for this thesis is presented below.

2.1 Characteristics

The immune microenvironment emerges during tumor development as a result of host-tumor interactions [55], and comprises an intricate network of stromal cells, vascular and lymphatic vessels, and the extracellular matrix. In addition, secreted, soluble factors such as cytokines, chemokines and a number of other signaling molecules mediate tumor-stroma interactions and can promote tumorigenesis through dysregulation of inter- and intra-cellular signaling cascades [56]. Cytokines are a diverse family of proteins that control autocrine or paracrine communication between individual cell types and can be induced by various factors such as hypoxia [57]. Chemokines are chemoattractant cytokines mostly induced by inflammatory cytokines such as tumor necrosis factor-α, and coordinate cell movement during inflammation as well as tumor cell growth, survival, migration, and angiogenesis

[58]. Our current understanding of immunity implies a dual role with both host-protective and tumor-promoting functions, a concept which is summarized under the term “cancer immunoediting” [59]. The contexture of the tumor microenvironment varies between different tumor types and within tumors of the same type, as well as within different sites of the same tumor, and has been shown to affect patient prognosis in various human cancers [60].

2.2 Tumor-infiltrating immune cells and immunotherapy

Immune cells are abundantly present in many human solid cancers including OSCCs, where the invasive margin of the tumor front is usually highly infiltrated by immune cells. Besides lymphoid cells (T cells, B cells and natural killer (NK) cells), myeloid cells such as macrophages, dendritic cells, granulocytes and mast cells have been found to infiltrate tumor tissues and can be used to predict prognosis [61]. Based on antigen specificity and timing of activation, immune cells can be divided into cells of the innate immunity (such as dendritic cells, NK cells, macrophages, neutrophils, basophils, eosinophils, and mast cells) and cells of the adaptive immunity (such as B cells and T cells). The first group represents the non-specific, first line of defense, and the latter is highly specific to a given antigen [50]. Cells of the innate and adaptive immunity crosstalk with each other to achieve functional adaptive immune responses. Antigen-presenting cells such as dendritic cells, macrophages and B cells stimulate antigen-specific T-cell populations, which further develop into effector T cells. Likewise, signals from T helper (Th) cells initiate affinity maturation and isotype switching of B cells [62]. It has long been believed that the tumor-draining lymph nodes are the only sites where the complex processes of orchestrating T- and B-cell anti-tumor responses can take place.

Further, infiltrating immune cells can be divided into a wide range of individual subtypes with distinct functional properties. However, dependent on environmental cues, the same types of immune cells sometimes accommodate different phenotypes with differing biological effects, emphasizing the complexity by which the immune

system directs anti-tumor responses [63]. An example of remarkable plasticity are macrophages, which may upon stimuli such as hypoxia switch between “classically activated” M1 macrophages with pro-inflammatory and anti-tumorigenic functions, and “alternatively activated” M2 macrophages with anti-inflammatory and pro-tumorigenic functions, of which the latter represent the majority of macrophages found in tumors [64, 65].

T cells are among the most prominent populations of infiltrating immune cells in head and neck cancers [66], and are classically divided into cluster of differentiation (CD)8+ cytotoxic T cells and CD4+ Th cells, which can further differentiate into a wide range of subclasses with distinct functional roles. Similar to NK cells, CD8+ T cells can produce interferon (IFN)-γ and directly kill target cells [63]. CD4+ Th cells can be further subdivided into Th1, Th2, T follicular helper, IL-17 expressing Th, and T regulatory cells. In contrast to the tumor-suppressive effects of Th1 responses, CD4+ Th2 cells and CD4+ T regulatory cells can downregulate CD8+ cytotoxity, a feature that has together with other immunosuppressive mechanisms such as impaired NK-cell activity and poor-antigen presenting function been linked to the progression and poor prognosis of head and neck cancers [54, 67, 68]. In addition, a wide range of individual, functionally distinct B cell and dendritic cell subtypes have been identified in the OSCC tumor microenvironment. The function and prognostic value of these cell types in OSCC are together with eosinophils and mast cells discussed later in this thesis and in the respective papers.

Targeting stromal components for therapeutic approaches is promising yet challenging due to the high immunologic heterogeneity of most of the tumors [69]. A pronounced immune cell infiltration has been found to be a predictor of favorable outcome, although the functionality of the individual immune cell subsets varies [60, 70]. Likewise, patients with ‘inflamed’ tumors, characterized by marked immune cell infiltration and high expression of cytokines, generally respond better to immunotherapies and show improved survival compared to those with ‘non-

inflamed' tumors, where recruitment of immune cells may be impaired [71, 72]. Head and neck cancers are mostly of the inflamed phenotype which makes them potentially susceptive for immunomodulatory therapies [73]. However, the generally poor survival of this patient group indicates that the tumor cells have developed immunosuppressive mechanisms that allow them to progress despite the apparent immune response [67]. It is now recognized that T cells in solid tumors are often functionally exhausted [74], and express inhibitory receptors such as programmed-death 1 (PD-1), which interacts with programmed-death ligand 1 (PD-L1)-expressing tumor cells and downregulates T-cell activation [75, 76]. In recent years, reversing immunosuppression through blockade of the PD-1/PD-L1 pathway has shown durable anti-tumor responses in 10-20% of oral cancer patients [77, 78].

2.3 Tertiary lymphoid structures

During chronic inflammation, B cells, follicular dendritic cells (FDCs), T cells and high-endothelial venules (HEVs) sometimes organize in clusters called tertiary lymphoid structures (TLSs), which have considerable morphological and functional similarity to secondary lymphoid organs, particularly lymph nodes (Figure 3). TLSs typically form in random, nonlymphoid tissues, and develop through a dynamic process called lymphoid neogenesis. During this process, TLSs develop from sparse lymphocytic infiltrates into more organized aggregates, and finally into mature TLSs with ectopic germinal centers [79]. This process is mechanistically similar to the development of lymph nodes, and requires a highly coordinated, lymphotoxin (LT)-mediated interplay between lymphocytes and non-lymphoid stromal cells. Upon stimulation by lymphocytes, the non-lymphoid stromal cells express a number of lymphoid chemokines including CC-chemokine ligands (CCL)19, CCL21 and CXC-chemokine ligands (CXCL)12 and CXCL13 that regulate lymphocyte recruitment and compartimentalization [80-82]. Persistent antigen exposure is essential for the initiation and maintenance of TLSs, which are in contrast to lymph nodes not

encapsulated and therefore highly susceptive for antigen-stimulation from their surroundings [79].

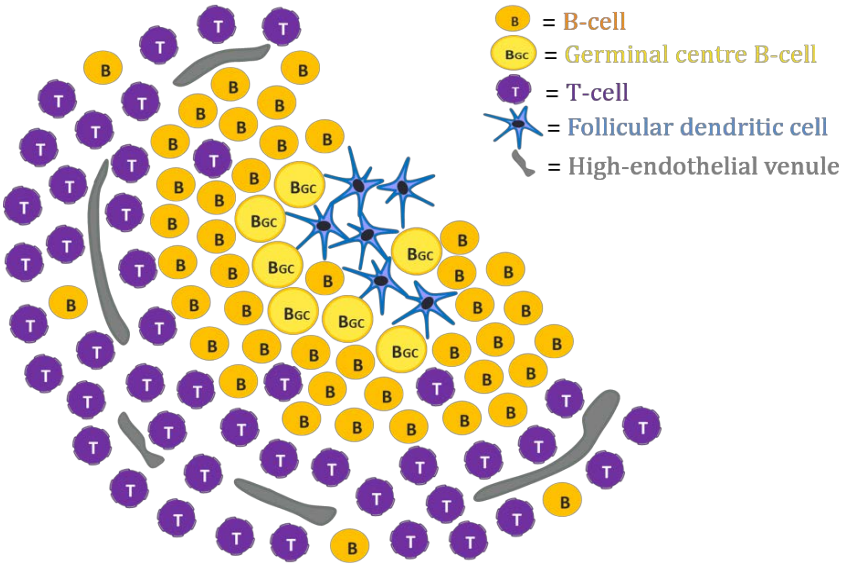

Figure 3. Schematic model of tertiary lymphoid structures. Specialized cell populations arrange themselves into distinct patterns forming a classical tertiary lymphoid structure (**Paper I**).

TLSs have initially been studied in conjunction with chronic inflammatory diseases such as chronic infection, chronic graft rejection or autoimmune disorders where they are thought to exacerbate disease [83]. Only recently, TLSs have been described in association with a variety of human solid cancers where they have shown beneficial effects, probably due to their ability to generate local anti-tumor responses [84]. Indeed, there is strong data suggesting that TLSs are privileged areas for T-cell recruitment and activation in primary tumors, with the germinal centers as putative sites for tumor-specific antigen production. This indicates that cellular and humoral immune responses can be initiated independently from secondary lymphoid organs, and supports an immune-modulating role for TLSs in the tumor microenvironment [80, 85]. However, much is still unknown about the formation and functional role of

TLSs in individual cancer types, and how these structures can be used for immune-modulating therapies [86].

2.4 High-endothelial venules

HEVs are specialized, post-capillary blood vessels that support lymphocyte recruitment into the tissue, and are abundantly present in all secondary lymphoid organs except the spleen [87]. HEVs have also been found in association with chronic inflammation, both as part of TLSs and independent of these structures, where they are believed to exert similar functions as in secondary lymphoid organs [88]. HEV endothelial cells have a typical 'plump', cuboidal morphology (Figure 4 A) with discontinuous, spot-welded junctions, and express lymphocyte-ligands such as the chemokine peripheral node addressin (PNAd) on their luminal surface, which binds to homing receptor L-selectin on circulating, naïve and memory lymphocytes [89, 90]. In addition, HEVs attract lymphocytes by presenting a number of different chemokines including CCL21, CCL19, CXCL12 and CXCL13 [88]. However, HEVs are highly plastic and may upon stimulation revert from a lymphocyte- to blood-carrying phenotype with flattened epithelium and lack of the surface marker PNAd [91-93] (Figure 4 B). In secondary lymphoid organs, LTα1β2 expression on dendritic cells and LTβ-receptor signaling from blood endothelial cells seem to be crucial for the development and stabilization of HEVs [94, 95].

It is now well recognized that HEVs play an important role in cancer immunology [89]. Whereas classical blood vessels are known to nurture tumor growth, the presence and density of tumor-related HEVs have been associated with tumor regression and favorable outcome in a variety of murine and human cancers, probably by enhancing influx of naïve T lymphocytes to the tumor [88]. However, remodeling of HEVs in the draining lymph nodes of breast and OSCC patients was found to be an early sign of metastatic spread and associated with poor prognosis [92, 93]. Although LT-engagement by dendritic or other cells appear to be important regulating factors, the exact mechanisms that drive HEV formation and stabilization

in tumors are yet to be elucidated, and may differ depending on the anatomical location of the tumor [86, 96]. Despite numerous unanswered questions about the physiology and function of tumor-associated HEVs, therapeutic HEV induction in tumors holds great promise to support efficient anti-tumor immune responses, as recently demonstrated by immune-modulating approaches [97, 98].

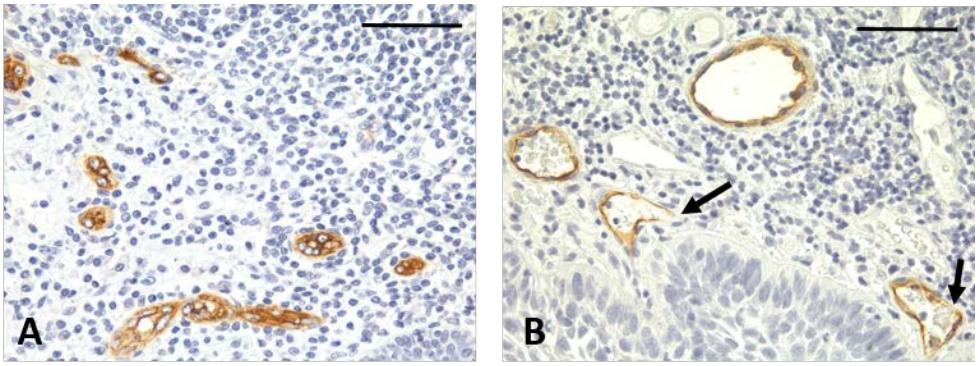

Figure 4. Different phenotypes of high-endothelial venules in oral squamous cell carcinoma. The micrographs show representative immunohistochemical stainings for high-endothelial venules (HEVs) found in our North Norwegian oral squamous cell carcinoma patient cohort (**Papers I-III**). Both HEVs with a thick wall and small or no lumen (a) and HEVs with a thinner wall and larger lumen (b) were found. Dilated vessels gradually lost their surface marker PNAd (arrows, b). PNAd-positive vessels are stained brown, and cell nuclei are stained blue by hematoxylin. Scale bars indicate 50 µm.

Hypothesis and aims

This work is based on the hypothesis that the composition and organization of the immune microenvironment affect the aggressiveness of OSCCs. Hence, we hypothesized that different components of the immune microenvironment can be used as prognostic markers. Based on our hypotheses, the main goal of the project was to describe how the tumor microenvironment in OSCC is organized, and to determine whether different subsets of the immune infiltrate can be used to predict patient outcome.

More specifically, we wanted to:

1. Determine whether TLSs can develop in OSCC and, if yes, whether these structures have prognostic value in a cohort of OSCC patients (**Paper I**).
2. Characterize morphological features of HEVs in OSCC and determine whether they have prognostic value in a cohort of OSCC patients (**Paper II**).
3. Characterize the association between HEVs and the presence of various tumor-infiltrating immune cells as well as expression of selected cytokines and the immune-regulatory protein PD-L1 (**Paper III**).
4. Determine whether the presence of various tumor-infiltrating immune cells as well as expression of selected cytokines and the immune-regulatory protein PD-L1 have prognostic value in a cohort of OSCC patients (**Paper III**).
5. Determine the prognostic value of some of the most commonly studied tissue-infiltrating immune cells in OSCC in the literature, and whether the studies identified in our literature search adhere to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [99, 100] (**Paper IV**).

Material and methods

This chapter provides an overview over the material and methods employed in the different papers and highlights general methodological considerations. Ethical and methodological considerations specific to this thesis are outlined in the discussion chapter. A more detailed description of the techniques and reagents can be found in the different papers. All papers are based on retrospective studies.

1. Patients

The study cohort that forms the basis of **Papers I-III** consists of 75-80 patients diagnosed with primary SCC of the oral cavity in the period 1986-2002 with the last day of follow-up being January 1, 2012. These patients derive from a larger North Norwegian cohort of patients with histologically verified oral and oropharyngeal carcinomas, where tissue material and clinicopathological data have been collected in a previous study from our group [101]. The clinicopathological data were retrieved from the patients' hospital files, pathology reports, and the Statistics of Norway, Cause of Death Registry. Clinicopathological variables included gender, age, smoking history, alcohol consumption, tumor site and differentiation, T-, N-, and M-statuses, treatment and HPV status according to p16 immunohistochemical staining. Tumors were staged following the latest (second to fifth) edition of the TNM classification system at the time of diagnosis [29, 102-104]. The classification of cancers of the lip and oral cavity remained unchanged between the different editions, so that the fifth edition, as summarized in Table 1, is relevant for all cases [29]. FFPE biopsies or surgical resections were collected from the archives of the Department of Clinical Pathology at the University Hospital of North Norway (UNN), and only patients with both primary tumor tissue and clinical data available were included. Rikardsen and colleagues demonstrated that this North Norwegian patient cohort correlated well with other European cohorts with regard to tumor location, stage, and risk factors [101]. This is important when comparing and interpreting results from

different populations, as exposure to etiological factors such as tobacco use may differ between oral cancer cohorts around the globe [3].

Cancers arising at different sites differ in their biology and response to treatment [105] and, if grouped, may skew results from prognostic marker studies [106]. Therefore, to obtain a more homogeneous cohort, we excluded several groups of patients from the original cohort. We excluded all patients with SCCs in the base of the tongue, the soft palate, and the tonsils, as these are often HPV-positive and should be regarded as a distinct entity [107]. Left were patients having SCCs in the mobile tongue, the floor of the mouth, buccal mucosa, gingiva and the hard palate, constituting the oral cavity proper. From this group, patients with verrucous carcinomas were excluded because these tumors generally show a less aggressive clinical behavior than conventional SCCs [108]. We also excluded patients who received radiotherapy prior to surgery, as this has been shown to influence the composition of the immune infiltrate and patient prognosis. Furthermore, very old patients, patients with co-morbidity and patients who did not receive treatment with curative intention were excluded, as they were likely to confound analyses of survival data. **Paper I** includes 80 patients, and **Papers II-III** are based on 75 of the 80 patients. For 5 of the 80 patients, the limited amount of tissue material available for our study was entirely used for the work on **Paper I**. A summary of the number of excluded patients with reasons for exclusion is given in Figure 5 below. The Regional Committee for Medical Health Research Ethics, Northern Norway (REK) approved the collection of the patients' tissue and clinical data (see appendix).

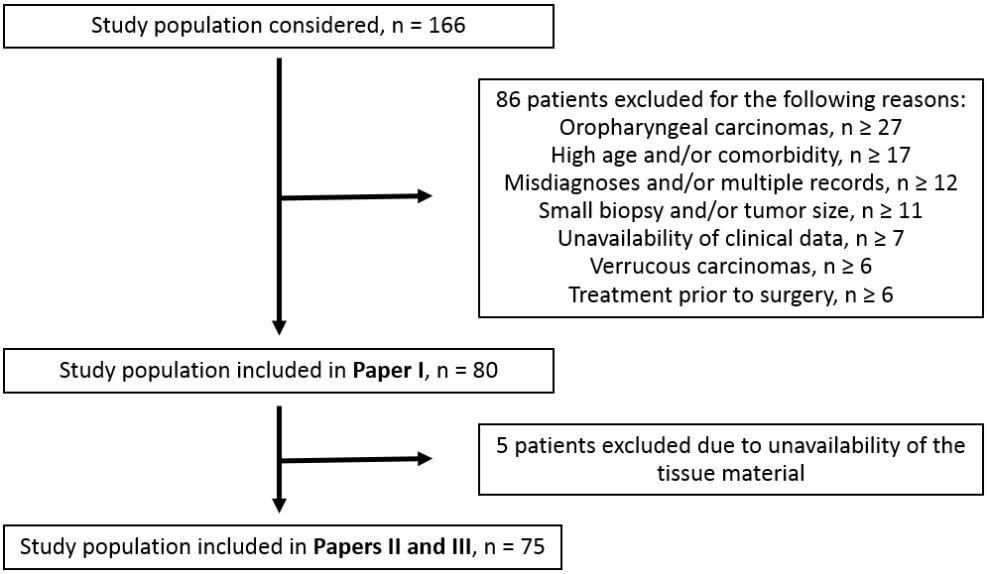

Figure 5. Patient flow chart. This figure describes the flow of patients included in Paper I and Papers II and III, respectively.

2. Immunohistochemistry

Immunohistochemistry (IHC), as employed in **Papers I-III** and reviewed in **Paper IV**, is the most widely used method for the detection of proteins in cells or tissues. IHC is based on the recognition of an antigen by the use of an antibody. Indirect IHC, the most common of IHC techniques, is a multi-step approach. In the first step, a primary antibody specifically binds to the antigen of interest. In the second step, an enzyme-labelled secondary antibody is applied for visualization. The most commonly used enzymatic label is horseradish peroxidase, which converts into a brown color after reaction with the substrate 3,3'-diaminobenzidine.

Several general methodological considerations have to be taken into account for correct interpretation and comparison of immunohistochemical results. Numerous pre-staining variables influence the IHC assay, as outlined in the discussion chapter.

In addition, the immunohistochemical staining results largely depend on the properties of the antibody used. The usefulness of an antibody cannot always be assumed from scientific publications or vendor specifications. Indeed, antibody validation for the specific application in individual laboratories is critical, especially if an antibody is tested for implementation in clinical routine. Antibody validation includes amongst others testing the specificity and reproducibility of an antibody in the context for which it is used [109]. There is no definition of a standard practice for antibody validation in IHC, but the use of blocking peptides and especially the use of appropriate positive and negative controls can provide valuable information. Whereas tissue with known positivity for the protein of interest is commonly accepted as a positive control, there is a wide range of negative controls reported in scientific works, of which some are highlighted in the discussion chapter of this thesis. Depending on the research question and material, one can choose between monoclonal and polyclonal antibodies. In contrast to monoclonal antibodies, polyclonal antibodies can recognize multiple independent epitopes. Although more often associated with non-specific background staining, polyclonal antibodies are usually favored over monoclonal antibodies for use in IHC as they have a higher chance of binding epitopes that are still present in the fixed material [110]. Reproducible antibodies show similar staining across different lots and assay runs over time, and for independent antibodies targeting the same protein [109].

As for all IHC procedures, there are no universal guidelines for evaluation of immunohistochemical staining. The huge variability of reported methods imposes challenges for comparison of studies, and stresses the importance of clear description of how the immunohistochemical evaluation was performed. Despite recent advances in computerized approaches such as digitalization of slides and automated image analysis [111], manual evaluation using conventional light microscopy is still the most widely used method for IHC evaluation in pathology departments worldwide. To assure reproducibility and assess the clinical usefulness of the

different markers, it is recommended to perform the scoring independently by several observers and to report the variability among the different scorings [106].

2.1 Immunohistochemical staining procedures

We stained 4-µm-thick whole tissue sections from FFPE archival material. For each patient, we chose a block with representative material from the available tumor-containing blocks based on hematoxylin and eosin staining, without specific evaluation of the inflammatory infiltrate. First, the sections were incubated overnight at 60°C, deparaffinised in xylene and rehydrated in graded alcohol baths before they were heat-treated in citrate buffer. Heat-treatment breaks down the protein cross-links generated by formaldehyde fixation thereby unmasking the antigen epitope of interest and allowing specific binding of the primary antibody. After antigen retrieval, we inactivated endogenous peroxidase activity by incubating the sections with 3% H2O2, thereby reducing non-specific background staining. We also used a protein-blocking reagent to block non-specific antibody binding. Then, we applied the primary and secondary antibody before visualization with 3,3'-diaminobenzidine, counterstaining with hematoxylin and dehydration of the sections in graded alcohol and xylene baths. Finally, the sections were mounted and evaluated in the microscope. PNAd staining for the detection of HEVs was done manually. Antibody specificity was tested by staining consecutive OSCC and normal oral mucosa sections for the endothelial cell marker PNAd as well as the blood vessel marker CD34 and the lymphatic endothelial cell marker D2-40. All other staining procedures were carried out in the automated slide stainer Ventana Benchmark, XT (Ventana, Tucson, AZ, USA) at the Diagnostic Clinic – Clinical Pathology, UNN, accredited according to the ISO/IEC 15189 standard for the respective staining. All staining runs included controls with known positivity for the different antibodies. For PD-L1 staining, negative controls with isotype-specific immunoglobulins were included for each patient sample. For the PNAd antibody, a specimen with the

primary antibody omitted was included in each run. Negative controls were included in the initial testing of the remaining antibodies.

2.2 Evaluation of immunohistochemical staining

We evaluated the immunohistochemically stained slides using a Leica DM2000 light microscope (Leica, Wetzlar, Germany). The staining was analyzed by a single observer for the morphological characteristics of HEVs, by two independent observers where an immunohistochemical score was to be obtained, and by three independent investigators for the presence of TLSs. All investigators were trained and blinded to the clinical outcome. In case of discrepancy, consensus was reached by reevaluating and discussing the results. We analyzed inter-observer variability in cases where slides had not been evaluated together.

TLSs, as studied in **Paper I**, were defined as accumulations of B cells containing FDCs, and were detected by staining consecutive sections of patients with obvious and indistinct B-cell aggregates for the FDC marker CD21. TLSs were subdivided into classical and non-classical, where FDCs displayed a continuous network in the classical type, and a more scattered distribution in the non-classical type. We also analyzed TLS-associated T cells and HEVs by staining consecutive sections with the CD3 and PNAd antibody, respectively. Consecutive sections from a subset of the TLS-positive patients were stained with BCL-6 for verification of germinal center B cells. In some patients, we evaluated TLSs at different levels in the tissue block, at a distance of 100 µm. This was done to analyze the intratumoral distribution of TLSs.

Assessment of HEVs, as first described in association with TLSs in **Paper I**, was based on PNAd-positive cell clusters forming a vessel-like structure. PNAd-positive single cells were not counted as HEVs. In **Paper II**, we assessed the presence of tumor-associated HEVs that we defined as HEVs located within 700-µm distance from the tumor front. For this, we carried out hotspot analyses and first scanned the PNAd-stained tissue sections at low power magnification to identify five areas with

high density of HEVs. Then, we took micrographs of the five hotspots at high power magnification, and calculated for each patient the mean number of HEVs per section by dividing the sum of HEVs in the five hotspots by five. The median number of HEVs per hotspot for all patients was used as cutoff for positive and negative HEV count, respectively. We also evaluated HEV morphology with regard to vessel wall thickness and lumen diameter. For this, we scanned the PNAd-stained sections, and analyzed the whole-slide digital images using digital measurement tools. Moreover, we semi-quantitatively assessed high and low level of inflammation in the digitized PNAd-stained sections at high power magnification with a cutoff of 50 lymphocytes/field of vision. To investigate the intratumoral distribution of HEVs, presence of HEVs was in some patients assessed at distinct levels throughout the tissue block, at a 100-µm distance.

In **Paper III**, we further characterized the inflammatory infiltrate. We identified mast cells and eosinophils in the tumor stroma by Giemsa staining using a similar hotspot approach as for HEVs. By immunohistochemical staining we recognized CD3-, CD4- and CD8-positive T cells, CD20-positive B cells and CD68-positive macrophages. Scoring of these cells was done in a semi-quantitative fashion, by capturing every second visual field at the tumor invasive margin and assigning the micrographs to a four-degree scale from no or almost no (0) to heavy infiltration (3). Then, a mean score for each section was calculated and dichotomized as low (0-1.49) or high (1.5-3). The PD-L1 and DC-lamp scores were also evaluated on a 0-3 scale, and classified as low (0 or 1) and high (2 or 3) expression. The PD-L1 score depended on the percentage of positively-labeled tumor cells in each tissue section, and DC-lamp expression in the tumor stroma was based on semi-quantitative evaluation of positive staining.

3. RNA extraction and quality control

Isolation of high-quality RNA is an important prerequisite for successful gene expression analyses. In **Paper III**, we used two different commercial kits for

extraction of total RNA from FFPE human oral cancer samples and freshly frozen (in liquid nitrogen) human lymphoma specimens, respectively. There are several methodological challenges related to RNA from formalin-fixed tissue, as further outlined in the discussion chapter. We have chosen a RNA isolation kit that has shown good results in previous studies [112, 113]. The freshly frozen samples were used as a positive control for subsequent real-time quantitative polymerase chain reaction (qPCR) analyses. Both procedures were based on silica columns that specifically bind the RNA and include the following steps: sample preparation, enzymatic digestion using proteinase K, purification and elimination of DNA and elution of the RNA in buffer or nuclease-free water. For the FFPE sections, we prepared the samples by deparaffinizing several consecutive 5-10 µm whole slide tissue sections. For the freshly frozen tissues, we mechanically disrupted and homogenized the material. Both the FFPE and freshly frozen samples were processed as they were, and contained both tumor material and surrounding tissue. We carried out spectrophotometric analysis via NanoDrop to assess RNA purity. The RNA integrity of our samples was evaluated using the RNA quality indicator (RQI) assessed by the automated electrophoresis system Experion (Bio-Rad Laboratories, Hercules, USA), which compares the electropherogram of RNA samples to a series of standardized degraded RNA samples assigning values between 1 for highly degraded RNA and 10 for intact RNA [114].

4. Real-time quantitative PCR

qPCR is generally a very sensitive and robust tool to amplify and detect nucleic acids, but the results may be strongly compromised by the low quality of input RNA [115], as further outlined in the discussion chapter.

In **Paper III**, we used qPCR to analyze the gene expression of inflammation-related cytokines and chemokines in FFPE oral cancer samples. We applied a two-step approach, where we first reverse transcribed total RNA into complementary DNA (cDNA), which we then used as a template for the qPCR reactions. The cDNA was

amplified in a thermal cycler through a series of cycles with repeated heating and cooling. The changing temperatures allow the double-stranded DNA to separate into single strands before binding of the primers and polymerization of the new DNA strands. After each cycle, the fluorescence intensity of a DNA-binding dye was detected in the thermal cycler. We designed primer sets to amplify short (~60bp) RNA segments within the target sequences. Partially based on findings from a previous study on FFPE oral cancer tissue [116], we identified the three most stably expressed genes in our samples using a publicly available software package. The choice of a panel of appropriate reference genes that are abundantly and stably expressed in the tissues is essential for accurate gene expression profiling [117].

Primer specificity was analyzed using melting curve analysis. In all qPCR runs, we included a control without reverse transcriptase and several non-template controls to test for contamination with genomic DNA and PCR products from previous runs, respectively. From the slope and correlation coefficient of regression curves from twofold serially diluted cDNA, we determined the qPCR amplification efficiency for each gene. We analyzed relative gene expression using the ΔΔCt method [118], where we normalized the relative amount of target mRNA against the geometric mean of the three reference genes. Results were presented as fold increase compared to the mean of the group with the lowest gene expression (+/- standard error of mean) or dichotomized in low and high expression with the median of fold increase as cutoff.

5. Systematic literature review

Summarizing and comparing existing knowledge in systematic reviews and meta-analyses can provide valuable information for both researchers and health care providers. However, the usefulness of these studies is often compromised due to poor reporting [119, 120]. In 2009, an international group of experienced authors and methodologists introduced the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines to enhance the transparency and completeness

of reporting in systematic reviews [119]. The choice of database, design of search strategy and eligibility criteria are important to retrieve relevant articles, and the results may or may not be summarized in meta-analyses. However, bias in the interpretation of results is inevitable also in thorough literature searches. A potential problem is a phenomenon called publication bias, which is the trend toward reporting positive results and is common to many research disciplines including cancer prognostic marker studies [121-123]. Reviewing biomarker studies with mainly statistically significant results may lead to overestimation of the prognostic value of a marker. Shifting the focus of journal editors from novelty and impact of findings to publication of negative results and follow-up of earlier studies may help to overcome this problem [106].

In **Paper IV**, we systematically searched the databases Ovid Medline (1946-2018 March 13) and Embase (1947-2018 March 13) for relevant literature on the prognostic value of some of the most commonly reported immune cells in oral cancer. With the help of a medical librarian, we developed a search strategy with controlled vocabulary based on Medical Subject Headings from Pubmed in addition to relevant free-text entry terms to retrieve relevant articles [124, 125]. We additionally employed the Cochrane Library and reference lists of reviews on related topics to identify relevant articles that were not retrieved through the combined Embase/Medline search. The two investigators independently screened the titles and abstracts of the retrieved records for relevance, and read the full-text articles in case of uncertainty and where the inclusion criteria were met. We designed a flow diagram with the excluded records according to the PRISMA guidelines [119]. Only studies with 40 or more OSCC patients and survival analyses based on immunohistochemical staining for tissue-infiltrating T cells, B cells, macrophages, dendritic cells, mast cells or NK cells were included. We also designed a checklist according to the REMARK guidelines [99, 100] to investigate the completeness in the reporting of study design and conduct. We used EndNote to manage the bibliographic records.

6. Statistical analysis

Critical evaluation of statistical methods is important for reliable conclusions and reproducibility of scientific results. Sample size is an important factor that influences the statistical outcome of a test. If the sample size is small, statistically significant differences are more likely to remain undetected [126]. In addition, the selection of appropriate cutoff points can bias the results. There is no generally accepted strategy how to choose the best cutoff point. Use of standard cutoff points from previous publications facilitates comparison between studies, but cannot be applied to novel markers or to different protocols [127,p. 461]. Here, one can instead select a cutoff based on the analyzed data such as the value that produced the smallest p-value – an approach that often overestimates the prognostic effect of a marker [128]. Despite numerous potential sources of bias, many prognostic marker studies rely on the p-value alone when judging the prognostic value of a suggested biomarker.

We used statistical package for the social sciences (SPSS) software for Windows (IBM, Armonk, NY, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA) to perform all calculations. We quantified inter-observer variability for the different scores by Spearman correlation test, and tested associations between the various variables using Pearson's Chi-square and Fisher's exact test. We used a Mann-Whitney U test for determination of significant morphological differences between HEVs. Univariate Kaplan Meier analysis was carried out to calculate disease-specific death rates and plot disease-specific survival curves. Only statistically significant variables from univariate analyses as estimated by the log rank test were entered into a stepwise forward multiple Cox regression model to identify independent prognostic determinants. We plotted log-minus-log plots to test the validity of the proportional hazards assumption. qPCR amplification efficiency was estimated by linear regression analysis of standard curves derived from serially diluted cDNA. Bivariate correlation analyses and the Pearson correlation coefficient

were used in the quantitative analyses of our systematic literature review. Results were considered significant if $P<0.05$, and borderline significant if $P<0.09$.

Summary of papers

Paper I

Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma.

Wirsing AM, Rikardsen OG, Steigen SE, Uhlin-Hansen L, Hadler-Olsen E.
BMC Clinical Pathology. 2014 Aug 23; 14:38. doi: 10.1186/1472-6890-14-38.

In **Paper I**, we used IHC to investigate the presence of tumor-associated TLSs in whole slide tissue sections from 80 OSCC patients. Some tissue blocks were sectioned at multiple levels to analyze intratumoral distribution of TLSs. We also correlated the presence of TLSs to the patient outcome. TLSs were detected in 17 (21 %) of the 80 OSCC patients, of which about a third remained undetected when analyzing only one level in the tissue block. TLSs were mainly located in the peri-tumoral stroma close to the invasive margin and were present as clusters of B cells containing FDCs that were surrounded by T cells and HEVs. B-cell aggregates with defined FDC meshwork showed distinct germinal centers (classical TLSs), whereas those with scattered FDCs did not (non-classical TLSs). The presence of TLSs correlated with increased survival rates, but only the T status was an independent prognostic factor.

Paper II

Presence of tumour high-endothelial venules is an independent positive prognostic factor and stratifies patients with advanced-stage oral squamous cell carcinoma.

Wirsing AM, Rikardsen OG, Steigen SE, Uhlin-Hansen L, Hadler-Olsen E.
Tumour Biology. 2016 Feb; 37(2):2449-59. doi: 10.1007/s13277-015-4036-4.

The HEVs identified in the stroma surrounding the tumor cells in **Paper I** formed the background for **Paper II**, which included 75 of the 80 OSCC patients from **Paper I**. We studied both the incidence and morphology of tumor-associated HEVs and analyzed their impact on patient survival. HEVs were present in 91% of the OSCC samples and, if present, were found at multiple levels within a tissue. HEVs displayed a heterogeneous morphology which was related to the inflammatory infiltrate. More specifically, we found HEVs with a thick vessel wall and small lumen in regions with high inflammation, and HEVs with a thinner vessel wall and larger lumen in areas with less infiltrating immune cells. The presence of HEVs was found to be an independent prognostic factor for improved survival of OSCC patients.

Paper III

Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma.

Wirsing AM, Ervik IK, Seppola M, Uhlin-Hansen L, Steigen SE, Hadler-Olsen E.
Modern Pathology. 2018 Feb 7. doi: 10.1038/s41379-018-0019-5.

The results on the presence and morphology of tumor-associated HEVs from **Paper II** indicated a close relationship between HEVs and the OSSC immune microenvironment and formed the basis for this paper. In **Paper III**, we more specifically characterized the inflammatory infiltrate in which HEVs arise with regard to the presence of different immune cell subsets and expression of

inflammatory cytokines and chemokines. We also explored a possible relationship between HEVs and PD-L1 as well as between PD-L1 and various cellular and molecular components of the OSCC immune infiltrate. Studying the same patient cohort as in **Paper II**, we employed IHC and qPCR, and correlated the results to each other as well as to clinicopathological data and patient survival. HEVs were associated with the infiltration of T- and B cells into the OSCC immune microenvironment and were predictive of an inflamed OSCC phenotype even at a high T stage. T-cell infiltration correlated with a tumor microenvironment rich in immune cells, and was associated with CCL19, CXCL12, and LTβ expression. Expression of PD-L1 correlated with increased infiltration of CD4+ cells and small tumor size, but not with the presence of HEVs. HEVs were more powerful prognostic markers than the N stage and the subsets of the immune infiltrate assessed in this study.

Paper IV

Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma – a systematic review.

Hadler-Olsen E, Wirsing AM. Manuscript submitted for publication.

For **Paper IV**, we systematically searched the literature for prognostic studies on some of the most commonly reported immune cells in OSCC. We also evaluated the completeness of the reporting of immunohistochemical staining and scoring procedures according to the REMARK guidelines [99, 100]. 1960 hits were identified through our searches, and 33 of them met our inclusion criteria. The study design and conduct of the published articles were very heterogeneous. CD163+ M2 macrophages and CD57+ mature NK cells showed the most promising results for the prediction of OSCC patient survival. The studies often lack important information on how the immunohistochemical assays were performed and analyzed.

Discussion

In this chapter, I will first address some of the key ethical considerations of my research with emphasis on ethical issues regarding the patient cohort. This is followed by an overview of the methodological considerations specific to this thesis. In the last section of this chapter, I will discuss the main findings from **Papers I-IV** and put them in the context of other findings from the literature.

1. Ethical considerations

People suffering from OSCC represent a particularly vulnerable patient group. Surgical resection of the tumor may lead to facial disfigurement, and radiation may cause severe problems with xerostomia, followed by speech- and eating disturbances, pain and dental problems. The often lifelong, treatment-related side effects may lead to stigma, shame and social exclusion of those who survive the disease. This, and the rather low incidence of OSCCs in Western countries, may be part of the reasons why groups representing the interests of OSCC patients are relatively small and may struggle to raise political attention. Today there is still relatively little public awareness of the need to develop new treatment strategies for this patient group compared to other cancer types such as breast and lung cancer. Enhancement of patient participation in research-related activities and popular dissemination of results in patient-focused publications may help to empower these patients and raise awareness for their concerns.

Every research involving human subjects faces ethical and legal requirements. In this thesis, all studies on human material adhered to The Declaration of Helsinki, which was adopted by the World Medical Association in 1964, and last revised in 2013 [129], and represents one of the key ethical guidelines for research with human subjects. Participants in research need to be provided with enough information for reasoned decision-making and be asked to give their consent. This concept is called informed consent and dates back to the Nuremberg Code from 1947 [130, pp. 151-

155]. It has been adopted in a revised version in the Norwegian Act of Patients' Rights from 1999 [131]. By decision from REK, Northern Norway (see appendix), we were obliged to send a letter to the patients with information about the study and the possibility to withdraw. However, we were granted exemption for written informed consent to minimize the burden of a consent process for the patients who were still alive and of high age by the start of the study. This implicated that patients who did not actively withdraw from the study were included. It has to be pointed out that there were no consequences related to participating in this study, and that all clinical data were kept anonymous. Confidentiality of patient information is essential as communication of individual biological data such as risk information may have severe social implications for the patients, e.g. on their rights to insurance or employment [132].

2. Methodological considerations

2.1 Collection of patient data and tissue

The retrospective design of our study has several limitations with regard to the collection of both patient information and tissue material. Patient information has originally been gathered for purposes other than research, and some of the parameters lacked standardized reporting in the patient files. Especially the self-reports on smoking and alcohol consumption should be treated with caution, also because various factors such as the desire to give socially appropriate answers or a perceived lack of confidentiality may affect these data [133]. Some patients in our study missed data on one or more variables which may reduce the power and precision of our results [134].

The study of archived FFPE tissue is an invaluable source to understand disease mechanisms, as it is often well-characterized with histological, pathological and follow-up clinical data. However, it also imposes major methodological challenges that may limit the reliability of results. The workflow from acquisition of the tissue sample to interpretation of the data is complex and comprises numerous pre-

analytical factors related to the collection and fixation of the samples that the researchers cannot influence retrospectively (Figure 6). Inadequate tissue processing may cause loss of antigenicity that negatively affects both histopathologic approaches and downstream analyses [135], as further outlined in sections 2.2 and 2.3 of this chapter.

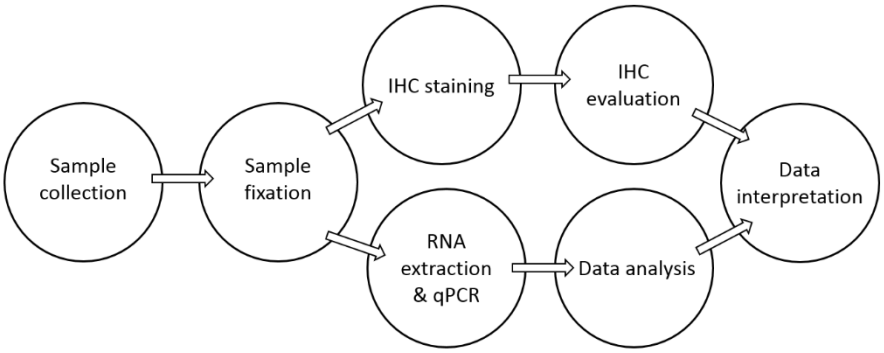

Figure 6. Workflow of immunohistochemistry (IHC) and qPCR experiments. This figure describes the complex workflow of IHC and qPCR analysis from the collection of the sample to the interpretation of the data. Pre-analytical factors regarding sample collection and fixation are often beyond the control of the researcher, but influence the assays and results.

In 2005, the REMARK guidelines were simultaneously published in different papers to encourage transparent and complete reporting and to improve the quality and usefulness of prognostic marker studies [99]. In 2012, an explanation and elaboration paper was published to accompany the REMARK guidelines [100]. The prognostic marker studies in **Papers I-III** broadly adhere to these guidelines. In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published [136], which outline the minimum information and analyses to include in qPCR experiments.

2.2 Immunohistochemistry

IHC is worldwide the most commonly used technique to study proteins of interest in archival FFPE patient tissue, and is often the method of choice for prognostic marker studies. However, many IHC-related procedures lack standardization of study design and conduct, which limits the reliability of the results and the comparability of biomarker studies [106]. A wide variety of pre-staining conditions such as the type and use of fixatives as well as tissue storage may affect the staining pattern and intensity [106, 137]. This information is however hardly reported in pathological records and was not available in our study. Besides, only precut slides were available in some cases, and these may have had altered immunoreactivity [138, 139].

Antibody validation, as outlined in the method section, is an important prerequisite for the interpretation of IHC results [109]. Most of the staining procedures in our studies have been performed according to standardized protocols at the Diagnostic Clinic – Clinical Pathology, UNN, and have been thoroughly validated for use in clinical routine. The anti-PNAd antibody, however, has so far only been used in research, and has been validated by us as specified in **Paper I**. We found that a few CD34+ blood vessels but no D2-40+ lymphatic vessels displayed sporadic PNAd positivity in consecutive OSCC tissue sections, indicating high specificity. Antibody specificity was also evaluated from the expected morphology of the positive staining, which in the case of PNAd staining was a vessel-like structure with or without lumen. PNAd-positive single cells were sporadically observed, but were not counted as HEVs. Human lymph nodes always showed positive staining. We used specimens with the primary antibody omitted as negative controls which is debatable as it controls for non-specific binding of the secondary antibody rather than specificity of the primary antibody itself [140]. In conclusion, our tests indicated a good specificity for the PNAd antibody. In case of more non-specific background staining or more staining of single cells, additional specificity testing would have been needed. In this case, including an isotype-specific immunoglobulin or ideally tissue from knockout

mice that does not express the protein of interest could have been suitable approaches [109]. We tested the anti-PNAd antibody extensively and found reproducible staining within different batches and assays. Especially in prognostic marker studies, it is recommended to identify the most reproducible among a panel of antibodies against different epitopes of the same target protein [106, 109]. In our study, we detected HEV solely with the anti-PNAd antibody (MECA-79), which appeared to be the only commercially available antibody for our purposes and has been well-described in the literature [141-143].

2.3 Real-time quantitative PCR

Analysis of archival patient tissue has historically been done by studying proteins using immunostaining techniques. Recent advances in the field of molecular research, however, have opened possibilities for genetic analysis of long-term preserved FFPE material. However, differences in sample acquisition as well as tissue processing and storage conditions often make comparison of gene expression data from archival FFPE tissue difficult [144]. For use in qPCR, the RNA quality of formalin-fixed tissue is often highly impaired due to cross-linking with proteins and other chemical modifications [145]. This requires careful controls and optimized procedures as outlined below.

Extraction of high-quality RNA is necessary for successful downstream analysis but can be demanding in the case of formalin-fixed tissue. In our study, the RQI values for the FFPE samples were low but above the cutoff set by the manufacturer of the RNA extraction kit, indicating an acceptable RNA quality. Employing qPCR in quantitative analysis of cytokines is challenging due to their naturally low expression levels [146, 147]. In extensively degraded tissue, amplicons with smaller size can increase the yield of quantifiable mRNA [148-150]. To determine the effect of amplicon size on the reliability of qPCR results, we used short, middle, long and extra-long amplicons for five high and five middle- to low-expressed genes, respectively, and compared quantified gene expression of samples stored in liquid

nitrogen with FFPE tissue. Our preliminary data show that amplicons ~60bp RNA yielded best results for quantification of both high and moderate- to low-expression gene transcripts in the FFPE tissue, both with regard to lower Ct values and higher amplification efficiency compared to larger amplicons [151]. Thus, we specifically designed primer sets to amplify short (~60bp) RNA segments within the target sequences.

Appropriate controls as outlined in the methods section are a prerequisite for correct interpretation of qPCR results. Genomic DNA contamination can greatly affect the qPCR signal of genes with low expression levels such as cytokines [146, 147], which is why each run included a control without the reverse transcriptase. We used RNA from frozen (in liquid nitrogen) lymphoid tissue as positive control in all qPCR runs. Compared to fixation with formaldehyde, freezing of tissue samples is considered a much better method to preserve nucleic acids [144]. This is reflected in our study by the three to four-times higher RQI values in the frozen compared to the FFPE samples.

Taken together, the acceptable quality of the extracted RNA as well as the optimized procedures strengthen the reliability of our results. Finally yet importantly, the biological importance of mRNA quantification remains questionable, as mRNA concentrations do not reflect the functional level of a protein [152]. Targeting cytokines at the protein level such as with IHC approaches may however be challenging as soluble, small peptides such as cytokines and chemokines may leak from the tissue [153].

2.4 Systematic literature review

A broad coverage of the published literature is the gold standard for every systematic research and helps to reduce bias in the datasets [154]. Numerous medical databases are available, each with specific scope in the coverage of journals and subjects [155]. As presented in **Paper IV**, we searched the two electronic databases Medline and Embase. Although many systematic reviews rely on Medline alone, searching

Medline in combination with Embase may be beneficial to cover a greater percentage of the available literature [156, 157]. In our study, the combined Embase/Medline search yielded comprehensive results as demonstrated in Figure 7. Embase provided almost twice as many hits as Medline (Figure 7, left). By searching Medline alone, we would have identified about 80% (n=26) of the 33 eligible articles (Figure 7, right). This indicates that more search hits did not necessarily mean many more relevant records, and that the additional value of employing the Embase database is marginal.

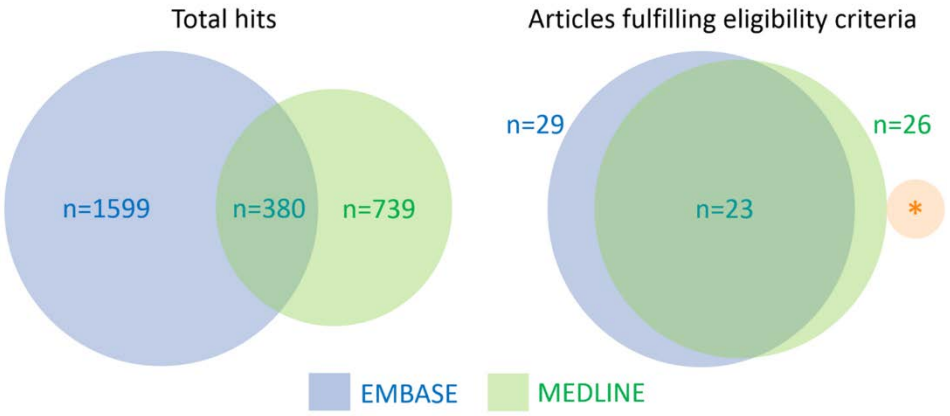

Figure 7. Venn diagrams showing the difference between hits retrieved (left) and articles fulfilling the eligibility criteria (right) from searching the Embase and Medline databases as presented in Paper IV. Overlap demonstrates duplicate records. * one article was retrieved through a review article. Figure made using www.meta-chart.com.

No search can identify all relevant articles. Articles with imprecise or uninformative abstracts and titles often lack adequate indexing in the databases [125] and may have been missed in our searches. Due to practical reasons, we excluded articles in languages other than English although this has been found to decrease the precision of study results [158]. Whereas narrow inclusion criteria impose the risk of excluding potentially relevant articles, defining broader criteria may increase the heterogeneity among the identified studies [154]. Due to the lack of standardized terminology for

oral cancer in the literature, we used relatively broad search criteria to identify publications on OSCC. This resulted however in a great amount of studies on head and neck cancers without specification of anatomical sites, as awareness of subsite-specific characteristics has only recently increased among researchers.

Due to the high heterogeneity of the studies and lack of reporting of statistical variables, we decided to not perform meta-analyses. Instead, we interpreted our results from the number of studies with highest statistical significance or largest "effect on survival", a quasi-quantitative approach which is poorly validated [119] and which may have biased our results as specified in the following section. More detailed reporting of the statistical data and the study conduct in general may help to facilitate data retrieval and synthesize the numerous published biomarker studies [106, 159].

2.5 Statistical analysis

Statistical tests are often misused or misinterpreted. There are many potential sources of bias in statistical analysis such as the size of the patient cohort [126]. In our study, non-significant results may have turned out to be statistically significant if the group size had been larger. Dividing cohorts in subgroups with critically small size may impair the validity of results from multivariate analysis. As a rule of thumb, a minimum of 10 events per variable should be used to assure reliable modeling in multivariate analysis [160]. In our study, the HEV-negative group comprised only seven patients which limits the quality of our results and calls for reevaluation in a larger cohort with more HEV-negative patients.

Cutoff values are another potential source of bias in statistical analysis. Our cutoff values were based on the median or mean of positively stained cells which increases the reproducibility of the scoring method and avoids bias in the datasets [100, 106]. Categorization of continuous variables, as used in our study, generally simplifies analyses, but is associated with loss of information, especially for dichotomized variables [100]. It is important to note that our cutoff points are data-dependent and

may be difficult to translate into a clinical setting. Finally yet importantly, discriminating patients into prognostic categories defined by cutoffs of a continuous prognostic factor only allows for superficial prognosis of individual patient outcome [161].

The usefulness of the widely implemented p-value has been extensively debated in the scientific community [126, 162, 163]. Despite being the dominant approach in many biomarker studies, leaning on the statistical significance of the p-value alone may be insufficient to measure the effect size or importance of a result [106, 159]. In the systematic review presented in **Paper IV**, we therefore based our conclusions on the "effect on survival" of a marker rather than its statistical significance. Only considering reported effect statistics such as the relative risk may have allowed for better comparison between the studies [163], but would have been impractical due to lack of reporting of these data in most of the studies. Replacing current statistical methods with approaches that more directly address the effect size of a parameter, along with complete reporting of study design and conduct, may help to avoid poor-decision making based on false conclusions and may promote good statistical and scientific practice [126, 162].

3. Discussion of main findings

It is generally accepted that the host immune system plays an important role in cancer development. Stromal cells surrounding the tumor crosstalk with cancer cells, and upon stimuli initiate an immune reaction that either prevents or promotes tumor growth and spread [164]. Significant effort has been directed towards understanding the roles of the cellular and molecular components that form the tumor microenvironment in OSCC, both with regard to their potential as prognostic markers and as tools for immunomodulatory therapies [1, 54, 165].

Development of TLSs has been extensively studied in chronic inflammatory diseases, where these structures are believed to contribute to disease progression

[79]. Only recently, TLSs have been acknowledged as a common feature in many human solid cancers, where they are often associated with prolonged patient survival [86, 166]. Accordingly, we detected TLSs in the tumor stroma of 21% of the 80 OSCC patient samples and found that the presence of these structures was associated with improved survival. It is widely recognized that the host can develop spontaneous immune responses against a tumor [49], and TLSs have been suggested to be responsible for orchestrating effective anti-tumor responses [167]. Thus, we suggest that the association with survival benefits in our patient cohort may be due to the ability of TLSs to generate local tumor-suppressive reactions in OSCC. Studies describing an independent effect for TLSs are scarce [168]. Also, in our Norwegian OSCC cohort, TLSs were found not to be independent prognostic markers. It has been speculated that TLSs simply support CD8+ T cell effector activity rather than exerting independent effects on patient survival [86]. Indeed, patients with an inflamed T-cell tumor microenvironment have been shown to have a better outcome and response to treatment despite the advanced disease [71, 72], and head and neck cancers are mostly of the 'inflamed' phenotype [73]. In our OSCC cohort, almost three-quarters of the tumors were highly infiltrated with CD3+ T cells, which again correlated with a tumor microenvironment rich in immune cells and inflammatory cytokines. However, neither the results from our Norwegian OSCC cohort nor the findings from our literature review indicated a strong positive prognostic effect for T cells in OSCC. It has however to be noted that many different T cell subsets with distinct functional properties exist, of which some have been shown to be functionally exhausted [74].

B cells can differentiate into antibody-producing plasma cells and have the potential to generate long-term T-cell antitumor responses [169]. We found that high infiltration of CD20+ B cells, as detected in 57% of our OSCC patients, was associated with increased survival rates. Pronounced CD20+ B-cell aggregates were also a characteristic feature of TLSs in our OSCC cohort. TLSs with distinct organizational levels have been described in the literature, from rudimentary B-cell

aggregates to fully developed structures with germinal centers [86]. We identified two distinct TLS phenotypes, whereof classical TLSs indeed presented all cells needed to generate a functional immune response. In classical TLSs, the CD20+ B-cell zones were found to comprise a network of CD21+ FDCs as well as Bcl6+ germinal center B cells, indicating sites for ongoing humoral response. However, functional studies are needed to elucidate whether TLSs are in fact sites for tumor-specific antibody production in our OSCC cohort. It would also be interesting to study the functional properties of the classical and non-classical TLS phenotypes found in our OSCC cohort. Our results indicate that the so-called non-classical phenotype may rather be a methodological artefact, as some TLSs classified as non-classical on one tissue level presented a classical pattern on another tissue level and *vice versa*. Detection of TLSs needed careful analysis, and inhomogeneous distribution of TLSs within the tumors increases the chance of false-negative results. To date, there is no standardized approach for the evaluation of the presence of tumor-associated TLSs. Developing common guidelines may help to synthesize future research, and shed light into the role of TLSs in cancer. Likewise**,** more and larger studies are needed to conclude about the prognostic value of CD20+ B cells, which are commonly understudied in OSCC.

In contrast, many studies assessing the prognostic value of CD68+ macrophages exist, although with conflicting results. Whereas high infiltration of CD68+ macrophages is in the literature mostly associated with decreased survival, we found an association with increased survival in our Norwegian OSCC cohort. However, the pan-macrophage marker CD68 does not differentiate between individual macrophage subtypes with distinct functionality. Although tumor-associated macrophages are often directed towards the tumor-supporting M2 phenotype [64, 65], it might be that many macrophages found in the tumor stroma of our OSCC cohort are M1 macrophages with tumor-suppressive functions.

More consistent findings in the literature are reported for CD163+ M2 macrophages and CD57+ mature NK cells, of which high numbers were associated with lower and higher survival rates, respectively. However, most of the studies were carried out in East-Asian patient cohorts, and differences such as in exposure to risk factors may limit the comparability of results derived from these cohorts to others. We demonstrated that most of the publications on biomarkers in OSCC lacked important information on how the studies were designed and conducted which limit the reliability and comparability of these results. Encouraging authors, editors and reviewers to enhance the transparency and completeness of reporting may improve the quality of biomarker studies and help to facilitate clinical implementation of individual markers.

Based on the immune cells assessed and variables included in our Norwegian OSCC cohort, we found that HEVs had superior prognostic value to the N stage and other components of the immune infiltrate. HEVs are specialized blood vessels that have been suggested to promote antitumor immunity in a variety of solid cancers through facilitating lymphocyte recruitment to the tumor site [89]. We found tumor-associated HEVs in 91% of the 75 OSCC samples, and their presence independently predicted improved patient outcome. HEVs were associated with an inflamed immune microenvironment both in early and advanced disease. The absence of HEVs in large tumors predicted lower survival compared to HEV-positive tumors of the same size. Thus, one can speculate that patients with HEV-negative, large tumors might benefit from more rigorous treatment. We showed a correlation between HEVs and high expression of inflammatory cytokines and chemokines as well as high influx of B and T cells, indicating that HEVs may be main gates for lymphocytes into the OSCC microenvironment. It is important to note here that the HEV-negative group was relatively small (n=7) which limits the reliability of our results. Besides its prognostic value, a good biomarker needs to exhibit clinical usefulness in terms of feasibility and reliability of immunohistochemical staining and scoring procedures [106]. HEVs were evenly distributed within the tumors,

suggesting that these vessels can be reliably detected on a single tissue level. Moreover, HEV detection is easy and only requires a single staining, indicating that implementation in diagnostic routine may be straightforward.

Our findings suggest a tumor-suppressive role for TLSs and HEVs in OSCC tumorigenesis. Both seem to develop early during OSCC progression, and some of the advanced tumors may have found ways to escape immune surveillance by disrupting the mechanisms that induce and stabilize TLSs and HEVs. HEV morphology has earlier been found to be highly versatile, depending on stimuli from the surrounding microenvironment [170]. We found HEVs resembling an immature phenotype with a relatively larger lumen and thinner vessel wall to be surrounded by fewer lymphocytes than those with the more mature morphology of a plump endothelial lining and a small lumen. Remodeling of HEVs from lymphocyte- to blood-carrying vessels has been associated with poor prognosis [92, 93] and HEVs with an immature phenotype may be in the process of differentiation or de-differentiation from/to normal blood vessels. Analyzing individual HEV subsets and their surroundings may give important clues to how HEVs can be induced and maintained as the tumor progresses. We did not find an association between HEVs and the presence of dendritic cells or between HEV-positive tumors and LTβ gene expression, although these parameters have shown key regulatory functions for HEV development and stabilization in other works [95].

Despite being generally rich in immune cells, many head and neck cancers have developed mechanisms that suppress the apparent immune response [66]. Cells expressing PD-L1 may impair effector T-cell functions, and blockade of the PD-1/PD-L1 pathway has shown promising results in many cancers including OSCC [77, 78, 171-173]. Knowledge about the prevalence and prognostic value of PD-L1 expression in OSCC is limited [75, 174], and we found no correlation between PD-L1-expressing tumor cells and patient survival. However, we observed an association between PD-L1 expression and high numbers of stromal CD4+ T cells

as well as CD8+ cytotoxic T cells and a number of inflammatory cytokines including IFN-γ. Cytotoxic T cells can induce PD-L1 expression in an IFN-γ-dependent manner, and the suggested negative feedback mechanism for PD-L1 in the pre-existing T-cell inflamed microenvironment [175, 176] may also be effective in the OSCC immune infiltrate. Our findings do not indicate a strong relationship between HEVs and PD-L1. Interestingly, OSCCs with high T stage, and particularly the HEV-positive ones, expressed lower PD-L1, suggesting that these tumors may have found ways to overcome PD-L1-mediated immunosurveillance despite tumor progression. Not all patients seem to benefit equally from PD-1/PD-L1 blockade therapy, and one can speculate that patients with T1/T2 tumors or HEV-negative T3/T4 tumors may be better responders. High PD-L1 expression correlated to an immature dendritic cell phenotype in lung cancer [177], and one could hypothesize that blocking PD-L1 may promote dendritic cell maturation which may again help to induce and stabilize HEVs. Likewise, it would be interesting to study the effect of PD-1/PD-L1 blockade on TLS formation and functionality. Indeed, absence of PD-1-signaling in mice has been shown to result in increased death of germinal center B cells, suggesting a role for this pathway in B-cell response [178]. There are still many unknown factors related to new-generation immunotherapies, and the highly adaptive and heterogeneous nature of the tumor microenvironment impedes direct targeting of individual components of the immune infiltrate [165]. A better understanding of the OSCC tumor microenvironment and how different components interact with each other and with the cancer cells may open for new therapeutic strategies to modulate antitumor immunity.

Conclusions and perspective

Based on the findings from our Norwegian OSCC patient cohort, we conclude that TLSs form in OSCC. The presence of these structures is associated with increased survival rates, but is not an independent prognostic factor. The inhomogeneous intratumoral distribution of TLSs along with their laborious detection limits the clinical usefulness of these structures. HEVs are found in relation to TLSs as well as independent from these structures, and appear in different phenotypes. The presence of HEVs is an independent prognostic marker for improved survival. Detection and scoring of HEVs is simple and reliable and could be easily used in clinical routine. Both early and advanced HEV-positive tumors are associated with high infiltration of T- and B lymphocytes, and with high expression of CCL19, CCL21, and CXCL12. T-cell infiltration correlates with high infiltration of a number of other immune cells as well as CCL19, CXCL12 as well as with LTβ expression. PD-L1 expression correlates with CD4+ T cells and small tumor size, but not with the presence of HEVs. High expression of CD20+ B cells and CD68+ macrophages are associated with improved patient survival, but are no independent prognostic markers. Based on the findings from our review, high expression of CD163+ M2 macrophages and CD57+ mature NK cells are promising prognostic markers for OSCC patients. Reporting of methods and data was found to be often incomplete in prognostic marker studies, which limits the usefulness of these studies.

We cannot draw any strong conclusions on the prognostic relevance of the markers identified in our literature review since important information is often missing. Thus, their prognostic value needs to be verified in larger and more standardized studies adhering to best-practice guidelines. Likewise, the reliability of the findings from our Norwegian OSCC patient cohort is limited by the small sample size and calls for verification of our results in larger studies. We are currently establishing a nationwide multicenter study with more than 500 OSCC patients. A next step will be to analyze the large cohort for promising prognostic markers identified in this

thesis. In addition, it would be interesting to study regulatory mechanisms of the immune microenvironment in functional studies, where we can follow the development of the tumor microenvironment during cancer progression. We are currently establishing a mouse model where we induce OSCC development by adding the carcinogen 4-nitroquinoline-1-oxide to the drinking water. This method is well-described in the literature [179-181] and gives predictable development of OSCC in the oral cavity via pre-malignant mucosal lesions.

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Appendices

Letter of approval from REK for the use of patient data and tissue (2007)

Renewal of approval from REK for the use of patient data and tissue (2015)

Paper I

Paper II

Paper III

Paper IV

Uhlin-Hansen Lars
Avdeling for patologi
Institutt for medisinsk biologi, Med fak, UiT
9037 TROMSØ

Deres ref.: 5.2007.32 Vår ref.: 200700623-7/IAY/400 Dato: 27.03.2007

P REK NORD 22/2007 MUNNHULEKREFT KLINISKE OG BIOLOGISKE ASPEKTER - SLUTTVURDERING - KOMITEEN HAR INGEN INNVENDINGER MOT AT PROSJEKTET GJENNOMFØRES

Vi viser til prosjektleders elektroniske brev av 26.3.2007 med vedlegg.

Prosjektleders tilbakemelding på komiteens merknader til prosjektet, sist i brev av 19.3.2007 tas til etterretning.

Regional komité for medisinsk forskningsetikk, Nord-Norge (REK Nord) har ingen innvendinger mot at prosjektet gjennomføres.

Det forutsettes at prosjektet er godkjent av aktuelle formelle instanser før det settes i gang.

Det forutsettes at prosjektet forelegges komiteen på nytt, dersom det under gjennomføringen skjer komplikasjoner eller endringer i de forutsetninger som komiteen har basert sin avgjørelse på.

Komiteen ber om å få melding dersom prosjektet ikke blir sluttført.

Komiteen oversender dokumenter til Sosial- og helsedirektoratet for behandling av søknad om oppretting av forskningsbiobank.

Vennlig hilsen

Ingunn Ytrehus
førstekonsulent
77645347

Kopi:
Sosial- og helsedirektoratet (postmottak@shdir.no) vedlagt biobankskjema og forespørsel om deltakelse i forskningsprosjektet.

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK nord			24.08.2015	2015/1383/REK nord
			Deres dato:	Deres referanse:
			16.06.2015	

Vår referanse må oppgis ved alle henvendelser

Lars Uhlin-Hansen

Institutt for medisinsk biologi

2015/1383 Munnhulekreft, -en retrospektiv studie av prognostiske biomarkører

Forskningsansvarlig: UiT Norges arktiske universitet
Prosjektleder: Lars Uhlin-Hansen

Vi viser til søknad om forhåndsgodkjenning av ovennevnte forskningsprosjekt. Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK nord) i møtet 13.08.2015. Vurderingen er gjort med hjemmel i helseforskningsloven (hfl.) § 10, jf. forskningsetikkloven § 4.

Prosjektleders prosjektomtale
Munnhulekreft er forbundet med plagsomme, behandlingsrelaterte bivirkninger og relativt høy dødelighet. Det er imidlertid stor variasjon i aggressivitet i disse svulstene. Av og til vokser små svulster fort og fører til død innen 1-2 år, mens andre pasienter med svulster i samme stadium blir varig helbredet. Det er derfor nødvendig med bedre individtilpasset behandling som tar høyde for at svulstene oppfører seg forskjellig. Hovedmålet med prosjektet er å påvise molekyler i kreftcellene som kan brukes som grunnlag for valg av behandling, både for å begrense de behandlingsrelaterte bivirkningene og for å bedre sjansen for helbredelse. Prosjektet er en videreføring av PhD-prosjektet til overlege Oddveig Rikardsen som disputerte i 2014. De innsamlede data er svært verdifulle og er godt egnet for videre studier. Flere undersøkelser er allerede påbegynt og disse bør fullføres. Prosjektet vil inngå som en del av doktorgradsarbeidene til de 3 studentene som er oppført som medarbeidere.

Vurdering

Design
Målet med prosjektet er å kartlegge biomarkører som kan benyttes til å gi fremtidige pasienter med munnhulekreft en mer individtilpasset behandling. Dette innebærer at man hos noen pasienter kan redusere bivirkningene, mens andre pasienter får ekstra behandling som øker sjansen for helbredelse.

Unntak fra samtykke
Prosjektet er en videreføring av prosjektet" Munnhulekreft, -en histopatologisk studie av tumorstroma interaksjoner" som dannet grunnlaget for PhD-prosjektet til overlege Oddveig Rikardsen. Hun laget et prosjektregister for pasienter behandlet for munnhulekreft ved UNN i perioden 1986- 2002. Alle pasientopplysninger er avidentifiserte og det er kun overlegene Oddveig Rikardsen og Sonja Steigen som har tilgang på koblingsnøkkelen. Det samme prosjektregisteret og kodenøkkel skal brukes i dette prosjektet.

Det søkes om fritak fra å innhente samtykke for alle pasientene som inngår i studien, med unntak av de som

eventuelt inngår i registeret «Biologisk forskningsreservasjon».

Fritak gjelder for nye analyser på biologisk materiale som er samlet inn 1986- 2002.

Vurdering av unntak fra å innhente samtykke for bruk av biologiske materiale i studien
Helseforskningslovens § 28 regulerer adgang til bruk av biologisk materiale som er innsamlet i helsetjenesten til forskning. Vilkårene for å kunne gi unntak fra å innhente samtykke er at forskningen er av vesentlig interesse for samfunnet, at hensynet til deltagernes velferd og integritet er i varetatt. Det må også innfortolkes et krav om at det vil være vanskelig å innhente samtykke. Komiteen vurderer prosjektet til å være av vesentlig interesse for samfunnet. I vurderingen av om hensynet til deltagernes integritet er i varetatt har komiteen lagt vekt på at prøvene behandles avidentifisert. I vurdering av om det vil være" vanskelig å innhente samtykke", fremgår det både av forarbeider og av kommentarer til helseforskningsloven at kvalifiserte og legitime årsaker til dette kan være dersom deltager er død, flyttet, sykdom, stort antall deltagere, samt at et stort frafall vil svekke forskingens validitet. Det kan også tas hensyn til hvor gamle prøvene er. I dette prosjektet er det omsøkte materialet hentet inn mellom 1986 og 2002. Prøvene er samlet inn før helseforskningsloven trådte i kraft. Selv om det ikke foreligger samtykke åpner loven for at materialet kan brukes. Munnhulekreft er en kreftform som i hovedsak rammer eldre personer. Den er dessuten forbundet med høy dødelighet (kun 50-60% overlever 5 år). De aller fleste pasientene som inngår i studien er døde, enten på grunn av kreften eller av annen årsak. Nesten alle de som fortsatt lever er svært gamle, for noen av pasientene vil det være ca. 13-29 år siden prøvene var tatt. Det må også forventes at mange av disse ikke responderer på henvendelsen om samtykke. Dersom det kreves samtykke må en derfor anta at det det blir en betydelig skjevfordeling av kohorten i forhold til tidligere undersøkelser som er gjennomført på dette materialet i prosjektet.

Unntak samtykke for gjenbruk av helseopplysninger
Det fremgå av hfl §15 «Dersom det er vanskelig å innhente nytt samtykke, kan den regionale komiteen for medisinsk og helsefaglig forskningsetikk godkjenne ny eller endret bruk av tidligere innsamlet humant biologisk materiale eller helseopplysninger uten at nytt samtykke innhentes. Dette kan bare skje dersom slik forskning er av vesentlig interesse for samfunnet og hensynet til deltakernes velferd og integritet er ivaretatt. Den regionale komité for medisinsk og helsefaglig forskningsetikk kan sette vilkår for bruken.

Vilkårene for å kunne gi unntak fra å innhente samtykke er at forskningen er av vesentlig interesse for samfunnet, at hensynet til deltagernes velferd og integritet er i varetatt. Det må også innfortolkes et krav om at det vil være vanskelig å innhente samtykke. Komiteen vurderer prosjektet til å være av vesentlig interesse for samfunnet.

I vurderingen av om hensynet til deltagernes integritet er i varetatt har komiteen lagt vekt på at opplysningene behandles avidentifisert. I vurdering av om det vil være" vanskelig å innhente samtykke", fremgår det både av forarbeider og av kommentarer til helseforskningsloven at kvalifiserte og legitime årsaker til dette kan være dersom deltager er død, flyttet, sykdom, stort antall deltagere, samt at et stort frafall vil svekke forskingens validitet. Det kan også tas hensyn til hvor gamle opplysningene er. I dette prosjektet er de omsøkte opplysninger hentet inn mellom 1986 og 2002. Opplysningene er samlet inn før helseforskningsloven trådte i kraft. Munnhulekreft er en kreftform som i hovedsak rammer eldre personer. Den er dessuten forbundet med høy dødelighet (kun 50-60% overlever 5 år). De aller fleste pasientene som inngår i studien er døde, enten på grunn av kreften eller av annen årsak. Nesten alle de som fortsatt lever er svært gamle, for noen av pasientene vil det være ca. 29 år siden prøvene var tatt. Det må også forventes at mange av disse ikke responderer på henvendelsen om samtykke. Dersom det kreves samtykke må en derfor anta at det det blir en betydelig skjevfordeling av kohorten i forhold til tidligere undersøkelser som er gjennomført på dette materialet i prosjektet.

Komiteen har vurdert at prosjektet har stor samfunnsnytte, risikoen for at prosjektet skal krenke deltagernes integritet ert liten og det er en reell fare for betydelig skjevfordeling dersom man skal innhente et aktivt samtykke. Fritak fra samtykke etter

Helseforskningsloven, gjelder personopplysningslovens bestemmelser om opplysningsplikt, jf. personopplysningsloven § 20. Hovedregelen ved unntak fra samtykke er at de involverte opplyses om

bruken av personopplysninger. Informasjon om registrering, her inklusjon i et forskningsprosjekt, er et viktig prinsipp det skal gode grunner til for å fravike.

Når det gjelder vilkåret om at informasjon skal være «umulig eller uforholdsmessig vanskelig», jf. § 20 annet ledd b), vurderer komiteen se at dette vilkår ikke er oppfylt for de av deltagerne som er i livet.

REK nord setter derfor som vilkår at deltagerne som er i live skal informeres om bruken av helseopplysningene og det biologiske materialet, samt informeres om muligheten til å reserverer seg.

Oppbevaring av data
Det fremgår av søknaden at data skal oppbevares i 10 år. Opplysninger skal ikke oppbevares lenger enn det som er nødvendig for å gjennomføre prosjektet. Den regionale komiteen for medisinsk og helsefaglig forskningsetikk kan bestemme at dokumenter som er nødvendig for etterkontroll av prosjektet, skal oppbevares i fem år etter at sluttmelding er sendt komiteen, jf hfl.38.

Komiteen imøteser en eventuell nærmere begrunnelse for hvorfor data skal oppbevares i 10 år.

Vedtak
Med hjemmel i helseforskningsloven §§ 2,9 10, samt forskningsetikkloven § 4 godkjennes prosjektet. Før prosjektet kan settes i gang ber REK nord om å få seg forelagt informasjonsskrivet til deltagerne.

Sluttmelding og søknad om prosjektendring
Prosjektleder skal sende sluttmelding til REK nord på eget skjema senest 30.06.2021, jf. hfl. § 12. Prosjektleder skal sende søknad om prosjektendring til REK nord dersom det skal gjøres vesentlige endringer i forhold til de opplysninger som er gitt i søknaden, jf. hfl. § 11.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningsloven § 28 flg. Klagen sendes til REK nord. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK nord, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Med vennlig hilsen

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Paper I

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RESEARCH ARTICLE Open Access

Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma

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Abstract

Background: Oral squamous cell carcinomas are often heavily infiltrated by immune cells. The organization of B-cells, follicular dendritic cells, T-cells and high-endothelial venules into structures termed tertiary lymphoid structures have been detected in various types of cancer, where their presence is found to predict favourable outcome. The purpose of the present study was to evaluate the incidence of tertiary lymphoid structures in oral squamous cell carcinomas, and if present, analyse whether they were associated with clinical outcome.

Methods: Tumour samples from 80 patients with oral squamous cell carcinoma were immunohistochemically stained for B-cells, follicular dendritic cells, T-cells, germinal centre B-cells and high-endothelial venules. Some samples were sectioned at multiple levels to assess whether the presence of tertiary lymphoid structures varied within the tumour.

Results: Tumour-associated tertiary lymphoid structures were detected in 21 % of the tumours and were associated with lower disease-specific death. The presence of tertiary lymphoid structures varied within different levels of a tissue block.

Conclusions: Tertiary lymphoid structure formation was found to be a positive prognostic factor for patients with oral squamous cell carcinoma. Increased knowledge about tertiary lymphoid structure formation in oral squamous cell carcinoma might help to develop and guide immune-modulatory cancer treatments.

Keywords: Oral squamous cell carcinoma, Prognostic factor, Tertiary lymphoid structure, B-cell, High-endothelial venule, Follicular dendritic cell, Germinal centre

Background

Oral squamous cell carcinomas (OSCCs) are tumours known to metastasize to lymph nodes at an early stage of their development [1]. Despite current improvements in clinical management of this cancer type, mortality and morbidity rates of OSCC patients have remained high over the last decades, with an average 5-year survival rate of about 50% [2,3]. The TNM staging of the tumour, and especially the presence and extent of lymph node metastasis (N stage), have considerable prognostic importance for patients with OSCC [4] and are used to guide treatment strategies. However, tumours of the same clinical stage may respond differently to the same treatment and may also have distinct clinical outcomes [5].

Considerable interest has been devoted to the complex interplay between tumour cells and host-immune response, and especially to how infiltrating immune cells might affect the clinical outcome of cancer patients. Anti-tumour functions of tumour-infiltrating lymphocytes (TILs), particularly of T-cells, have been observed in numerous types of cancer [6]. Accumulating evidence indicates that infiltrating immune cells may also be involved in the development and progression of oral cancer, where they have shown both favourable and detrimental effects [7]. It is well established that immune cells infiltrating to sites of chronic inflammation organize themselves both anatomically and functionally similar to secondary lymphoid organs (SLOs), a phenomenon called tertiary lymphoid structure (TLS) formation [8]. Similar to lymphoid follicles, TLSs typically comprise aggregates of B-cells in a meshwork of follicular dendritic cells (FDCs) that are then surrounded by T-cells as well as specialized blood vessels

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referred to as high-endothelial venules (HEVs) [9]. HEVs express the lymphoid chemokine peripheral node addressin (PNAd), which binds to L-Selectin on naive lymphocytes and thus promote lymphocyte recruitment to sites of chronic inflammation [10]. Furthermore, a complex interplay between different lymphotoxin- and chemokine-induced signalling pathways is required for the initiation of TLS formation [9]. In contrast to lymph nodes, TLSs are not encapsulated, resulting in constitutive, direct antigenic stimulation from their surrounding microenvironment [11]. Lymphatic vessels have also been found in association with TLSs, but their functional interplay is not yet fully clarified [12]. The presence of ongoing germinal centre (GC) reactions in B-cell clusters of ectopic lymphoid structures has been reported, indicating that adaptive immunity can be triggered at sites different from SLOs [11]. In autoimmune disorders, formation of ectopic lymphoid tissue is associated with disease progression [11], whereas TLS development in breast, ovarian, non-small-cell lung, renal and colorectal cancer is reported to be associated with a favourable prognosis [13-23].

The aim of the present study was to evaluate the incidence of TLSs in OSCCs, and if present, analyse whether they were associated with clinical outcome. The study included tissue samples and clinical data from 80 patients diagnosed with OSCCs between 1986 and 2002 at the Diagnostic Clinic – Clinical Pathology, University Hospital of North Norway (UNN). The presence of TLSs was determined based on immunohistochemical staining patterns of B-cells, FDCs, GC B-cells, T-cells and HEVs. We established that the presence of TLSs is a positive prognostic factor for patients with OSCC. Understanding and interpreting TLS formation in OSCC might help to implement and guide immunotherapeutic interventions. In terms of individual clinical management, reliable prognostic markers together with targeted anticancer therapies might improve the consistently low survival rates in patients with oral cancer.

Methods

Patients

The study broadly follows the REMARK recommendations for tumour marker prognostic studies [24]. Eighty patients with histologically verified primary SCC of the oral cavity in the period 1986–2002 were selected from the archives of the Diagnostic Clinic – Clinical Pathology, UNN. The last day of follow-up was January 1st, 2012. The specimens were formalin-fixed, paraffin-embedded tumour resections or biopsies from the mobile tongue, floor of the mouth, buccal mucosa, gingiva and soft and hard palate. We excluded specimens from the base of the tongue and the tonsils – sites naturally rich in lymphatic tissue. Patients with a history of former head and neck cancer were also excluded from the study. Clinical data, including tumour staging according to the TNM-classification and treatment modalities, were retrieved from the patients' hospital files, pathology reports and the Statistics of Norway, Cause of Death Registry, and are listed in Table 1. Information on the HPV status determined by p16 immunohistochemical staining was obtained from the Diagnostic Clinic – Clinical Pathology, UNN, and is also presented in Table 1. In addition to the patient samples, formalin-fixed, paraffin-embedded normal oral tissue was used as control. The study was approved by the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007), which also gave the permission to access patient files containing the clinical data. All clinical data were kept anonymous.

Immunohistochemistry

Four-micrometer-thick sections of formalin-fixed, paraffin-embedded tissue of patients with OSCC on Superfrost Plus slides were subjected to immunohistochemical staining. From patients where several tumour-containing paraffin-blocks were available, a block with representative material, based on H/E staining, was chosen without specific evaluation of the inflammatory infiltrate. Before staining, all specimens were incubated overnight at 60°C, deparaffinised in xylene, rehydrated in graded alcohol baths and subjected to heat-induced antigen retrieval in 0.01 M sodium citrate buffer at pH 6.0. Prior to antibody incubation, inherent peroxidase activity in the tissue was blocked with 3% H_2O_2 (Ventana Medical Systems, France or Dako Glostrup, Denmark). The following primary antibodies were used: Mouse anti-CD20, clone L26; Mouse anti-CD21, clone 2G9; Mouse anti-bcl-6, clone GI191E/A8; Mouse anit-CD34, clone QBEnd/10; Rabbit anti-CD3, clone 2GV6 (all from Ventana Medical Systems, France); Mouse anti-Podoplanin, clone D2-40 (Dako, Glostrup, Denmark) and Rat anti-PNAd, clone MECA-79, (Biolegend, San Diego). Dilutions and incubation times are listed in Table 2. Except for the PNAd antibody, all immunohistochemical stainings were done in the automated slide stainer Ventana Benchmark, XT (Ventana) at the Diagnostic Clinic – Clinical Pathology, UNN, which is accredited according to the ISO/IEC 15189 standard for the respective stainings, using the same protocols, positive and negative controls as in the clinical routines. For these automated stainings, a cocktail of HRP labelled goat anti-mouse IgG/IgM and mouse anti-rabbit secondary antibodies together with diaminobenzidine from the Ventana UltraView Universal DAB Detection Kit (#760-500, Ventana) were applied for visualization.

Manual staining with the PNAd primary antibody was performed as previously described [25], using HRP-labelled goat anti-rat light chain secondary antibody (#AP202P, Millipore, Temecula, CA) and diaminobenzidine (Dako EnVision + System-Horseradish Peroxidase,

Table 1 Comparison of clinicopathological variables between 80 OSCC patients with and without TLSs using Pearson's Chi-square test

	TLS-negative (N = 63) (no. (%))	TLS-positive (N = 17) (no. (%))	P-value
Gender			
Male	35 (55.6)	11 (64.7)	0.498
Female	28 (44.4)	6 (35.3)	
Age at diagnosis, years			
Mean	63.19	63.71	0.178
0-59	23 (36.5)	6 (35.3)	0.926
≥ 60	40 (63.5)	11 (64.7)	
Smoking history			
Never smoker	14 (22.2)	4 (23.5)	
Former smoker	10 (15.9)	1 (5.9)	0.722
Current smoker	34 (54.0)	11 (64.7)	
Unknown	5 (7.9)	1 (5.9)	
Alcohol consumption			
Never	12 (19.0)	1 (5.9)	
≤ 1 times weekly	24 (38.1)	6 (35.3)	0.114
> 1 times weekly or daily	17 (27.0)	3 (17.6)	
Unknown	10 (15.9)	7 (41.2)	
Tumour site			
Mobile tongue	29 (46.0)	9 (52.9)	
Floor of mouth	17 (27.0)	5 (29.4)	
Soft palate	1 (1.6)	0 (0.0)	0.956
Buccal mucosa	7 (11.1)	1 (5.9)	
Alveolar ridge	8 (12.7)	2 (11.8)	
Unknown	1 (1.6)	0 (0.0)	
Tumour differentiation			
Well	20 (31.7)	10 (58.8)	
Moderate	39 (61.9)	5 (29.4)	0.058
Poor	4 (6.3)	2 (11.8)	
T stage			
T1	23 (36.5)	6 (35.3)	
T2	18 (28.6)	9 (52.9)	0.187
T3, T4	21 (33.3)	2 (11.8)	
Unknown	1 (1.6)	0 (0.0)	
N stage			
N0	41 (65.1)	13 (76.5)	0.670
N+	17 (27.0)	3 (17.6)	
Unknown	5 (7.9)	1 (5.9)	
M stage			
M0	57 (90.5)	17 (100.0)	
M+	1 (1.6)	0 (0.0)	0.417
Unknown	5 (7.9)	0 (0.0)	
Treatment			
Surgery local +/− neck resection	7 (11.1)	2(11.8)	
Surgery and radiotherapy	41 (65.1)	12 (70.6)	
Radiotherapy +/− chemotherapy	8 (12.7)	3 (17.6)	0.700
None or palliative	5 (7.9)	0 (0.0)	
Unknown	2 (3.2)	0 (0.0)	
HPV/p16			
Negative	52 (82.5)	16 (94.1)	
Positive	5 (7.9)	1 (5.9)	0.386
Unknown	6 (9.5)	0 (0.0)	

Dako) for detection. Counterstaining was done with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO). Finally, the sections were dehydrated in graded alcohol and xylene baths, and mounted with Histokit (Chemiteknikk, Oslo, Norway). Negative controls were treated identically but with the primary antibodies replaced by the antibody diluting solution. Formalin-fixed, paraffin-embedded human lymph nodes served as positive controls for the PNAd staining. Negative control sections never showed any staining, whereas the positive control sections (lymph nodes) always showed positive staining confined to the cells that were supposed to be positive (data not shown). The specificity of the PNAd antibody was evaluated on consecutive sections from six different OSCC samples and three samples of normal oral mucosa. These OSCC and normal tissue sections were assessed for

Table 2 Antibodies for immunohistochemistry

Antibody	Dilution	Incubation time
Mouse anti-CD20, clone L26, Ventana Medical Systems, France	Pre-diluted	16 min
Mouse anti-CD21, clone 2G9, Ventana	Pre-diluted	32 min
Mouse anti-bcl-6, clone GI191E/A8, Ventana	Pre-diluted	40 min
Mouse anti-Podoplanin, clone D2-40, Dako, Glostrup, Denmark	1:25	32 min
Mouse anit-CD34, clone QBEnd/10, Ventana	Pre-diluted	32 min
Rabbit anti-CD3, clone 2GV6, Ventana	Pre-diluted	16 min
Rat anti-PNAd, clone MECA-79, Biolegend, San Diego	1:25	30 min
Goat anti-rat light chain antibody, #AP202P, Millipore, Temecula, CA	1:250	30 min

overlapping immunohistochemical staining for the PNAd antibody, the blood vessel marker CD34 and the lymphatic endothelial cell marker D2-40. In the OSCC samples, sporadic CD34+ blood vessels were to a minor degree positive for PNAd, whereas no D2-40+ lymphatic vessels were positive, indicating a high degree of antibody specificity. No HEV staining was seen in the three samples from normal oral mucosa.

Immunohistochemical evaluation

Eighty patients were included in the study. In 25 of the patients, the presence of TLSs was evaluated at a single level in the tumour tissue block. In 45 of the patients – randomly chosen from the 80 patients – TLS formation was evaluated at two discrete levels at about 100 μm distance in the tissue block. Additionally, tumour tissue blocks from 10 of the patients – nine of them negative for TLSs in the superficial level – were cut down completely and presence of TLSs was evaluated at 100 μm distance throughout the tumour sample.

We used a two-step method for TLS detection. First, the tissue sections were immunohistochemically stained for the pan B-cell marker CD20 and assigned to three different groups based on their staining pattern: obvious B-cell aggregates, indistinct aggregates of B-cells and no or scattered B-cells. Second, staining for the FDC marker CD21, the T-cell marker CD3 and the HEV marker PNAd was performed on consecutive sections of those with obvious and indistinct B-cell aggregates. For FDC evaluation, areas with clusters of B-cells were examined at high-power magnification (400×). All tumours that had one or several accumulations of B-cells containing CD21 positive FDCs were defined as TLS-positive. All TLSs also contained HEVs and T-cells. The TLS-positive tumours were further subdivided into classical and non-classical TLSs. A classical TLS was defined as a B-cell aggregate containing a continuous FDC meshwork, and a non-classical TLS as a B-cell aggregate with a more diffuse distribution of the FDCs. Sections from seven of the TLS-positive tumours were stained with BCL-6 to verify the presence of GC B-cells in B-cell clusters of TLSs.

Statistical analysis

All statistical analyses were performed with the SPSS software version 22.0 for Windows (IBM, Armonk, NY). The association between various clinicopathological variables was examined by the Pearson's Chi-square test. Disease-specific death (DSD) and disease-specific survival (DSS) curves were estimated in univariate analyses and by Kaplan Meier method. The log-rank test was used to evaluate significant differences between the groups of patients. Variables that were statistically significant in the univariate analysis were entered into multivariate Cox regression analyses to identify independent prognostic factors in the presence of other variables. Validity of the proportionality assumption was verified by plotting log-minus-log plots. P-values less than 0.05 were considered statistically significant.

Results

Presence of TLSs in OSCC

TLSs are highly organized structures that typically appear as clusters of B-cells containing FDCs. These clusters are then surrounded by T-cells and HEVs as shown schematically in Figure 1. We investigated the presence of TLSs in tumour specimens from 80 patients with OSCCs using immunohistochemistry. Sections with distinct or more diffuse B-cell aggregates were considered likely to have TLSs, and their consecutive sections were stained for FDCs, T-cells and HEVs, whereas sections without B-cell aggregates were not further analysed. At the first level assessed, TLSs were found in 13 of the 80 specimens. Eleven of these TLSs were found in sections with distinct B-cell aggregates, and only two in sections with diffuse B-cell aggregates. Pictures of a classical TLS are shown in Figure 2. One more TLS-positive tumour was identified by staining for TLSs at an additional level about 100 μm deeper in the tissue blocks from 45 of the patients. Three additional TLS-positive tumours were detected by assessing the whole tissue sample from 10 patients. These three TLS-positive tumours showed TLSs at multiple levels. Altogether, TLSs were found in 17 (21 %) of the 80 patients included in the study. The maximum number of TLSs in a single section was four, but usually not more than two TLSs were detected in each of the positive sections. The TLSs were mainly found in the peri-tumoural stroma within 0.5 mm distance from the tumour front, in lymphocyte-rich subepithelial areas.

Within the B-cell aggregates, FDCs were found in either of two patterns: distinct meshworks (Figure 3A) or diffuse accumulations (Figure 3B) of CD21 positive cells. Only B-cell aggregates with contiguous FDC meshworks showed distinct accumulations of BCL6+ GC B-cells (Figure 3C) and are here referred to as *classical TLSs*. In the B-cell aggregates with diffuse accumulations of FDCs, GC B-cells were either absent (Figure 3D) or dispersed throughout the follicle, and these are here referred to as *non-classical TLSs*. Sometimes both classical and non-classical TLSs were found in the same section. Analyses of multiple tissue levels showed that some TLSs classified as non-classical on one tissue level presented a classical pattern on another tissue level and vice versa.

Clinicopathological characteristics and prognostic value of TLSs

Clinicopathological data of the patients were analysed for correlation with the presence of TLSs, and the

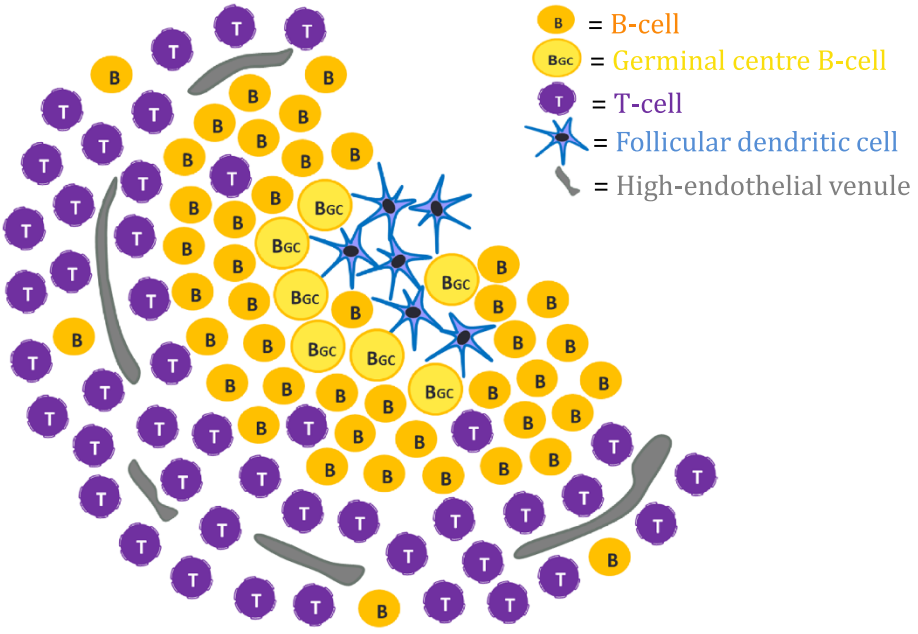

Figure 1 Schematic model of tertiary lymphoid structures. Specialized cell populations arrange themselves into distinct patterns forming a classical tertiary lymphoid structure (TLS).

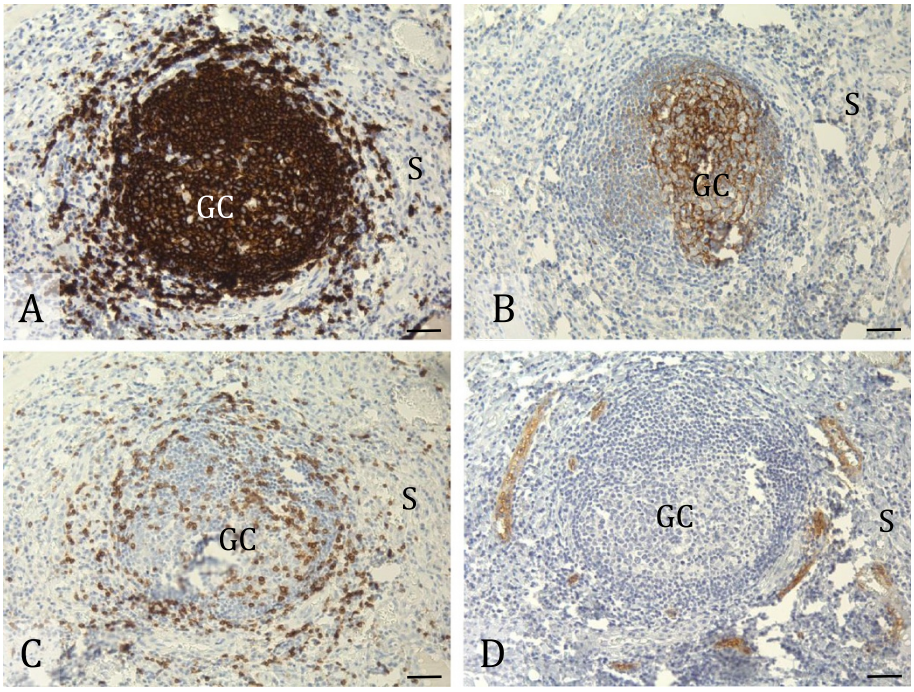

Figure 2 Tertiary lymphoid structures in oral squamous cell carcinoma. The pictures show representative immunohistochemical stainings on consecutive sections of the same oral squamous cell carcinoma (OSCC) tissue sample for detection of classical tertiary lymphoid structures (TLSs). A section that presents clusters of CD20+ B-cells **(A)** typically shows organized accumulations of follicular dendritic cells (FDCs) in a consecutive section stained for CD21 **(B)**. T-cell areas within and around the B-cell follicle are found by staining another consecutive section for CD3 **(C)**. High-endothelial venules (HEVs) adjacent to the B-cell follicle are detected when staining a consecutive section for PNAd, as shown in **(D)**. CD20+, CD21+ and CD3+ cells as well as PNAd + vessels are stained brown, and cell nuclei are stained blue by hematoxylin. Germinal centres are labelled "GC" and stroma surrounding the TLS is labelled "S" in the micrographs. Scale bars indicate 40 μm.

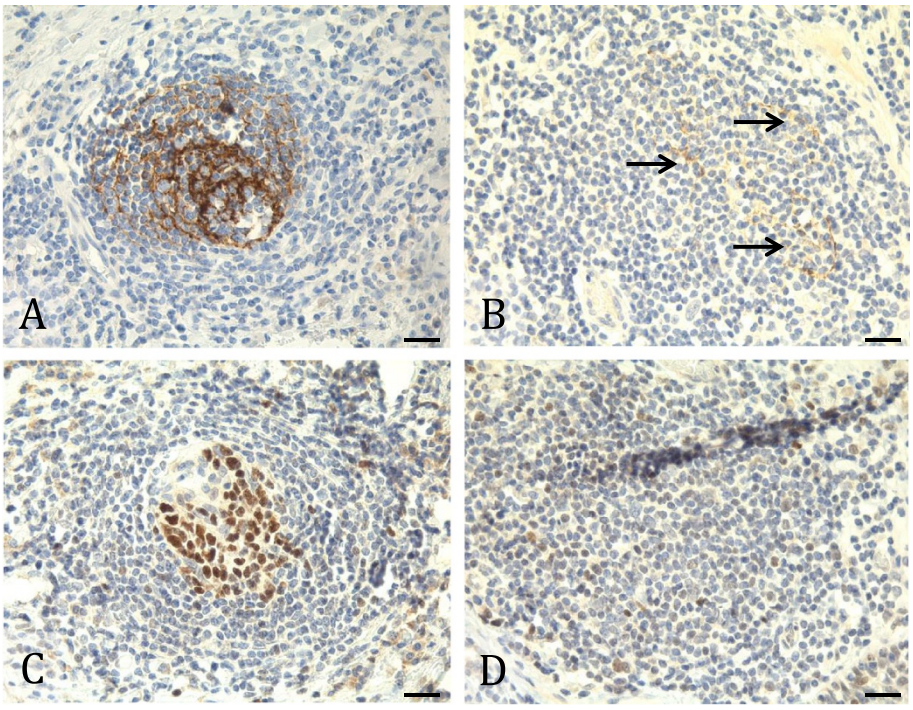

Figure 3 Classical and non-classical tertiary lymphoid structures. The pictures show representative immunohistochemical stainings on consecutive sections of two different oral squamous cell carcinoma (OSCC) tissue samples (A/C *vs.* B/D) for detection of classical (A/C) and non-classical (B/D) tertiary lymphoid structures (TLSs). B-cell follicles of classical TLSs normally comprise contiguous meshworks of CD21+ follicular dendritic cells (FDCs), as indicated in **(A)**, and show distinct accumulations of germinal centre (GC) B-cells when staining a consecutive section for BCL6, as presented in **(C)**. B-cell follicles of non-classical TLSs usually contain scattered FDCs, as shown in (**B**; arrows), and lack GC B-cells on a consecutive section stained for BCL6 **(D)**. In some cases, non-classical TLSs also show abnormal GCs with BCL6+ cells dispersed throughout the follicle (data not shown). CD21+ and BCL6+ cells are stained brown, and cell nuclei are stained blue by hematoxylin. Scale bars indicate 40 μm.

results are presented in Table 1. Although not statistically significant, the majority of TLSs were found in patients with well-differentiated tumours. Further, there were more TLSs in T1 and T2 tumours compared to T3/T4 tumours. TLSs showed no statistically significant association with the other variables examined. The prognostic value of various clinicopathological variables in OSCC was investigated in univariate analysis using the log-rank test (Table 3). Based on the assessment of one tissue level, TLS-positive tumours indicated a trend toward improved survival. When the assessment of TLSs was based on multiple tissue levels, a significant association between the presence of TLSs and favourable outcome in OSSC patients was found, as shown in Figure 4. As patients presented various TLS subtypes (either classical, non-classical or both classical and non-classical), we analysed whether the TLS subtype influenced 5-year DSD. As presented in Additional file 1: Table S1, there was a tendency towards lower 5-year DSD for all patients with TLSs, regardless of the subtype. However, the differences were not statistically significant. Presence of classical TLSs alone or in combination with non-classical TLSs seemed to be associated with better prognosis compared to the presence of only non-classical TLSs, but again, no statistical significant difference between the subtypes was found ($P = 0.304$; data not shown). Furthermore, our results also confirmed the prognostic value of the T, N and M stages. The variables that showed statistically significant association with DSD in the univariate analyses (T, N stage and TLS) were entered into multivariate Cox regression analyses. The M status was excluded from multivariate analyses as there was only one M + patient. Proportional hazards assumptions were satisfied for multivariate analyses as shown by parallel curves for different categories of prognostic variables on log-minus-log plots (Additional file 1: Figure S1). In multivariate analyses, only the T status remained independently associated with disease-specific death ($P < 0.001$, Table 4).

Discussion

In the present study, we have demonstrated TLSs in OSCC by immunohistochemical analyses. To the best of our knowledge, this is the first report of TLSs in OSCCs. TLSs were found in 16% of the patients when a single level of the tumour was assessed, and in 21% of the patients when multiple levels of the tumours were analysed. This is a rather low occurrence compared to what

Table 3 Clinicopathologic variables as predictors for 5-year disease-specific death in univariate analysis for 80 patients with OSCC

	Patients (N = 80) (no. (%))	5-Year death (%)	P-value
Gender			
Male	46 (57.5)	37.0	0.403
Female	34 (42.5)	29.4	
Age at diagnosis, years			
0-59	29 (36.3)	31.0	0.637
≥ 60	51 (63.8)	35.3	
Smoking history			
Never smoker	18 (22.5)	27.8	
Former smoker	11 (13.8)	27.3	0.897
Current smoker	45 (56.3)	37.3	
Unknown	6 (7.5)	33.3	
Alcohol consumption			
Never	13 (16.3)	23.1	
≤ 1 times weekly	30 (37.5)	33.3	0.633
> 1 times weekly or daily	20 (25.0)	35.0	
Unknown	17 (21.3)	41.2	
Tumour site			
Mobile tongue	38 (47.5)	21.1	
Floor of mouth	22 (27.5)	40.9	0.074
All others*	20 (25.0)	50.0	
Tumour differentiation			
Well	30 (37.5)	26.7	
Moderate	44 (55.0)	36.4	0.296
Poor	6 (7.5)	50.0	
T stage**			
T1	29 (36.7)	20.7	
T2	27 (34.2)	11.1	<0.001
T3, T4	23 (29.1)	78.3	
N stage			
N0	54 (67.5)	22.2	
N+	20 (25.0)	70.0	<0.001
Unknown	6 (7.5)	16.7	
M stage			
M0	74 (92.5)	33.8	
M+	1 (1.3)	100.0	0.021
Unknown	5 (6.3)	20.0	
HPV/p16			
Negative	68 (85.0)	35.3	
Positive	6 (7.5)	16.7	0.720
Unknown	6 (7.5)	33.3	
TLS single level			
Negative	67 (83.8)	37.3	0.156
Positive	13 (16.3)	15.4	
TLS multiple level			
Negative	63 (78.8)	39.7	0.039
Positive	17 (21.3)	11.8	

P-values were calculated using the log-rank test.
*For univariate survival analysis, the tumour sites were grouped into three categories.
**Only 79 patients were analysed because the unknown case was taken out from the calculations.

has previously been reported in colorectal cancer and lung cancer, suggesting that the occurrence of TLSs varies among different types of tumours [14,19,26]. When assessed at a single level, presence of TLSs was not a significant predictor of survival. However, when analysed at multiple levels, their presence in the tumour was a positive prognostic factor. This indicates that the prognostic value of TLSs depends on the type of analysis, probably due to their rather infrequent occurrence and tumour heterogeneity. In multivariate analyses, only T stage turned out to be an independent prognostic factor. TLS status, however, performed better than N stage, which is recognized as one of the best prognostic factors in OSCCs [4]. In the TLS-positive tumours, either single or multiple TLSs were found in the same tissue section. In some of the TLSs, GC B-cells and FDC meshworks were

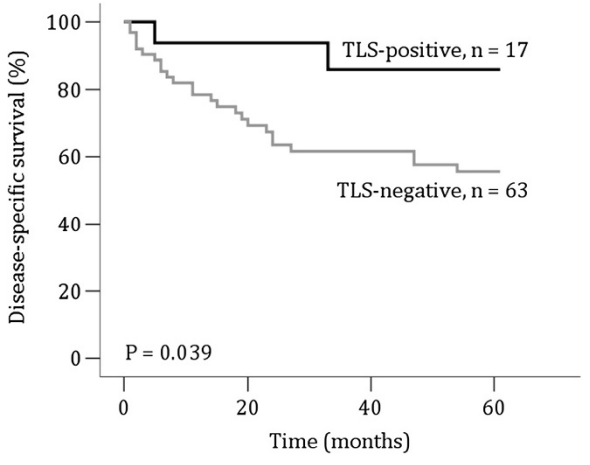

Figure 4 Results from multiple level analysis: Kaplan Meier analysis of 5-year disease-specific survival for 80 patients with oral squamous cell carcinoma with and without tertiary lymphoid structures. The presence of tertiary lymphoid structures (TLSs) is associated with improved survival in patients with oral squamous cell carcinoma (OSCC) (P = 0.039). The Kaplan-Meier curve shows a 5-year disease-specific survival rate of 88.2% for TLS-positive patients and 60.3% for TLS-negative patients. The P-value was calculated using the log-rank test.

Table 4 Results from multiple level analysis: multivariate analysis of 5-year disease-specific death according to Cox's proportional hazards model*

Variable	Hazard ratio	95% C.I.	P-value
T stage	—	—	< 0.001
T stage (1) (T1 [n = 29] v. T2 [n = 27])	0.538	0.134 - 2.151	0.381
T stage (2) (T1 [n = 29] v. T3/T4 [n = 23])	7.237	2.814 - 18.612	< 0.001
N stage	—	—	0.359
N stage (1) (N0 [n = 54] v. N + [n = 20])	1.820	0.742 - 4.461	0.191
N stage (2) (N0 [n = 54] v. unknown [n = 5])	2.342	0.290 - 18.900	0.424
TLS (negative [n = 62] v. positive [n = 17])	2.409	0.556 - 10.448	0.240

*Only 79 patients were analysed because the case with unknown T stage was taken out from the calculations.

observed, providing evidence that the TLSs comprised all cells needed to generate a functional immune response. We called lymphoid structures with defined FDC meshworks and GCs *classical TLSs*, as this phenotype has been mostly described for TLSs in literature. Besides the classical TLSs, we also found TLSs with diffuse accumulations of FDCs and scattered or absent GC B-cells that we termed *non-classical TLSs*. It remains elusive whether non-classical TLSs have the same immunological properties as classical TLSs. Immunohistochemistry on multiple tissue planes of the same tumour showed in some cases that classical and non-classical phenotypes corresponded to the same ectopic lymphoid structure. This implies that the two different patterns may be artefacts of the methodical approach of TLS detection. This is also supported by the fact that both classical and non-classical TLSs were found on the same tissue plane. Moreover, patients with TLSs showed prolonged survival regardless of TLS subtype, indicating that none of the TLS subtypes alone are particularly associated with survival. However, we found a trend towards better prognosis for patients with classical TLSs or with both classical and non-classical TLSs compared to patients with non-classical TLSs only. This indicates that, in some cases, non-classical TLSs could also represent immature follicles that may later develop into classical TLSs with full immunogenic properties. Previous studies have already reported the presence of fully and not fully mature TLSs in cancer and other inflammatory diseases [27].

Many questions about TLS development in oral cancer remain to be elucidated. Ectopic lymphoid formation is a common feature in chronically inflamed tissues and has been found in a number of different diseases at various anatomical sites [11]. After the switch from acute to chronic inflammation, gradual accumulation of lymphocytes as well as promotion of lymphangiogenesis and transformation of blood vessels into lymphocyte-guiding HEVs has been observed [28]. In oral cancer, chronically inflamed tissue precedes most of the tumours [29], providing favourable sites for TLS formation. In our OSCC samples, the TLSs were mainly located in the subepithelial lymphocytic infiltrate close to the tumour front. It would be of great interest to find out why the chronic infiltrate sometimes organizes into these structures. Disclosing the mechanisms that regulate TLS development may give important information on how to improve immune-modulating cancer therapy. Lymphoid neogenesis has been most extensively studied in autoimmune disorders such as rheumatoid arthritis, Sjögrens' syndrome and Hashimoto's thyroiditis, where TLSs might contribute to disease progression [11]. In some ectopic GCs, B-cells producing antibodies against self-antigens have been recognized, but data are still sparse [28]. In OSCC, it is not yet clear which antigenic targets the lymphocytes might react to and whether auto-antigens play a role in the induction of TLSs. In terms of viral agents that are linked to human tumours, human papillomavirus (HPV) has become a topic of interest during the last years. While HPV is a known risk factor for oropharyngeal cancer, it probably plays only a minor role in cancers arising in the oral cavity [30]. In the present study, there was no correlation between HPV-status and TLS formation. Although the antigenic stimuli directing TLS formation in OSSC are unknown, it seems likely that the immune-modulating factors that promote TLS development derive from the cancer cells rather than from autoimmunity or infection. Our results show that TLSs are most likely to form in well-differentiated tumours. It has been proposed that tumour growth might be related to stem and amplifying cell patterns, and that dedifferentiation may play a role in the origin of cancer stem cells (CSCs) in OSCC [31]. CSCs are a minority of malignant cells that are thought to be able to attenuate host anti-tumour immune responses [32]. Thus, one could speculate that dedifferentiation makes the tumour cells less antigenic and thereby elicits a milder inflammatory reaction with lower induction of TLSs. Previous studies on lymphoid neogenesis have revealed that clearance of the inflammation-inducing antigen or clinical therapy are able to cause a complete remission of the ectopic lymphoid structure [9]. This might be advantageous in autoimmune diseases to stop aggravation of the disease. However, as TLSs are thought to be conducive for patient survival in OSCC, characterization of stimulating agents might be used therapeutically to promote TLS formation by presentation of the causative agent. Investigation of circulating lymphocytes in blood samples of OSCC patients may provide new insights into the

involvement of the host immune reaction in TLS development. A long-lasting chronic inflammation, as in larger tumours, could promote TLS development. In the present study, however, more TLSs were found in smaller tumours, clearly indicating that TLS formation can also take place in the early phases of tumour growth.

Conclusion

We found TLS formation to be a positive prognostic factor for patients with OSSC when tumours were analysed at multiple levels. Thus, patients with TLS-positive tumours might benefit from more restrictive treatment while a closer follow-up and more aggressive therapy should be considered for patients with TLS-negative tumours. However, before we can envisage TLSs as prognostic factors in individual clinical management of OSCC patients, larger studies on ectopic lymphoid structures are needed. Our study shows that correct assessment of TLS by immunohistochemistry requires careful analyses. When assessing CD20 B-cell staining, both dense and more diffuse aggregates of B-cells should be considered as putative TLSs. We found however, that about a third of the TLS-positive patients were missed when analysing only one level in the tissue block. This may be due to the fact that we selected blocks with representative tumour material rather than the tumour blocks with most intense inflammation. By selecting differently, the chance of discovering TLSs on a single tissue level might have increased. PCR-based approaches, such as combining analyses of a combination of mature FDC markers, HEV markers and TLS associated chemokines such as CCL19, CCL21 and CXCL13 [21], could also decrease the chance of missing TLS-positive tumours. Furthermore, analyses of TLS associated chemokines in serum from cancer patients could be a possible indicator of TLS formation.

The future trend in clinical cancer management points to personalized treatment. The use of biomarkers to guide treatment decisions along with development of immunotherapy may benefit the patient. Thus, understanding TLS formation in OSCC might help to guide targeted anti-cancer therapies and improve the dismal survival rates of patients with oral cancer.

Additional file

Additional file 1: Table S1. Results from multiple level analysis: Univariate Kaplan Meier analysis of 5-year disease-specific death for 80 patients with oral squamous cell carcinoma with various subtypes of tertiary lymphoid structures (TLSs). **Figure S1.** Results: Log minus log plots for proportional hazards checking; (A) T stage; (B) N stage; (C) tertiary lymphoid structure (TLS).

Abbreviations

OSCCs: Oral squamous cell carcinomas; TILs: Tumour-infiltrating lymphocytes; SLO: Secondary lymphoid organ; TLS: Tertiary lymphoid structure; FDCs: Follicular dendritic cells; HEV: High-endothelial venule; PNAd: Peripheral node addressin; GC: Germinal centre; CSC: Cancer stem cell.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

AW carried out the manual immunohistochemical staining, participated in interpretation and scoring of the immunohistochrmical stainings and the statistical analyses and drafted the manuscript. OR retrieved the clinical information from patient journals, participated in the statistical analyses and critically reviewed the manuscript. SES participated in interpretations of the immunohistochemical stainings and the statistical analyses, and critically reviewed the manuscript. LUH participated in design of the study and in interpretations of the immunohistochemical stainings, and critically reviewed the manuscript. EHO participated in design of the study, scoring of the immunohistocehemical stainings and helped to draft the manuscript. All authors read and approved the final manuscript.

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Additional file

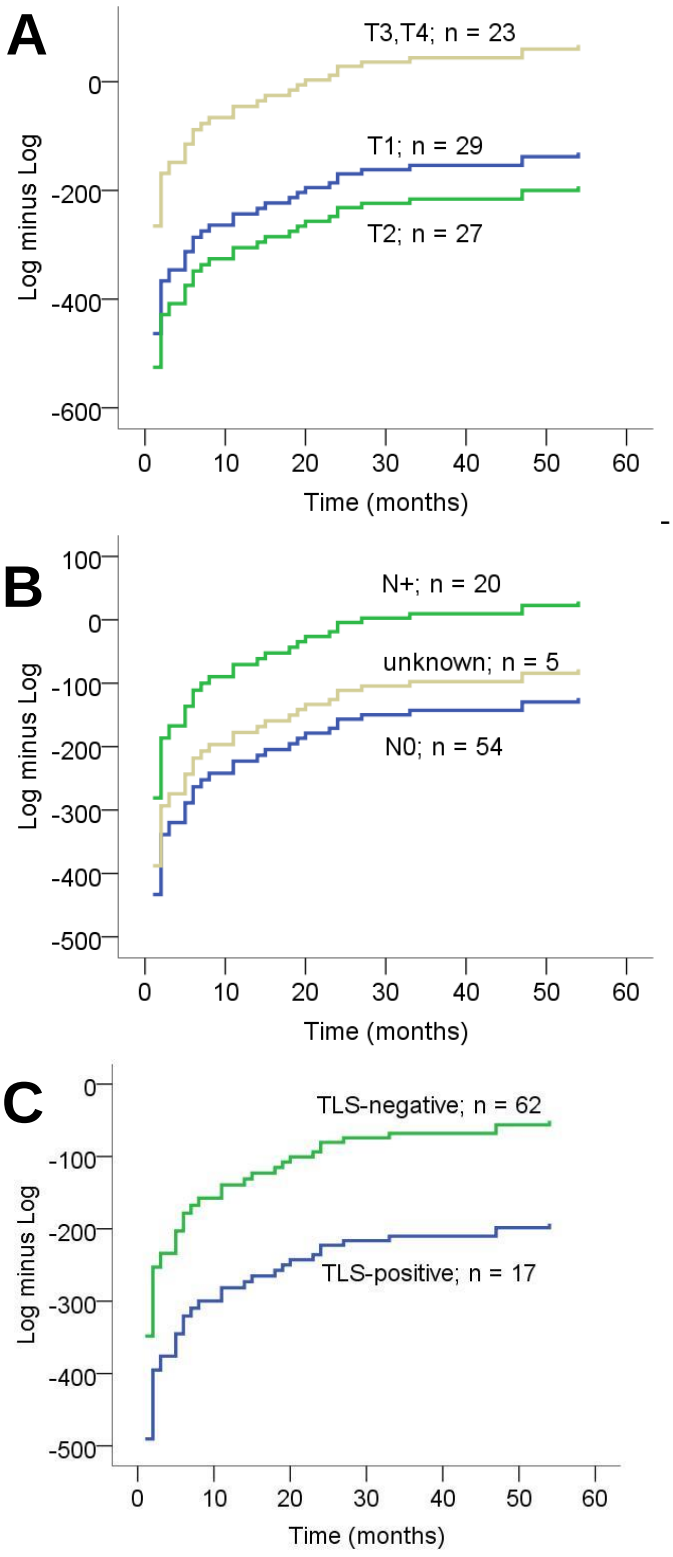
Table S1

Results from multiple level analysis: Univariate Kaplan Meier analysis of 5-year disease-specific death for 80 patients with oral squamous cell carcinoma with various subtypes of tertiary lymphoid structures (TLSs).

Patients	**N=80 (no. (%))**	**5-Year death (%)**	**P-value**
classical TLS	5 (6.3)	0.0	0.150
all others	75 (93.8)	36.0	
non-classical TLSs	8 (10.0)	25.0	0.574
all others	72 (90.0)	34.7	
both classical and non-classical TLS	4 (5.0)	0.0	0.165
all others	76 (95.0)	35.5	

P-values were calculated using the log-rank test.

Figure S1



Results: Log minus log plots for proportional hazards checking; (A) T stage; (B) N stage; (C) tertiary lymphoid structure (TLS).

Paper II

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ORIGINAL ARTICLE

Presence of tumour high-endothelial venules is an independent positive prognostic factor and stratifies patients with advanced-stage oral squamous cell carcinoma

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Abstract Staging of oral squamous cell carcinoma is based on the tumour-node-metastasis (TNM) system, which has been deemed insufficient for prognostic purposes. Hence, better prognostic tools are needed to reflect the biological diversity of these cancers. Previously, high numbers of specialized blood vessels called high-endothelial venules have been reported to be associated with prolonged survival in patients with breast cancer. In this study, we analysed the prognostic value and morphological characteristics of tumour-associated high-endothelial venules in oral cancer. The presence of tumour-associated high-endothelial venules was evaluated by immunohistochemistry in 75 patients with oral squamous cell carcinoma and analysed with correlation to clinicopathological parameters, patients' survival and vessel morphology. Ten of the samples were analysed at multiple levels to evaluate intratumoural heterogeneity. The presence of tumour-associated high-endothelial venules was found to be associated with lower disease-specific death in multivariate regression analyses (P=0.002). High-endothelial venules were present in all (n=53) T1-T2 tumours, but only in two thirds (n=14) of the T3-T4 tumours. The morphology of high-endothelial venules was heterogeneous and correlated with lymphocyte density. High-endothelial venules were found to be distributed homogeneously within the tumours. We found the presence of tumour-associated high-endothelial venules to be an easy-to-use, robust, and independent positive prognostic factor for patients with oral cancer. Absence of these vessels in advanced-stage tumours might identify patients with more aggressive disease. Evaluating the presence of tumour-associated high-endothelial venules might help to tailor the treatment of oral cancer patients to their individual needs.

Keywords Oral squamous cell carcinoma · Prognostic factor · High-endothelial venules · Peripheral node addressin · Inflammation · Lymphocyte trafficking

Introduction

The far majority (>90 %) of malignancies in the oral cavity and the oropharynx are squamous cell carcinomas (SCCs), which are generally aggressive and frequently metastasize to lymph nodes at an early phase [1]. The tumour-node-metastasis (TNM) staging system, which describes tumours based on the size and invasion features of the primary tumour (T), the presence of lymph node metastases (N), and distant metastases (M) [2], is currently the most reliable way to predict the outcome for patients with oral SCCs (OSCCs) [3, 4]. However, the TNM grading does not reflect the considerable biological diversity of these tumours [5]. Numerous studies on potential prognostic markers to foresee the outcome and therapeutic needs of individual OSCC patients have been performed [6–8]. Despite concerted efforts, none of these biomarkers are routinely used in clinical practice. Thus, there is a great need to identify new and better prognostic tools that are

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both reliable and easy to implement in clinical routines. Furthermore, a better understanding of the biology of OSCCs could guide the development of new, targeted therapies for OSCC patients.

Angiogenesis is one of the hallmarks of cancer and, as such, generally associated with tumour progression and poor clinical outcome [9]. However, formation of specialized blood vessels called high-endothelial venules (HEVs) has recently been found to be associated with a favourable prognosis in breast cancer patients, probably by facilitating anti-tumour responses through recruitment of cytotoxic lymphocytes to the tumour site [10, 11]. HEVs appear normally in lymph nodes where they support high levels of lymphocyte extravasation from the blood [10, 12]. As their name implies, HEVs are characterized by cuboidal endothelial cells, which express specialized ligands for lymphocytes such as the chemokine peripheral node addressin (PNAd) on their luminal surface [13]. By binding to L-selectin, PNAd anchors circulating, naive lymphocytes to the HEV wall [14]. Lymphocyte extravasation is mediated through discontinuous, 'spot-welded' junctions, which are characteristic for HEV endothelial cells [12, 15, 16] and which differ from the tight junctions that characterize capillary and arterial endothelium [17]. High density of tumour-infiltrating lymphocytes (TILs) has earlier been shown to have beneficial effects on patient survival in several human solid tumours [18, 19]. TILs are sometimes organized in so-called tertiary lymphoid structures (TLSs), which resemble lymphoid follicles in lymph nodes, but typically appear in non-lymphoid tissue under terms of chronic inflammation [20]. HEVs are thought to be key players in the recruitment of lymphocytes to the TLSs, and TLS formation has been associated with improved survival rates in lung, breast, colorectal and oral cancer [11, 21–23]. In human cancers, presence of HEVs, both in TLSs and independent of these structures, is a recent discovery [10]. Tumour HEV density and phenotype have been found to be highly heterogeneous and dependent on the surrounding tissue, suggesting that HEVs shape their tissue microenvironment and vice versa [24]. HEVs were seen to form independently from T- and B-lymphocytes but strongly required signalling from dendritic cells [13, 25, 26]. Remodelling of tumour HEVs from lymphocyte-carrying vessels into dilated, blood-carrying vessels with thin walls has been proposed as an early prognostic marker of sentinel lymph node metastasis in breast and oral cancer patients [27, 28]. Different from their counterparts within peripheral lymph nodes, tumour-associated HEVs and HEVs within TLSs are still poorly understood [29]. However, a better understanding of the mechanisms regulating tumour HEVs might have a promising potential in modulating tumour growth and developing new therapeutic strategies for cancer patients [24].

The present study was performed to assess the morphology and prognostic value of tumour-associated HEVs in OSCCs as well as their correlation to clinicopathological characteristics. Immunohistochemical staining for HEVs was conducted on tumour samples from 75 OSCC patients. We found that the presence of HEVs is an independent prognostic marker for lower disease-specific death (DSD) and that lack of these vessels in advanced-stage tumours identifies patients with more aggressive disease. Thus, the presence of HEVs might be a useful hallmark to stratify OSCC patients and to help select patient subsets for individual therapeutic approaches.

Materials and methods

Patients

The study broadly adheres to the REMARK recommendations for tumour marker prognostic studies [30]. Formalin-fixed, paraffin-embedded tumour samples from 75 patients with histologically verified primary SCC of the oral cavity were collected from the archives of the Department of Clinical Pathology, University Hospital of North Norway (UNN). Inclusion criteria were availability of both tissue from the primary tumour and clinical information. Patients with prior radiotherapy to the head and neck area and patients with previous oral and pharyngeal cancer were excluded from the study. Large biopsies covering the subepithelial areas were evaluated from the patients who were not resected. Data on clinicopathological features, including treatment procedures and the HPV status, were acquired from the patients' hospital files, pathology reports and the Statistics of Norway, Cause of Death Registry, and are presented in Table 1. The tumours were staged according to the newest TNM classification at the time of diagnosis [31–34]. The classification of cancers of the lip and oral cavity remained unchanged between the different editions during the registration periods thus giving no consequence for the study. The patients were diagnosed in the period 1986–2002, and the last day of follow-up was January 1, 2012. The Regional Committee for Medical and Health Research Ethics, Northern Norway, approved the use of the patients' tissue and the collection of the clinical information (REK-number 22/2007).

Immunohistochemistry

Immunohistochemical studies of HEVs were performed on formalin-fixed, paraffin-embedded, 4-μm-thick tumour tissue sections. Manual staining for HEVs, including evaluation of antibody specificity, were carried out as previously described [23, 35]. In brief, after rehydration, heat-induced antigen retrieval, and blocking steps, sections were incubated with the PNAd primary antibody (#120801, Rat anti-PNAd, clone MECA-79, Biolegend, San Diego, dilluted 1:25) for 30 min. Afterwards, HRP-labelled goat anti-rat light chain secondary

Table 1 Comparison of clinicopathological variables between 75 OSCC patients with and without high-endothelial venules (HEVs) using Pearson's chi-squared test

	HEV-negative (*N*=7) (no. (%))	HEV-positive (*N*=68) (no. (%))	*P* value
Gender			
Male	6 (85.7)	37 (54.4)	
Female	1 (14.3)	31 (45.6)	0.227
Age at diagnosis, years			
Mean	68.29	62.26	0.795
0–59	2 (28.6)	26 (38.2)	
≥60	5 (71.4)	42 (61.8)	0.706
Smoking history			
Never smoker	1 (14.3)	16 (23.5)	
Former smoker	1 (14.3)	9 (13.2)	
Current smoker	4 (57.1)	39 (57.4)	
Unknown	1 (14.3)	4 (5.9)	0.821
Alcohol consumption			
Never	1 (14.3)	11 (16.2)	
≤1 times weekly	0 (0.0)	27 (39.7)	
>1 times weekly or daily	3 (42.9)	17 (25.0)	
Unknown	3 (42.9)	13 (19.1)	0.156
Tumour site			
Mobile tongue	1 (14.3)	34 (50.0)	
All others	6 (85.7)	34 (50.0)	0.071
Tumour differentiation			
Well	3 (42.9)	25 (36.8)	
Moderate	2 (28.6)	40 (58.8)	0.036
Poor	2 (28.6)	3 (4.4)	
T stage			
T1/T2	0 (0.0)	53 (77.9)	
T3/T4	7 (100.0)	14 (20.6)	
Unknown	0 (0.0)	1 (1.5)	<0.001
N stage			
N0	3 (42.9)	48 (70.6)	
N+	3 (42.9)	15 (22.1)	
Unknown	1 (14.3)	5 (7.4)	0.326
M stage			
M0	6 (85.7)	63 (92.6)	
M+	0 (0.0)	1 (1.5)	
Unknown	1 (14.3)	4 (5.9)	0.668
HPV/p16			
Negative	4 (57.1)	61 (89.7)	
Positive	1 (14.3)	5 (7.4)	
Unknown	2 (28.6)	2 (2.9)	0.011

antibody (#AP202P, Millipore, Temecula, CA, dilluted 1:250, incubated 30 min) and diaminobenzidine (Dako EnVision + System-Horseradish Peroxidase, Dako) were used for detection. Counterstaining was done with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO). As previously described [23], formalin-fixed, paraffin-embedded human lymph nodes served as positive controls. Antibody specificity was evaluated by immunohistochemical staining of consecutive sections of OSCC tissue from six different patients as well as three normal oral mucosa samples with the PNAd antibody, the blood vessel marker CD34 and the lymphatic endothelial cell marker D2-40. A few CD34+ vessels but no D2-40+ lymphatic vessels displayed sporadic PNAd staining in consecutive OSCC tissue sections, whereas the three normal oral

mucosa tissue sections were entirely negative for the PNAd antibody. No other HEV markers were used for verification of PNAd-positive vessels, as the PNAd antibody displayed only little unspecific staining. Sections where the primary antibody was omitted were used as negative controls.

High-endothelial venule count and morphological analysis

Seventy-five patients were included in the study. In 65 of them, the presence of HEVs was assessed based on evaluation of the PNAd staining at a single level in the tumour tissue block. In the remaining 10 patients, the blocks were cut down completely and presence of HEVs was assessed at 100-μm distance throughout the tumour sample. All sections were stained with the endothelial cell marker PNAd as described above. The cut-off value for PNAd positivity was obtained from hot spot analyses, as a modification of the method published by Weidner et al. [36]. The sections were assessed by light microscopy at low power magnification (100×) to recognize the areas with highest HEV density (hot spots). Only distinct brown PNAd staining in clusters of more than one cell were considered as HEVs. Micrographs of the five areas with highest HEV density were taken with a Leica DFC 420 camera on a Leica DM2000 microscope (Leica, Wetzlar, Germany) at high power magnification (400×), and the number of HEVs in the photographs was counted manually. For each tumour, the number of HEVs in each hot spot was added and the total number was then divided by five, giving a mean number of tumour-associated HEVs per section. Some tumours had less than five HEV-positive areas. In these cases, the total sum of HEVs was also divided by five. The median number of HEVs per hot spot for the whole group of patients was used as cut-off value for positive and negative HEV count, respectively. To verify PNAd staining, whole-slide digital images of the same tumour sections were manually reinvestigated for presence of HEVs at very high magnification under the virtual objective of the virtual microscope using the same cut-off value for HEV positivity as derived by hot spot analyses. This approach mainly aimed to avoid false-negative results due to weak PNAd staining in sporadic sections, which might be hard to detect using conventional light microscopy. Moreover, there is a growing interest in using whole-slide imaging for different applications in pathology practice [37], and we wanted to test practical issues including efficiency of PNAd detection. For these reasons and for being able to study HEV morphology, all slides were scanned with a Zeiss Mirax Scanner at 400× magnification, and the whole-slide digital images were investigated at high magnification (up to 63.76×) using the image analysis Mirax Viewer Software (3DHISTECH, Budapest, Hungary). Digital line measurement tools, developed in the Mirax Viewer Software, were used to analyse (minimal) distances between outer vessel wall of HEVs and nearest tumour cells, vessel wall thickness and inner vessel diameter. For quantitative estimation of the association between morphologic alteration of HEVs and level of inflammation, vessel wall thickness and lumen diameter of 100 HEVs were analysed in areas with low and high levels of inflammation, respectively. Level of inflammation was evaluated semi-quantitatively in the digitized PNAd-stained sections with a cut-off of 50 lymphocytes/field of vision at high power magnification. Branched vessels and vessels without evident lumina were not taken into account when evaluating vessel wall thickness and inner lumen diameter. In general, no PNAd-positive single cells were counted as HEVs. HEVs have earlier been found in the same tissue sections in relation to TLSs [23], and these lymph node-like structures might also arise in the salivary glands, unrelated to the tumour. Thus, to exclude HEVs that were not tumour-associated, only HEVs within 700-μm distance from the tumour front were taken into account. A trained pathologist histologically evaluated this. All studies were carried out manually.

Statistical analysis

Statistical analyses were performed using the SPSS software version 22.0 for Windows (IBM, Armonk, NY) and Microsoft Excel 2013 (Microsoft, Redmond, WA). Inter-observer variability for HEV count was quantified by the Spearman correlation test. Vessel wall and lumen diameter data produced by image analysis deviated from a normal distribution. Therefore, a Mann–Whitney *U* test was used to determine if there was a significant difference in vessel wall thickness and lumen diameter between HEVs found in areas with high and low levels of inflammation, respectively. Correlation analyses for possible associations between different variables were performed using the Pearson's chi-squared test. Univariate Kaplan–Meier analyses were used to evaluate disease-specific death (DSD) rates and disease-specific survival (DSS) curves. Significant differences between the groups of patients were estimated by the log-rank test. Statistically significant determinants in the univariate analysis were entered into multivariate Cox regression analyses. A stepwise forward multiple Cox regression analysis was carried out to determine independent prognostic factors. Validity of the proportional hazards assumption was tested by plotting log-minus-log plots. *P* values <0.05 were considered statistically significant.

Results

High-endothelial venules were present in the majority of oral squamous cell carcinomas and homogeneously distributed within each tumour

Seventy-five patients with SCC of the oral cavity were evaluated for presence of HEVs by immunohistochemical staining

for the MECA-79-reactive ligand PNAd. We first defined a cut-off value for PNAd positivity by analogue hot spot analysis. The total number of tumour-associated HEVs was counted in five hot spots from each section. According to a median count of 0.5 HEVs per hot spot, we defined tumours with a total number of <3 tumour-associated HEVs per section as HEV-negative and those with a total number of ≥3 HEVs per section as HEV-positive. The reproducibility for hot spot analysis between the two investigators (EHO and AMW) was very good with the Spearman's rho correlation coefficient for mean HEV count per tumour section being 0.970. The cut-off value was subsequently applied to whole-slide digital images of the same tissue sections. Digital image analyses at high-power magnification using the designed cut-off value for PNAd positivity revealed that 68 (90.7 %) of the OSCC samples were positive and the remaining 7 (9.3 %) were negative. The final evaluation of the sections with high magnification revealed more vessels than the first hot spot analyses. We therefore recommend using high-power magnification to detect vessels with less intensive staining and avoid false-negative results. HEVs were preferentially located peripherally in the tumour stroma within marked accumulations of inflammatory cells. The tissue samples that were investigated for HEVs on multiple levels showed a full correlation in HEV score (positive/negative) between the levels. Nine of the 10 tissue blocks were positive for HEVs on multiple levels throughout the tumour sample, whereas one tissue block was entirely negative for HEVs on multiple levels.

High-endothelial venules in oral squamous cell carcinoma displayed heterogeneous morphology related to the inflammatory tumour microenvironment

The HEVs in our OSCC patient cohort displayed a heterogeneous morphology, and the three main phenotypes were vessels with a thick wall and a small lumen (Fig. 1a), vessels with a thin wall and a larger lumen (Fig. 1b), and branched vessels (Fig. 1c). Dilated and especially branched vessels were characterized by a gradual loss of their specific marker PNAd (Fig. 1b, c, arrows). Morphologic alterations of HEVs were also evaluated in association to their surrounding tissue microenvironment (Fig. 2). In highly inflammatory regions, mainly HEVs with a thick wall and a small or no lumen were seen (Fig. 2a), whereas HEVs in regions with less infiltrating immune cells usually presented with thinner walls and a larger lumen (Fig. 2b). Quantitative analysis of vessel wall thickness and lumen diameter of 100 HEVs in areas with low and high level of inflammation, respectively, showed that vessel walls of HEVs were significantly thicker in areas with high compared to areas with low grade of inflammation (U=1724.5; P<0.001; Fig. 2d). Lumen diameter, however, was significantly larger in HEVs located within low-grade inflammatory regions (U=3219.0; P<0.001; Fig. 2e). Branched vessels were found in locations with various levels of inflammation (Fig. 2, asterisks). Only few HEVs were seen in areas with very low inflammation (Fig. 2c). Each of the tumour samples usually contained a heterogeneous mixture of the three HEV morphologic phenotypes (Fig. 2, overview picture), and no single phenotype was exclusively found in any of the tumours. In association with the TLSs, however, only thick-walled HEVs with a very small or no lumen were found (data previously presented in [23]).

Presence of tumour-associated HEVs is an independent positive prognostic factor in patients with oral squamous cell carcinoma

Presence of HEVs was analysed with correlation to the patients' clinicopathological features and 5-year DSD (Tables 1 and 2). As presented in Table 1, presence of HEVs was significantly associated with tumour differentiation, T stage and HPV status. In univariate analysis, positive HEV status was significantly associated with longer 5-year survival rate (73.5 % compared to 14.3 % in HEV-negative patients, P<0.001; Fig. 3b). Significant adverse prognostic factors in univariate analysis included tumour site different from the mobile tongue as well as increasing T stage and positive N and M stages, as indicated in Table 2. The T and the N stages as well as the tumour site and the HEV status were entered

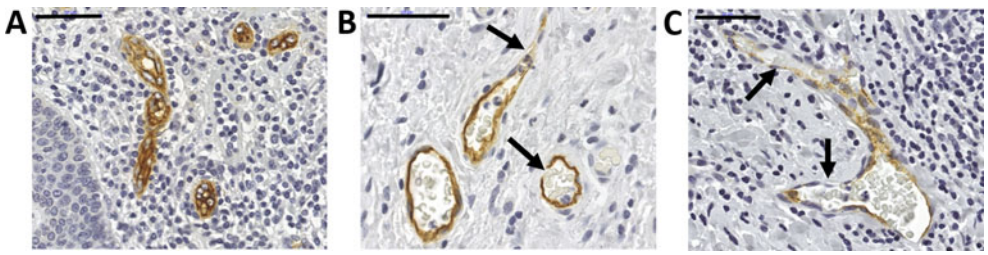

Fig. 1 Different phenotypes of high-endothelial venules in oral squamous cell carcinoma. The pictures show representative immunohistochemical stainings for high-endothelial venules (HEVs). Both HEVs with a thick wall and a small or no lumen (**a**) as well as HEVs with a thinner wall and a larger lumen (**b**) were found. Some of the HEVs were also seen to branch out (**c**). Dilated and branched vessels gradually lost their specific marker PNAd (*arrows*, **b**, **c**). PNAd + vessels are stained *brown*, and cell nuclei are stained *blue* by hematoxylin. *Scale bars* indicate 50 μm

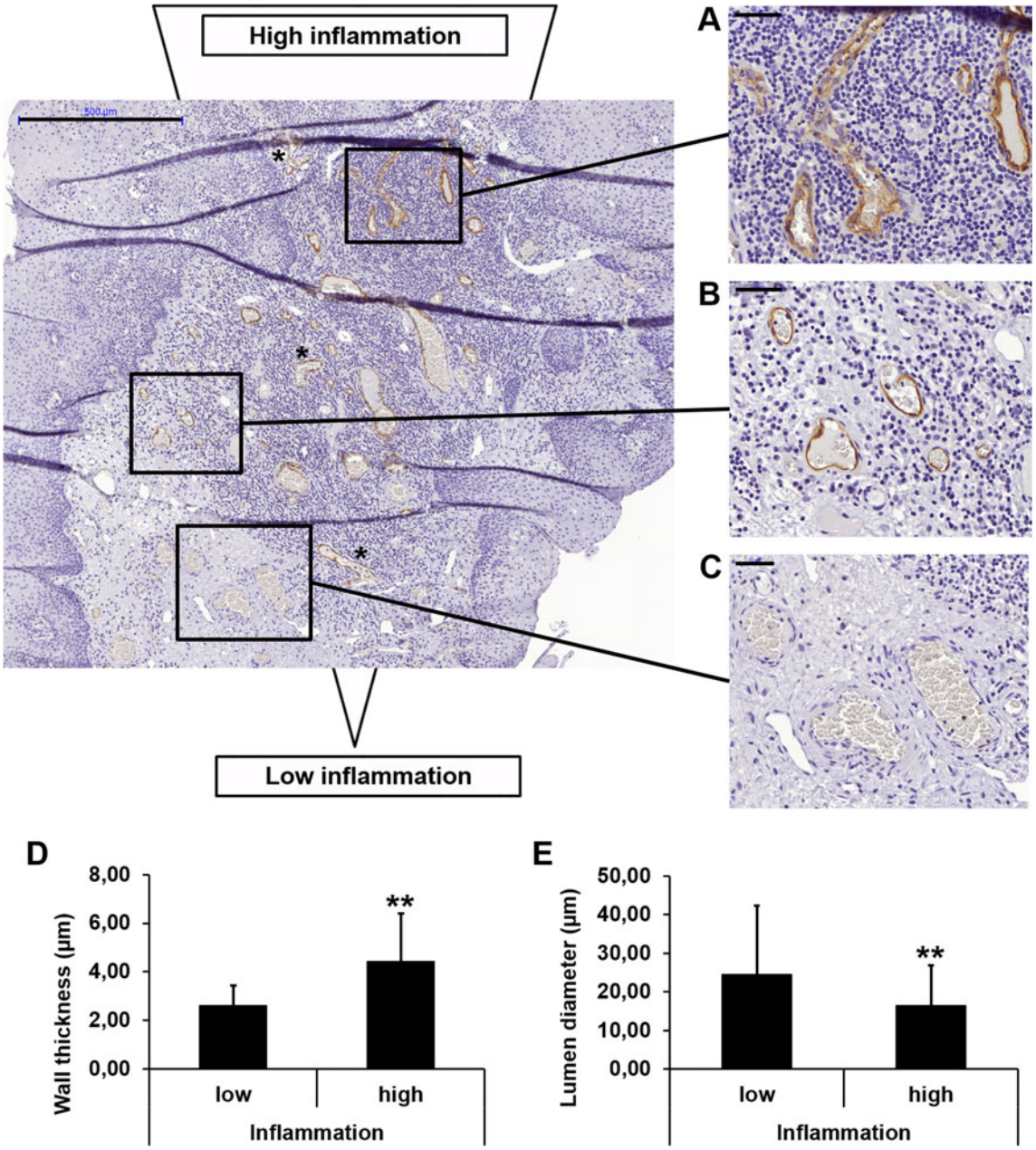

Fig. 2 Qualitative and quantitative analysis of distribution of morphologic altered high-endothelial venules in the tumour microenvironment of oral squamous cell carcinoma. **a–c** Representative immunohistochemical stainings for high-endothelial venules (HEVs) in areas with various levels of lymphocyte infiltration. In sections with high level of inflammation (**a**), HEVs had a significantly thicker wall (**d**) and smaller lumen (**e**) compared to sections with lower level of inflammation (**b**). In regions with very low or no inflammation, very few or no PNAd + vessels were seen, respectively (**c**). Branched vessels were found in areas with various levels of inflammation (*asterisks* in overview picture). PNAd + vessels are stained *brown*, and cell nuclei are stained *blue* by hematoxylin. *Scale bars* indicate 500 µm in the overview picture and 50 µm in the enlarged sections (**a–c**). *Columns* indicate average vessel wall thickness (**d**) and lumen diameter (**e**). *Error bars* represent one standard deviation. $^{**}P<0.001$ relative to HEVs found in areas with low level of inflammation

into multivariate Cox regression analyses. The cases with the unknown T and N stages as well as the M status were taken out from the calculations because of unequal group size. All variables satisfied the proportional hazards assumption (Fig. S1). Both the T stage ($P<0.001$) and the HEV status ($P=0.002$) were independently predictive for DSD (Table 3). HEVs were found in all ($n=53$) of the T1-T2 tumours, but only in two thirds ($n=14$) of the T3-T4 tumours (Table 1). For this reason, separate univariate analyses were conducted on the 21 patients with T3-T4 OSCCs. In these tumours, the absence of HEVs was a statistically significant predictor for lower 5-year survival (14.3 % compared to 28.6 % in HEV-positive patients, $P=0.004$; Fig. 3a). No multivariate analyses were carried out on this patient subset due to small sample size.

Table 2 Clinicopathologic variables as predictors for 5-year disease-specific death in univariate Kaplan–Meier analysis for 75 patients with OSCC

	Patients (N=75) (no. (%))	5-Year DSD (%)	P value
Gender			
Male	43 (57.3)	37.2	0.206
Female	32 (42.7)	25.0	
Age at diagnosis, years			
0–59	28 (37.3)	28.6	0.578
≥60	47 (62.7)	34.0	
Smoking history			
Never smoker	17 (22.7)	23.5	0.654
Former smoker	10 (13.3)	20.0	
Current smoker	43 (57.3)	37.2	
Unknown	5 (6.7)	40.0	
Alcohol consumption			
Never	12 (16.0)	16.7	0.347
≤1 times weekly	27 (36.0)	29.6	
>1 times weekly or daily	20 (26.7)	35.0	
Unknown	16 (21.3)	43.7	
Tumour site			
Mobile tongue	35 (46.7)	17.1	0.024
All others	40 (53.3)	45.0	
Tumour differentiation			
Well	28 (37.3)	28.6	0.679
Moderate	42 (56.0)	33.3	
Poor	5 (6.7)	40.0	
T stage			
T1/T2	53 (70.7)	15.1	<0.001
T3/T4	21 (28.0)	76.2	
Unknown	1 (1.3)	0.0	
N stage			
N0	51 (68.0)	21.6	<0.001
N+	18 (24.0)	66.7	
Unknown	6 (8.0)	16.7	
M stage			
M0	69 (92.0)	31.9	0.014
M+	1 (1.3)	100.0	
Unknown	5 (6.7)	20.0	
HPV/p16			
Negative	65 (86.7)	32.3	0.570
Positive	6 (8.0)	16.7	
Unknown	4 (5.3)	50.0	
HEV			
Negative	7 (9.3)	85.7	<0.001
Positive	68 (90.7)	26.5	

P values were calculated using the log-rank test

Discussion

In this study, we have presented tumour-associated HEVs as a robust and easy-to-use, independent positive prognostic marker for patients with oral cancer. Sixty eight (90.7 %) of the 75 OSCC samples were defined as HEV-positive and the remaining 7 (9.3 %) were negative. Univariate analysis showed that patients with HEVs had a significantly lower 5-year DSD rate compared to patients without HEVs (P<0.001). In multivariate analyses, both the HEV status and the T stage were significant independent predictors of DSD (P=0.002 and P<0.001, respectively). HEVs were found in all T1-T2 tumours, but only in two thirds of the T3-T4 tumours, suggesting tumour-associated HEV status as a promising prognostic factor in

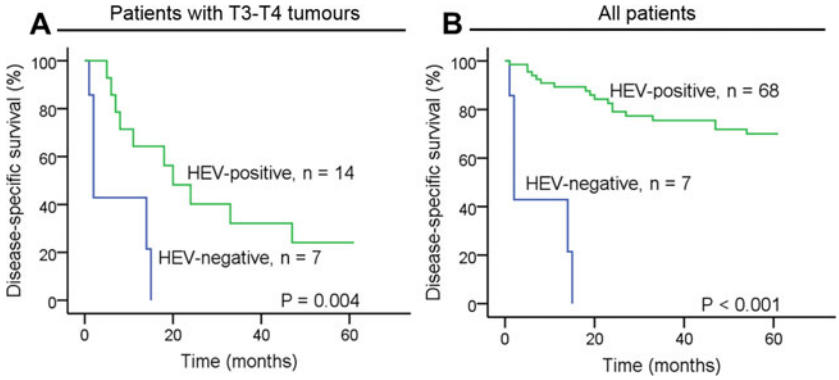

Fig. 3 Kaplan–Meier analysis of 5-year disease-specific survival for patients with oral squamous cell carcinoma with and without high-endothelial venules. The presence of high-endothelial venules (HEVs) is associated with improved survival in **a** 21 patients with T3-T4 oral squamous cell carcinoma (OSCC) (P=0.004) and in **b** all 75 OSCC patients in our cohort (P<0.001). In the T3-T4 tumours (**a**), the Kaplan–Meier curve shows a 5-year disease-specific survival (DSS) rate of 28.6 % for HEV-positive patients and 14.3 % for HEV-negative patients. The 5-year DSS rate for all HEV-positive patients in our cohort (**b**) was 73.5 %. The P value was calculated using the log-rank test

patients with advanced cancer. Previously, tumour HEVs were found to be independently associated with favourable clinical outcome in breast cancer [10]. To the best of our knowledge, this is the first study to report HEVs as an independent positive prognostic factor for primary OSCC cancer patients.

Tumour HEVs have earlier been shown to display a broad heterogeneity [10, 38]. Previously, tumour HEVs were observed in human solid cancers, such as melanomas, breast cancers, colon, lung and ovarian carcinomas in approximately 70 % of the patients [10, 11, 24, 39]. In oral cancer, however, we found approximately 90 % of the patients to be HEV-positive. This might be due to inter-tumoural heterogeneity as well as different methodical approaches including individual cut-off points for HEV positivity. The majority of the HEV negative tumours were found in oral locations other than the tongue, suggesting that HEVs may form more readily in some environments than others. In non-small-cell lung cancer, HEVs have been exclusively associated with ectopic lymphoid accumulations of immune cells called TLSs [40]. We have previously reported the presence of HEVs in association with TLSs in the same OSCC patient cohort as in the present study [23]. In contrast to the report from non-small-cell lung cancer, we here demonstrate that the majority of HEVs were unrelated to TLSs, as 68 of the 75 patients were HEV-positive while TLSs were only found in 17 of the tumours [23]. Presence of HEVs was significantly associated with HPV status, assessed by p16 immunohistochemical staining, where a higher proportion of the HPV-negative tumours were HEV-positive. Due to the low number of HPV-positive tumours in our study, this correlation needs to be confirmed in larger studies. Intratumour heterogeneity displays great challenges for the identification of suitable prognostic markers [41]. In our study, we therefore performed multiple level assessments of 10 tissue blocks showing that analysis for HEV positivity on a single level can be considered representative for the whole tumour. These results underpin the value of HEVs as a simple and robust, positive prognostic marker that might be easily implementable in clinical routines for patients with oral cancer.

Recent findings indicate that HEVs have an important role in cancer progression and that their plasticity might be a critical feature [24, 27, 28]. Martinet et al. conducted a study on breast carcinoma sections, where the highest density of tumour HEVs was found within the in situ and not the invasive component [42]. In our study, HEVs were found in all of the T1-T2 tumours but were not observed in one third of the T3-T4 tumours, suggesting that these vessels might play an important role for the progression of oral cancer, perhaps by suppressing tumour growth. HEVs have however been shown to be plastic and able to differentiate and dedifferentiate upon various stimuli, such as from the tumour microenvironment [13]. Thus, in larger tumours, HEVs might transdifferentiate to normal blood vessels and gradually lose their specific marker PNAd. Several studies have reported that such a remodelling is associated with poor prognosis [27, 28]. Dendritic cells

Table 3 Multivariate analysis of 5-year disease-specific death according to Cox's proportional hazards model[a]

Variable	Hazard ratio	95 % CI	P value
T stage (T1/T2 [n=49] vs. T3/T4 [n=20])	8.296	3.120–22.062	<0.001
HEV (negative [n=6] vs. positive [n=63])	0.147	0.044–0.490	0.002

[a] Only 69 patients were analysed because the cases with the unknown T and N stages were taken out from the calculations

are thought to be important regulators of HEV-mediated lymphocyte trafficking to lymph nodes [24]. In the absence of dendritic cells, mature HEVs were seen to revert to an immature phenotype, and HEV-mediated lymphocyte recruitment to lymph nodes was inhibited [26]. Thus, it might be that more advanced tumours have found ways to disarm the dendritic cells causing HEV regression. Generation and maturation of dendritic cells, however, might vary with tumour grade and also within the tumours, which might then result in heterogeneous HEV phenotypes. We found dilated and especially branched HEVs to be characterized by a gradual loss of their PNAd staining. Moreover, these morphologically altered HEVs were generally associated with low grade of inflammation. Accordingly, HEVs with a thick wall and a small lumen—resembling the lymphocyte trafficking functional phenotype—were mostly located in areas with more prominent lymphocytic infiltration. These findings are consistent with previous results, where HEVs lined by a cuboidal endothelium were surrounded by more lymphocytes than those lined by a flat endothelium [39]. In our patient cohort, HEVs with different morphologies were found in the same tumours. Tumour growth from a single cell is a stepwise progression, and the heterogeneity of HEV morphology within a single tumour might be due to transition processes in tumour immunogeneity.

It has earlier been shown that remodelling of HEVs in lymph nodes of human breast cancer and OSCC patients is a poor prognostic factor [27, 28]. In the present study, we have not investigated the presence and morphology of HEVs in metastatic lymph nodes of these patients. However, in future studies, it would be of great interest to investigate the HEV status in the respective lymphatic basin. Further, we did not evaluate the association between specific morphological phenotypes and clinicopathological characteristics, but rather, the presence of HEVs irrespective of their morphologic features, as many tumours displayed several morphological HEV phenotypes. Hence, we can only hypothesize that morphologically altered vessels might have adverse effects on tumour progression. Additionally, it would be interesting to see if HEVs with weak PNAd staining start expressing blood vessel markers and if HEV phenotype is associated with presence or absence of dendritic cells. Another weakness of our study is that we conducted semi-quantitative analyses of HEV surrounding lymphocytes but did not distinguish between different subsets of lymphocytic cells. However, future studies are needed to better characterize the inflammatory infiltrate in which HEVs arise, as cell-type-specific transcription factors are probably involved in the development and maintenance of HEVs [13]. Studying different subsets of immune cells and specific chemokines might be an interesting supplement to better understand possible interactions between HEVs and the tumour microenvironment. Finally yet importantly, our results have to be confirmed in larger studies.

In conclusion, we report that the presence of tumour-associated HEVs in OSCCs is significantly correlated with lower DSD. Tumour HEVs were commonly found in early stage OSCCs, but only in two thirds of the T3-T4 tumours. Thus, evaluation of this biomarker might be important to identify patients with more aggressive T3-T4 OSCCs. Our results on HEV morphology indicate an intricate relationship between HEVs and the surrounding microenvironment. HEVs are highly plastic, and shifting from a lymphocyte- to a blood-carrying vessel might be associated with tumour progression. Thus, identification of markers and genes for HEV development and regulation in cancer-associated inflammation may open new opportunities for targeted therapies and genetic manipulation. As immunohistochemical detection of HEVs is simple, and the distribution of these vessels seems to be even throughout a tumour, this may be a valuable supplement to the TNM staging for prognosis assessment and treatment stratification in OSCC patients.

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Ethical approval All procedures performed in this study involving human material were in accordance with the ethical standards of the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required. This article does not contain any studies with animals performed by any of the authors.

Compliance with ethical standards

Conflicts of interest None

Research involving human participants and/or animals All procedures performed in this study involving human material were in accordance with the ethical standards of the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not present any animal studies.

Informed consent The Regional Committee for Medical and Health Research Ethics, Northern Norway approved the study without requiring informed consent from the patients, as many of them were dead when the study was initiated.

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Supplementary material

Supplementary figure

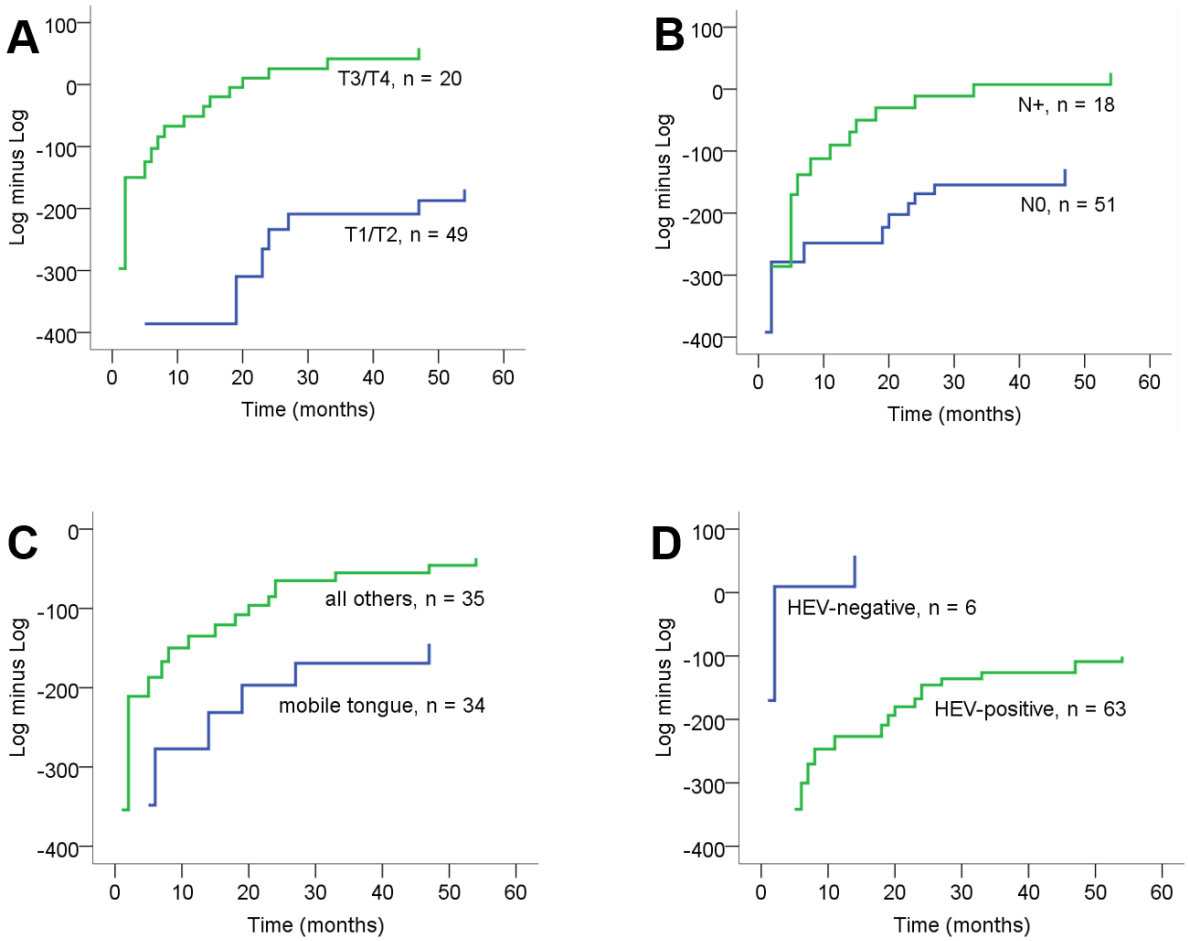

Figure S1. Log minus log plots for proportional hazards checking; (A) T stage, (B) N stage, (C) tumour site, (D) high-endothelial venules (HEVs).

Paper III

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ARTICLE

Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma

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Abstract

Oral squamous cell carcinomas are associated with a poor prognosis, which may be partly due to functional impairment of the immune response. Lymphocyte recruitment to the tumor site is facilitated by high-endothelial venules, whereas expression of programmed-death ligand 1 (PD-L1) can impair T-cell function. Thus, we hypothesize that these factors are important in shaping the immune response in oral squamous cell carcinoma. In the present study, we characterized the immune infiltrate in formalin-fixed, paraffin-embedded tumor samples from 75 oral squamous cell carcinoma patients. We used immunohistochemistry to determine the distribution of immune cell subsets, high-endothelial venules, and PD-L1, as well as quantitative real-time polymerase chain reaction to assess the expression of inflammatory cytokines and chemokines associated with lymphocyte trafficking. Finally, we calculated correlations between the presence of immune cell subsets, the gene expression patterns, high-endothelial venules, PD-L1, and the clinicopathological parameters, including patient survival. The presence of high-endothelial venules correlated with increased number of CD3+ T cells and CD20+ B cells, higher levels of the chemokines CXCL12 and CCL21, and lower levels of CCL20, irrespective of the tumors' T stage. In univariate analysis, high levels of CD20+ B cells and CD68+ macrophages, positive high-endothelial venule status, and low T and N stages predicted longer patient survival. However, only the presence of high-endothelial venules and a low T stage were independent positive prognosticators. This indicates that high-endothelial venules are important mediators and a convenient marker of an antitumor immune response in oral squamous cell carcinoma. Our findings suggest that these vessels are a potential immunomodulatory target in this type of cancer. PD-L1 staining in tumor cells correlated with lower T stage, increased infiltration of CD4+ cells, and higher expression of several inflammation-related cytokines. Thus, oral squamous cell carcinomas rich in CD4+ cells may preferentially respond to PD-1/PD-L1 blockade therapy.

Introduction

The majority of oral cancers are squamous cell carcinomas, and their incidence is increasing in many Western countries [1]. Oral squamous cell carcinomas are regarded as aggressive cancers, but tumors of the same stage show substantial heterogeneity in progression and response to treatment. Unlike many other cancers, such as breast, lung, and colorectal cancer, there are no reliable biomarkers that predict the aggressiveness or treatment response of an individual oral squamous cell carcinoma [2–4].

The immune system is an important and complex regulator of tumor evolution. Immune suppression promotes tumorigenesis in murine models [5]; and likewise, a number of cancers occur with increased frequency and aggressiveness in immunocompromised patients [6]. Mutated cells express altered proteins or tumor-specific antigens that usually evoke an immune response [7]. However, the intensity and composition of the tumor-associated inflammation vary between patients and may affect prognosis, as demonstrated in melanoma, breast, and ovarian cancer

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Table 1 Primary antibodies for immunohistochemistry

Target	Primary antibody	Dilution	Incubation time (min)
T cells	Rabbit monoclonal anti-CD3, clone 2GV6; Ventana Medical Systems, Tucson, AZ	Pre-diluted	16
T helper and T regulatory cells	Rabbit monoclonal anti-CD4,clone SP35; Ventana Medical Systems	Pre-diluted	32
Cytotoxic T cells	Rabbit monoclonal anti CD8, clone SP57; Ventana Medical Systems	Pre-diluted	32
B cells	Mouse monoclonal anti-CD20, clone L26; Ventana Medical Systems	Pre-diluted	16
Macrophages	Mouse monoclonal anti-CD68, clone KP-1; Ventana Medical Systems	Pre-diluted	12
Dendritic cells	Mouse anti-DC-LAMP/CD208, clone 104G4, #DDX0190; Dendritics, Dardilly, France	1:50	32
PD-L1+ tumor cells	Rabbit monoclonal anti-PD-L1, clone SP263, #790–4905; Ventana Medical Systems	Pre-diluted	16
High-endothelial venules	Rat anti-PNAd, clone MECA-79; Biolegend, San Diego, CA	1:25	30

[8–10]. For some cancers, including melanoma and head and neck cancer, an inflamed and a non-inflamed phenotype have been described [11, 12]. Inflamed tumors are characterized by marked T-cell infiltration and high expression of chemokines that can recruit effector T cells, and are generally associated with a favorable prognosis [13]. Non-inflamed tumors, on the other hand, may be linked to defective recruitment of immune cells to the tumor microenvironment, and predict a poor outcome. Lymphocyte extravasation can be regulated by high-endothelial venules, which are blood vessels expressing peripheral node addressin that binds L-selectin on circulating naive lymphocytes [14]. High-endothelial venules are present in secondary lymphoid organs, and develop regularly in chronically inflamed tissue [15]. We have previously reported that the presence of high-endothelial venules is associated with improved survival in oral squamous cell carcinomas [16], which is in line with results from studies on melanoma and breast cancer [17, 18]. However, these vessels are plastic, and their remodeling into regular blood vessels may impair lymphocyte recruitment and precede sentinel lymph node metastasis [19, 20].

Although oral squamous cell carcinomas are mostly of the inflamed phenotype suggesting effective immune surveillance [13], these cancers are generally associated with poor survival. This could be explained by immune editing mechanisms that impair infiltrating lymphocytes and allow tumor progression despite the apparent immune response [21]. Programmed-death ligand 1 (PD-L1)-expressing cells can induce immune suppression by binding the PD-1 receptor (PD-1) on activated T cells and downregulate effector T-cell functions [22–24]. Reversing immunosuppression through blockade of the PD-1/PD-L1 pathway has shown clinical efficacy in various cancers, including oral squamous cell carcinoma [25–27]. However, studies on the prevalence and prognostic role of PD-L1 expression in oral squamous cell carcinoma are limited, and conflicting results are reported [22, 28, 29]. To date, it remains unclear why only a subset of patients respond to PD-1/PD-L1 blockade treatment.

In a cohort of 75 oral squamous cell carcinoma patients, we recently demonstrated that advanced-stage tumors with high-endothelial venules present were associated with a more pronounced inflammatory response and longer patient survival compared to their high-endothelial venule-negative counterpart [16]. In the present study on the same patient cohort, we explored the hypothesis that high-endothelial venules are master regulators of tumor immunity in oral squamous cell carcinoma by characterizing the immune cell infiltration and cytokine expression in relation to presence of such vessels. Furthermore, we analyzed the association between the tumors' PD-L1 expression and the immune response, and examined a potential relationship between high-endothelial venules and PD-L1 statuses.

Materials and methods

Patients and material

In this retrospective study, we included 75 patients diagnosed with primary oral squamous cell carcinoma in the period 1986–2002 from the archives of the Department of Clinical Pathology, University Hospital of North Norway. Follow-ups were continued until January 1, 2012. Patients were included in the study if both clinical data and formalin-fixed, paraffin-embedded tumor specimens (biopsy or surgical resection) were available. Only patients with cancers in the oral cavity proper were included, and they were distributed among the following locations: mobile tongue ($n = 36$); floor of the mouth ($n = 21$); the alveolar ridge ($n = 9$); buccal mucosa ($n = 7$); palatal arches (n = 1) and unspecified oral cavity ($n = 1$). Exclusion criteria were prior radiotherapy to the head and neck area as well as previous oral or oropharyngeal cancer. Sixty-eight percent of the patients received surgery in combination with radiotherapy,

15% concomitant radiotherapy and chemotherapy, 11% surgery in combination with local neck-dissection, 5% none or palliative therapy, and 1% were unknown. We retrieved clinical and histopathologic information from the patients' hospital files, pathology reports, and the Statistics of Norway, Cause of Death Registry, and have earlier published these data in studies analyzing the same patient cohort [16, 30]. Pathological tumor staging was determined according to the most recent (second to fifth) TNM classification from the American Joint Committee on Cancer at the time of diagnosis. There were no changes in the classification of cancers of the lip and oral cavity between the different editions used, so that the description in the fifth edition is relevant for all cases [31]. We conducted the study in line with the REMARK guidelines for tumor marker prognostic studies [32], and following approval from the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007).

Immunohistochemistry

We used 4-µm-thick sections of formalin-fixed, paraffin-embedded tumor specimens from oral squamous cell carcinoma patients for immunohistochemical analyses, and performed both automated and manual staining methods. Specifications of the primary antibodies as well as their incubation conditions are listed in Table 1. We used CD3 as a pan T-cell marker. Various subtypes of T helper and T regulatory (Treg) cells express CD4 strongly, whereas monocytes, macrophages, and dendritic cells may show weak expression. Cytotoxic T cells, and to less extent, natural killer cells and subsets of dendritic cells express CD8. CD20 is a pan B-cell marker, and CD68 is a pan macrophage marker, but it may also stain subsets of lymphocytes, fibroblasts, and endothelial cells. Mature dendritic cells and, to some extent, pneumocytes express DC-lamp/CD208.

Automated staining

Staining for CD3, CD4, CD8, CD20, CD68, and PD-L1 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, as previously published [30]. Briefly, for antigen retrieval, deparaffinized and blocked sections were heat-treated in 0.01 M sodium citrate buffer at pH 6.0. A cocktail of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG/IgM and mouse anti-rabbit secondary antibodies (Ventana UltraView Universal DAB Detection Kit, #760–500, Roche, Mannheim, Germany) were used for visualization with diaminobenzidine. As these procedures are automatized, the manufacturer controls the incubation time of secondary antibodies. In every run, one slide with known positivity for the different antibodies (tonsil or lymph node) was added as control. Automated staining runs for PD-L1 included in addition a negative control slide with rabbit monoclonal negative control Ig (#790–4795, Ventana) for each patient. DC-lamp staining was performed in the Ventana Discovery ULTRA autostainer (Ventana). After dewaxing, cell conditioning-1 solution (#950–124, Ventana) was applied for antigen retrieval for 32 min at 95 °C. Endogenous peroxidase was blocked by discovery inhibitor CM (#760–4840, Ventana). After incubation with the DC-lamp primary antibody, the pre-diluted secondary antibody (OmniMap anti-mouse HRP; #760–4310, Ventana) was loaded for 20 min followed by HRP amplification and visualization by ChromoMap DAB (#760–159; Ventana). Counterstaining was performed using the hematoxylin II counterstain reagent (#790–2208, Ventana). Metastatic lymph node tissue microarray slides of lung cancer were used as positive control. Giemsa staining was performed using BenchMark Special Stains, an automated slide stainer from Ventana. In every run, one slide with known positivity for the stain was added as control.

Manual staining

Peripheral node addressin staining for the detection of high-endothelial venules was performed as previously described [30]. In brief, sections were deparaffinized, rehydrated, subjected to heat-induced antigen retrieval, blocked, and incubated with the peripheral node addressin primary antibody. Next, the sections were incubated with HRP-labeled goat anti-rat light chain secondary antibody (#AP202P, Millipore, Temecula, CA, diluted 1:250, incubated 30 min) and diaminobenzidine (Dako EnVision+ System-Horseradish Peroxidase, Dako) before being counterstained with Harry's hematoxylin (Sigma-Aldrich, St. Louis, MO). Human lymph node specimens were used as positive control, and specimens with the primary antibody omitted served as negative control.

Immunohistochemical evaluation

Two trained, independent observers (either EHO and AMW, or SES and IKE) who were blinded to the clinical outcome evaluated the immunohistochemical staining quantitatively and semiquantitatively, as illustrated in the flow chart of Figure S1. We assessed inter-observer variations for all staining except DC-lamp and PD-L1, and the results are listed in Table S1. Agreement was reached by reevaluation and discussion in case of differing scores. For DC-lamp and PD-L1, the two investigators reached consensus when evaluating the slides together. Micrographs

were taken with a Leica DFC 420 camera on a Leica DM2000 microscope (Leica, Wetzlar, Germany). Presence of high-endothelial venules was assessed as published earlier [16]. Briefly, we scanned the peripheral node addressin stained tumor-adjacent tissue at low power magnification (×100) to identify five areas with high density of high-endothelial venules (hotspots). Micrographs of these hotspots were taken at high power magnification (×400), and the mean number of high-endothelial venules per section was calculated for each patient by dividing the sum of these vessels in the five hotspots by five. The median number of high-endothelial venules per hotspot for all patients served as cutoff for positive and negative count. Figure S2 shows a micrograph of tumor-associated high-endothelial venules. Giemsa staining was used to identify mast cells and eosinophils as cells with round nuclei and blue/purple granules or cells with lobulated nuclei and bright orange/pink granules, respectively. For each of the cell types, five hotspots in the tumor stroma were identified at low power magnification, and micrographs were taken at ×400 magnification. The total cell number in the hotspots was counted and the mean number calculated. The cutoff for high vs. low count was defined as the median number for the patient cohort, which was 10 for eosinophils and 3 for mast cells. We scored the CD3+, CD4+, CD8+, CD20+, and CD68+ staining semiquantitatively, as it was impossible to apply a quantitative scoring system in densely stained areas. First, we identified the invasive margin of the tumor at low power magnification (×100), and micrographs were taken at ×400 magnification, capturing every second visual field of this area. Thus, the number of microscope fields depended on the tumor size. We developed a four-degree scoring scale for the different cell subsets as illustrated in Figure S3, and assigned each micrograph a score: 0 = no or almost no infiltration; 1 = mild infiltration; 2 = moderate infiltration; and 3 = heavy infiltration. In cases where evaluation was difficult because of weak staining or nonspecific background staining, the positively stained cells were counted, and the scores determined according to the respective cutoff. We calculated a mean score for each section before dichotomizing as low or high if it was 0–1.49 or 1.5–3, respectively. PD-L1 expression was assessed based on the percentage of positively labeled tumor cells in each section, and was classified as follows: 0 (labeling in ≤5% of cells), 1 (labeling in >5% and ≤10% of cells), 2 (labeling in >10% and ≤50% of cells), and 3 (labeling in >50% of cells). Expression of DC-lamp was found only in inflamed areas of the tumor stroma and was scored as not expressed (0), slightly expressed [1], moderately expressed [2], or strongly expressed [3] based on semiquantitative evaluation. Both PD-L1 and DC-lamp scores were subsequently grouped into two categories: low expression (0 or 1); and high expression (2 or 3).

RNA extraction and quality control

RNA extraction was performed to evaluate gene expression of cytokines. In cases with sufficient residual tumor material, we isolated total RNA from formalin-fixed, paraffin-embedded oral squamous cell carcinoma tissue blocks using the High Pure FFPET RNA Isolation Kit (Roche) following the manufacturer's instructions. In brief, up to four consecutive, 5–10 µm-thick sections from each block were deparaffinized and digested with proteinase K, followed by multiple silica-based column purification steps and DNAse I treatment. The RNA on the column was washed several times before being eluted in 20–35 µl elution buffer. We used a mixture of RNA isolated from three different fresh frozen (in liquid nitrogen) human lymphoma specimens as a positive control for further real-time quantitative PCR (qPCR) analyses. The RNeasy Fibrous Tissue mini Kit (Qiagen) was used for RNA isolation according to the manufacturer's protocol. Briefly, the tissue was homogenized using a TissueLyser (Qiagen) before digestion with proteinase K and centrifugation. The supernatant was washed and treated with DNAse I on a miniature column, and the RNA eluated in 50 µl nuclease-free water. We measured total RNA quantity and purity on the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and assessed RNA integrity using the Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, USA).

Real-time quantitative PCR

We used the QuantiTect Reverse Transcription kit (Qiagen) to reverse transcribe 100–200 ng total RNA into cDNA, which was subsequently diluted 1:15 in nuclease-free water. QPCR was performed in duplicates or triplicates using the Light Cycler 96 instrument (Roche). Target cDNA was amplified through 40 cycles in 20 µl reactions containing 1× FastStart Essential DNA Green Master (Roche), 10 µl diluted cDNA (1:15), and 300 nM primers (Table S2). Melting curve analysis was used to verify the specificity of the primers. Controls with the reverse transcriptase omitted and non-template controls were included to test for genomic DNA contamination and carry-over products, respectively. The qPCR amplification efficiency for each gene was calculated from the slope and correlation coefficient (R2) of regression curves from twofold serially diluted cDNA. The ΔΔCt method [33] was used to calculate the relative amount of target mRNA normalized against the geometric mean of the reference genes elongation factor 1 alpha, ribosomal protein L27, and ribosomal protein S13. We used geNorm analyses [34] to select three stably expressed reference genes. For association with high-endothelial venules and PD-L1 statuses, we present the results as fold increase compared to the mean of the group with the lowest gene expression (±standard error of mean).

Table 2 Comparison of low vs. high counts of infiltrating immune cells in OSCC patients[a] with and without HEVs using Fisher's exact test

	All tumors			T1/T2	T3/T4					
	HEV+	HEV−		All HEV+	All (HEV+/−)		HEV+		HEV−	
	No. (%)	No. (%)	*P*	No. (%)	No. (%)	*P*	No. (%)	*P*	No. (%)	*P*
CD3										
Low	15 (22.4)	6 (85.7)	0.002	11 (21.2)	10 (47.6)	0.026	4 (28.6)	0.396	6 (85.7)	0.002
High	52 (77.6)	1 (14.3)		41 (78.8)	11 (52.4)		10 (71.4)		1 (14.3)	
CD4										
Low	44 (65.7)	5 (100.0)	0.170	34 (65.4)	14 (73.7)	0.359	9 (64.3)	0.587	5 (100.0)	0.138
High	23 (34.3)	0 (0.0)		18 (34.6)	5 (26.3)		5 (35.7)		0 (0.0)	
CD8										
Low	35 (52.2)	5 (100.0)	0.061	28 (53.8)	12 (63.2)	0.336	7 (50.0)	0.517	5 (100.0)	0.057
High	32 (47.8)	0 (0.0)		24 (46.2)	7 (36.8)		7 (50.0)		0 (0.0)	
CD20										
Low	26 (38.2)	6 (85.7)	0.038	20 (37.7)	12 (57.1)	0.104	6 (42.9)	0.478	6 (85.7)	0.022
High	42 (61.8)	1 (14.3)		33 (62.3)	9 (42.9)		8 (57.1)		1 (14.3)	
CD68										
Low	33 (48.5)	6 (85.7)	0.109	22 (41.5)	17 (81.0)	0.002	11 (78.6)	0.014	6 (85.7)	0.034
High	35 (51.5)	1 (14.3)		31 (58.5)	4 (19.0)		3 (21.4)		1 (14.3)	
DC-lamp										
Low	32 (51.6)	5 (71.4)	0.279	25 (53.2)	11 (52.4)	0.579	6 (42.9)	0.354	5 (71.4)	0.314
High	30 (48.4)	2 (28.6)		22 (46.8)	10 (47.6)		8 (57.1)		2 (28.6)	
Eosinophils										
Low	37 (55.2)	6 (85.7)	0.227	26 (50.0)	16 (76.2)	0.035	10 (71.4)	0.129	6 (85.7)	0.082
High	30 (44.8)	1 (14.3)		26 (50.0)	5 (23.8)		4 (28.6)		1 (14.3)	
Mast cells										
Low	32 (47.8)	6 (85.7)	0.108	25 (48.1)	13 (61.9)	0.209	7 (50.0)	0.568	6 (85.7)	0.068
High	35 (52.2)	1 (14.3)		27 (51.9)	8 (38.1)		7 (50.0)		1 (14.3)	
PD-L1										
Low	23 (59.0)	4 (66.7)	0.544	15 (50.0)	12 (85.7)	0.024	8 (100.0)	0.010	4 (66.7)	0.386
High	16 (41.0)	2 (33.3)		15 (50.0)	2 (14.3)		0 (0.0)		2 (33.3)	

HEV high-endothelial venule

[a]Analyses for CD3+ cells, eosinophils, and mast cells were carried out in 74 patients, for CD4+ and CD8+ cells in 72 patients, for DC-lamp+ cells in 69 patients, and for PD-L1+ cells in 45 patients. All other analyses included 75 patients

For survival and correlation analyses, we dichotomized the results in low and high expression based on the median of fold increase as cutoff.

Statistical analysis

We used SPSS software version 22.0 for Windows (IBM, Armonk, NY) and Microsoft Excel 2013 (Microsoft, Redmond, WA) for all calculations. Inter-observer variability for the various quantitative and semiquantitative cell counts was analyzed using the Spearman correlation test, and correlation between two variables was assessed by the Fisher's exact test. We used univariate Kaplan–Meier analyses to calculate disease-specific death rates and plot disease-specific survival curves, and the log-rank test to evaluate the statistical significance. Multivariate analyses were done using a stepwise forward multiple Cox regression model. Linear regression analyses of standard curves derived from serially diluted cDNA were used to estimate qPCR amplification efficiency. The significance level was set at $P < 0.05$, and the borderline significance level at $P < 0.09$.

Results

High-endothelial venules predict T- and B-cell infiltration into oral squamous cell carcinomas

We recently demonstrated a correlation between the presence and morphology of high-endothelial venules and the

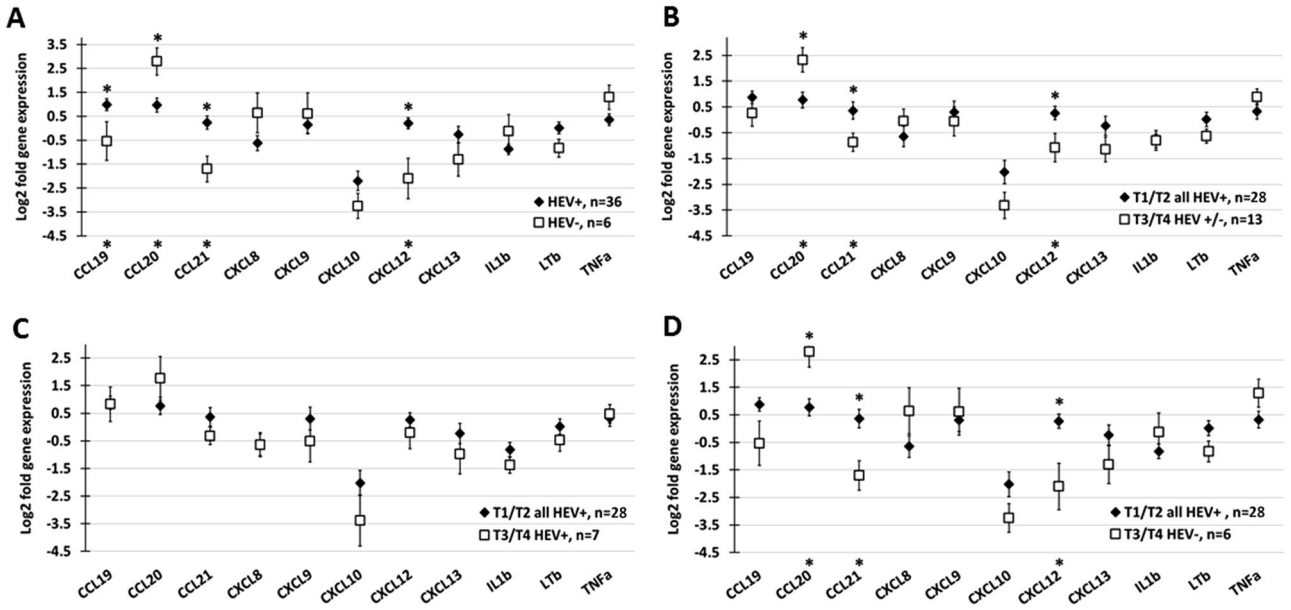

Fig. 1 Log2-fold gene expression of various chemokines in formalin-fixed paraffin-embedded oral squamous cell carcinoma tissue samples. Comparison of the following groups: **a** high-endothelial venule (HEV)-positive vs. HEV-negative tumors; **b** T1/T2 vs. T3/T4 tumors; **c** T1/T2 vs. T3/T4 tumors with HEVs; and **d** T1/T2 vs. T3/T4 tumors without HEVs. All T1/T2 tumors were HEV-positive. Error bars indicate ±standard error of the mean, and $*P < 0.05$. The *P*-value was calculated using two sample *T*-test

intensity of the inflammatory infiltrate in oral squamous cell carcinomas [16]. Now, to determine the composition of the immune infiltrate, we immunohistochemically stained tumor sections from 75 oral squamous cell carcinoma patients with antibodies against the following immune cell markers: CD3 (pan T cell); CD4 (T helper, Treg cell); CD8 (cytotoxic T cell); CD20 (pan B cell); CD68 (pan macrophage), and DC-lamp/CD208 (mature dendritic cell). We also performed Giemsa staining to identify eosinophils and mast cells. The high-endothelial venule-negative tumors were generally less infiltrated with immune cells compared to the high-endothelial venule-positive tumors (Table 2). The differences were statistically significant for CD3+ ($P = 0.002$) and CD20+ ($P = 0.038$) cells, and borderline significant for CD8+ cells ($P = 0.061$).

To determine cytokines associated with high-endothelial venules, we conducted qPCR analysis of 42 of the 75 oral squamous cell carcinoma tissue samples, of whom 36 were high-endothelial venule-positive and 6 were negative (Fig. 1a). The lymphoid chemokines CCL19, CCL21, and CXCL12 were significantly higher expressed in the high-endothelial venule-positive compared to the tumors without these vessels ($P = 0.04$, 0.02, and 0.001, respectively). The inflammatory chemokine CCL20, however, was significantly lower expressed in the high-endothelial venule-positive than in the -negative tumors ($P = 0.02$). Taken together, our results shows that high-endothelial venule-positive tumors are more heavily infiltrated with lymphocytes than tumors without such vessels, which may be promoted by the chemokines CCL19, CCL21, and CXCL12.

Oral squamous cell carcinomas with high-endothelial venules retain an inflamed phenotype even at high T stage

In the same oral squamous cell carcinoma patient cohort, we have earlier found that high-endothelial venules were present in all T1/T2 tumors, compared to two-thirds of the T3/T4 tumors [16], and hypothesized that absence of these vessels in advanced tumor stages may suppress the immune reaction. To elaborate this theory, we compared the immune cell infiltrate and cytokine expression in the T1/T2 tumors with the T3/T4 tumors, as well as the T3/T4 tumors subdivided into high-endothelial venule-positive and -negative. We found a significantly higher amount of infiltrating CD3 + T cells, CD68+ macrophages, and eosinophils in the T1/T2 tumors (all high-endothelial venule-positive) compared to the T3/T4 tumors (high-endothelial venule-positive and -negative) as presented in Table 2 ($P = 0.026$, 0.002, and 0.035, respectively). Dividing the T3/T4 tumors into high-endothelial venule-positive and -negative revealed that the positive had an immune cell infiltrate that resembled the T1/T2 tumors, except for fewer CD68+ cells in the T3/T4 tumors ($P = 0.014$). In contrast, the high-endothelial venule-negative T3/T4 tumors showed lower scores than T1/T2 tumors for all immune cell subsets analyzed, except CD4+ cells and DC-lamp+ dendritic cells that were not significantly different.

QPCR analysis of 28 T1/T2 tumor samples (all high-endothelial venule-positive) and 13 T3/T4 tumor samples (high-endothelial venule-positive, $n = 7$; high-endothelial

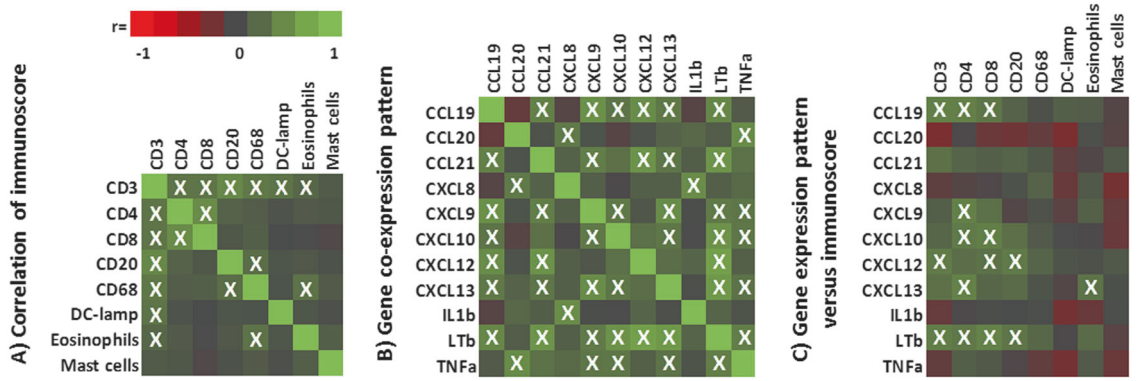

Fig. 2 Heatmaps of Pearson correlation coefficients. The heatmaps show results from bivariate correlation analyses of the following parameters in the oral squamous cell carcinomas: **a** immune cell immunohistochemical scores; **b** gene expression pattern; and **c** gene expression pattern and immune cell immunohistochemical scores. White crosses in the heatmaps show significant correlation between the different variables with $P < 0.05$

venule-negative, $n = 6$) revealed higher levels of CCL21 and CXCL12, and lower levels of CCL20 in the T1/T2 compared to the T3/T4 tumors ($P = 0.04$, 0.02, and 0.01, respectively; Fig. 1b). In accordance with the results from immune cell analyses, the high-endothelial venule-positive T3/T4 tumors displayed no statistically significant or borderline significant differences to the T1/T2 tumors' cytokine expression (Fig. 1c). The high-endothelial venule-negative T3/T4 tumors, however, had significantly lower expression of CCL21 and CXCL12 and higher expression of CCL20 ($P = 0.02$, 0.04, and 0.01, respectively; Fig. 1d) than the T1/T2 tumors. Together, our results suggest that the presence of high-endothelial venules supports an inflamed phenotype even in large (T3/T4) tumors.

T-cell infiltration predicts an immune cell-rich tumor microenvironment and is associated with CCL19, CXCL12, and lymphotoxin b expression

Cytokines, chemokines, and cells of the innate and adaptive immune system interact in complex attraction, activation, and inhibition networks. To reveal putative regulatory mechanisms in the oral squamous cell carcinoma immune infiltrate, we performed correlation analyses between the expression level of cytokines, the various immune cell scores, and the clinicopathological variables. The immunohistochemical score for CD3+ T cells showed statistically significant, positive correlations with all other immune cells analyzed except for mast cells (Fig. 2a). There were few significant correlations between the other immune cell subsets. With the exception of CXCL8, CCL20, and interleukin-1β (IL1b), we found numerous positive, significant correlations between the expression levels of the different cytokines analyzed (Fig. 2b). Lymphocyte score (CD3+, CD4+, CD8+, and CD20+ cells) showed significant positive correlation with one or several of the cytokines CCL19, CXCL9, CXCL10, CXCL12, CXCL13, and lymphotoxin b (LTb; Fig. 2c). None of the cytokines was significantly correlated with the number of CD68+ macrophages, DC-lamp+ dendritic cells, or mast cells, suggesting that these cells are regulated by mechanisms distinct from the other studied immune cell populations. Correlation data of the patients' clinicopathological variables with immune cell scores and cytokine expression levels are presented in Table S3. Of note, the group size for many of the variables is small, smoking history and alcohol consumption are patient-reported data, and treatment choice is strongly influenced by tumor stage and comorbidity. Altogether, our results show that high numbers of CD3+ T cells in oral squamous cell carcinoma are correlated with increased infiltration of a number of other immune cells, and suggest that the cytokines CCL19, CXCL12, and LTb may have a prominent role in promoting immune cell infiltration through their association with T cells.

PD-L1 expression in oral squamous cell carcinoma cells correlates with increased infiltration of CD4+ cells and small tumor size

Cancer cells may escape immune surveillance by expressing PD-L1, which induces apoptosis in PD-1 expressing T lymphocytes, most notably cytotoxic T cells. PD-1/PD-L1 checkpoint blockade has shown promising results in various types of cancer, but only subgroups of patients respond to the treatment. To increase the understanding of PD-L1 expression and its association with the immune microenvironment, we analyzed PD-L1 expression in 45 tumors, randomly chosen from the 75 oral squamous cell carcinoma patient cohort, and determined correlation to infiltrating immune cells, cytokine expression, and the presence of high-endothelial venules. In all, 18 (40%) of the 45 tumor sections stained positive for PD-L1. The staining was either

Table 3 Comparison of low vs. high numbers of infiltrating immune cells in oral squamous cell carcinoma patients[a] with low and high PD-L1 scoring using Fisher's exact test

	PD-L1 low No. (%)	PD-L1 high No. (%)	*P*
CD3			
Low	10 (37.0)	4 (22.2)	0.237
High	17 (63.0)	14 (77.8)	
CD4			
Low	21 (80.8)	8 (47.1)	0.025
High	5 (19.2)	9 (52.9)	
CD8			
Low	18 (69.2)	7 (41.2)	0.066
High	8 (30.8)	10 (58.8)	
CD20			
Low	11 (40.7)	9 (50.0)	0.379
High	16 (59.3)	9 (50.0)	
CD68			
Low	15 (55.6)	9 (50.0)	0.475
High	12 (44.4)	9 (50.0)	
DC-lamp			
Low	14 (58.3)	9 (50.0)	0.411
High	10 (41.7)	9 (50.0)	
Eosinophils			
Low	17 (63.0)	8 (44.4)	0.179
High	10 (37.0)	10 (55.6)	
Mast cells			
Low	13 (48.1)	8 (44.4)	0.525
High	14 (51.9)	10 (55.6)	

[a]Analyses for CD4+ and CD8+ cells were carried out in 43 patients and for DC-lamp+ cells in 42 patients. All other analyses included 45 patients

membranous and/or cytoplasmic as demonstrated in Figure S4. We did not assess PD-L1 positivity of stromal cells because of diffuse staining. Tumors with high PD-L1 expression showed increased infiltration of CD4+ and CD8+ cells compared to those with low PD-L1 expression ($P = 0.025$ and 0.066, respectively; Table 3). They also expressed higher levels of a number of cytokines, including CCL19, CCL21, CXCL9, CXCL10, CXCL13, and LTb ($P = 0.006$, $P = 0.010$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P = 0.011$, respectively; Fig. 3). PD-L1 score was not correlated with patients' survival ($P = 0.207$; Table S4). The T3/T4 tumors had significantly lower PD-L1 immune score than the T1/T2 tumors ($P = 0.024$; Table 2). Dividing the T3/T4 tumors into high-endothelial venule-positive and -negative revealed that all positive T3/T4 tumors had low PD-L1 expression, whereas the negative showed no statistically significant difference in PD-L1 expression compared to the T1/T2 tumors ($P = 0.010$ and 0.386, respectively). Our results demonstrate that PD-L1 expression in tumor

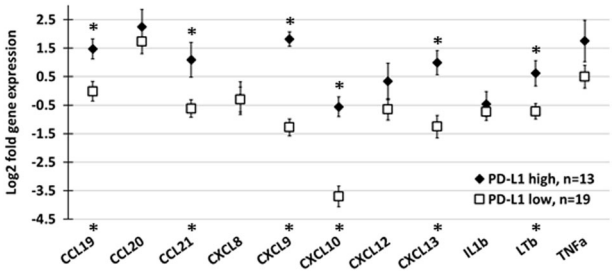

Fig. 3 Comparison of log2-fold gene expression of various chemokines and cytokines in formalin-fixed paraffin-embedded oral squamous cell carcinoma tissue samples with high and low programmed-death ligand 1 (PD-L1) score. Error bars indicate ±standard error of the mean, and $*P < 0.05$. The *P*-value was calculated using two sample *T*-test

cells is associated with a tumor microenvironment rich in CD4+ cells and inflammatory cytokines, and that high-endothelial venules are negatively correlated with PD-L1 in large tumors (T3/T4).

High-endothelial venules are more powerful prognosticators than the N stage and separate components of the oral squamous cell carcinoma immune infiltrate

Many components of the immune infiltrate have been suggested as potential prognosticators for oral cancer, but study results are contradictory. Univariate Kaplan–Meier survival analyses of the immune cell scores and cytokine expression levels in our oral squamous cell carcinoma cohort showed that high numbers of CD20+ B cells and CD68+ macrophages were significant prognosticators for longer 5-year survival ($P = 0.002$ and 0.027, respectively; Fig. 4 and Table S4). We have previously reported that the high-endothelial venule status, the tumor site as well as the T and N stages were significant prognostic factors in the same patient cohort [16]. All variables that were significantly associated with disease-specific death in univariate analyses were entered into multivariate Cox regression analyses. The proportional hazards assumption was fulfilled for all variables (Figure S5 and [16]). Only the T stage ($P < 0.001$) and the high-endothelial venule status ($P = 0.002$) were independent predictors for disease-specific death (Table 4). These results suggest that high-endothelial venules are more important prognostic factors than the N stage and the subsets of the immune infiltrate assessed in this study.

Discussion

In this study, we show that the presence of high-endothelial venules is indicative of a favorable immune

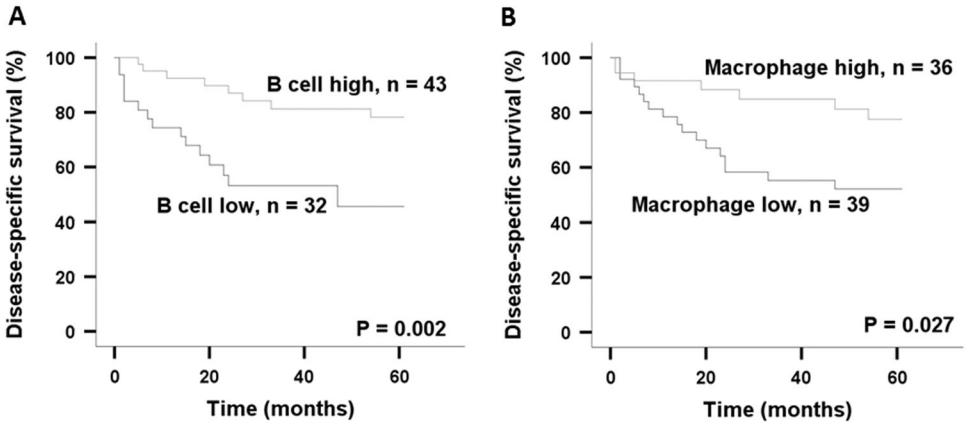

Fig. 4 Kaplan–Meier analysis of 5-year disease-specific survival for patients with oral squamous cell carcinoma. High counts of **a** CD20+ B cells and **b** CD68+ macrophages were associated with improved survival ($P = 0.002$ and 0.027, respectively). The Kaplan–Meier curves show a 5-year disease-specific survival rate of 81.4% for CD20 high vs. 50% for CD20 low tumors (**a**), and 80.6% for CD68 high vs. 56.4% for CD68 low tumors (**b**). The *P*-value was calculated using the log-rank test

Table 4 Multivariate analysis of 5-year disease-specific death according to Cox's proportional hazards model[a]

Variable	Hazard ratio	95% CI	*P*
T stage (T1/T2 ($n = 49$) vs. T3/T4 ($n = 20$))	0.121	0.045–0.321	<0.001
HEV (positive ($n = 63$) vs. negative ($n = 6$))	0.147	0.044–0.490	0.002

[a]Only 69 patients were analyzed because the cases with the unknown T and N stages were taken out from the calculations

microenvironment in oral squamous cell carcinoma, and that these vessels seem to counter-act immunosuppressive mechanisms and improve patient survival irrespective of the tumors' T stage. PD-L1-expressing tumor cells correlated positively to a tumor microenvironment rich in CD4+ cells, but had no prognostic significance. Our results suggest that high-endothelial venules have a pivotal role in shaping an antitumor immune response in oral squamous cell carcinoma, and that PD-1/PD-L1-targeted immunotherapy might be specifically successful in patients with tumors rich in CD4+ cells. To the best of our knowledge, this is the first study to investigate jointly the role of PD-L1 and high-endothelial venules in oral squamous cell carcinoma, two components of the immune infiltrate with important regulatory functions.

High density of tumor-associated high-endothelial venules has earlier been associated with improved survival and inflammation in several cancers, including melanoma, oral, and breast cancers [16, 17, 35], and in the present study, we aimed to determine the composition of the high-endothelial venule associated immune infiltrate in oral squamous cell carcinoma. We found that the high-endothelial venule-positive tumors were heavily infiltrated by CD3+ T cells, CD8+ cytotoxic T cells, and CD20+ B cells, which is in accordance with previous findings in melanoma and breast cancer [17, 18]. Infiltration of CD8+ cytotoxic T cells, and especially a high CD8+/Treg ratio have been associated with a favorable prognosis in a variety of human solid tumors including ovarian, cervical and oral cancer [36–38]. However, of the immune cell subsets analyzed in our study, only increased infiltration of CD20+ B cells and CD68+ macrophages were significantly correlated with improved patient outcome in univariate analyses. The presence of B cells has earlier been associated with positive outcome in head and neck cancer patients [39, 40]. B cells mediate a humoral immune response by producing tumor-specific antibodies, and these cells often localize and cooperate with T cells to facilitate potent, long-term antitumor responses [41]. In contrast to our results, previous studies have mainly found a negative association between macrophages and patient survival in oral cancer [42–44]. The conflicting results may be due to differences in immunohistochemical procedures and scoring. Besides, tumor-associated macrophages appear in different functional states that may vary between tumors and within specific tumor areas [45, 46], and the pan macrophage marker CD68 does not distinguish between these phenotypes. The CD68 antibody, as well as several of the other antibodies used in this study, is not strictly specific for a single cell type. Accurate assessment of immune cell subsets requires multiple markers for each subtype, which is laborious and costly to incorporate in a day-to-day routine clinical practice. In the present study, only the high-endothelial venule- and T-statuses were independent

positive prognosticators for 5-year disease-specific survival. This indicates that these vessels are more relevant as prognostic markers than other components of the tumor microenvironment analyzed, and strengthens their role as a potent surrogate marker of an effective antitumor immune response. Immunohistochemical detection of high-endothelial venules is simple and reliable [16], and implementation in clinical pathology practice could thus be straightforward.

Chemokines in and around high-endothelial venules are thought to be crucial for lymphocyte extravasation into lymphoid organs [47]. In the present study, the gene expression of the lymphoid chemokines CXCL12, CCL19, and CCL21 was upregulated in the high-endothelial venule-positive tumors. Expression of CXCL12 is an important attractant for naive T and B cells [48, 49], and has a potential role in high-endothelial venule-mediated T-cell trafficking into lymph nodes in vitro [50]. CCL19 and CCL21 interact with CCR7 on lymphocytes, and trigger efficient T-cell homing. Thus, increased expression of CCL19, CCL21, and CXCL12 in high-endothelial venule-positive tumors supports an important role for these vessels in homing of naive T cells into oral squamous cell carcinoma. Besides their role in lymphocyte homing, CCL19 and CCL21 stimulate migration and maturation of dendritic cells [51]. Through LT expression, dendritic cells promote maintenance of a mature high-endothelial venule phenotype [52]. Therefore, downregulation of CCL21 in T3/T4 tumors may be indicative of a tumor microenvironment with less infiltrating mature dendritic cells, causing dedifferentiation of high-endothelial venules into normal blood vessels [19, 20]. However, we did not find a significant association between DC-lamp, a marker for mature dendritic cells, and high-endothelial venule score. To further study the association between dendritic cells and these vessels, we also performed high-endothelial venule/dendritic cell double staining, but faced technical difficulties (data not shown). In future studies, assessing markers for tumor angiogenesis could be a valuable supplement to investigate high-endothelial venule plasticity, and to shed light on development and maintenance of these vessels in advanced tumors. Furthermore, validating the results of the present study in a larger patient cohort with a higher number of high-endothelial venule-negative tumors is important, as the low number of these tumors in the present study may skew the statistical results.

High-endothelial venule-negative tumors expressed higher levels of the chemokine CCL20 than -positive tumors. CCL20 attracts cells expressing the CCR6 receptor, such as dendritic cells and memory and effector T cells. Several studies have found that CCL20 is a chief attractant of Treg cells, a distinct lineage of CD4+ T cells that suppresses antitumor immune responses [53–55]. Interestingly, tumor cells may also express CCR6, and accordingly, CCL20 has been associated with migration and metastases of cancer cells [56]. We did not specifically stain for Treg cells nor CCR6, but speculate that the downregulation of CCL20 in high-endothelial venule-positive oral squamous cell carcinomas could help sustain an antitumor immune response by avoiding recruitment of Treg cells. This would be interesting to investigate in future studies. In summary, we show that both the early and the advanced high-endothelial venule-positive tumors displayed higher levels of tumor-suppressive components of the immune infiltrate than the negative. Along with the vessels' independent prediction of improved survival, this suggests that high-endothelial venules promote a tumor-suppressive immune response irrespective of the tumors' T stage.

Tumor progression often correlates with immune evasion. In several cancers, including oral squamous cell carcinoma, immune suppression may be facilitated by PD-L1 that inhibits T-cell functions [22, 23]. In the present study, tumors with high PD-L1 score showed a significant increase in CD4+ cell infiltration ($P = 0.025$). Increased numbers of immunosuppressive CD4+ Treg cells, as well as dysfunctional T lymphocytes have previously been found to predict immunosuppressive properties in oral cancer patients [57–59]. We also found a borderline significant association between PD-L1+ tumor cells and infiltrating CD8+ cells ($P = 0.066$), as well as the expression level of a number of inflammation-related cytokines. This suggests that PD-L1 expression by itself does not lead to a non-inflamed tumor environment, but may influence the efficacy of the immune reaction. This is in accordance with several other studies that report PD-L1 expression to be associated with cytotoxic T cells that can induce PD-L1 expression in an interferon (IFN)-γ dependent manner [60–62]. CXCL9 is an IFN-γ-inducible chemokine, and we found a perfect linear relationship between PD-L1 score and CXCL9 expression ($r = 1$; data not shown). Human papilloma virus (HPV)/p16-positive tumors had significantly lower CXCL9 expression than HPV/p16-negative tumors. A correlation between HPV-positivity and PD-L1 expression has previously been reported in oropharyngeal cancer [63]. Due to the low number of HPV/p16-positive cancers in our study, the correlation between the virus, CXCL9, and PD-L1 expression in oral cancer needs validation in a study with a higher amount of HPV-positive patients.

Tumors in the floor of the mouth and tumors of cigarette smokers showed significantly lower PD-L1 expression than tumors at other subsites in the oral cavity and tumors of patients who were non-smokers. In the same patient cohort, we have previously reported a correlation between alcohol and tobacco consumption and tumors in the floor of the mouth [64]. Furthermore, tumors in the floor of the mouth expressed less CCL21 than tumors in the other anatomical

sites (Table S5), and tumors with low PD-L1 expression expressed lower levels of CCL21 than tumors with strong PD-L1 expression (Fig. 3). Whether there is a direct effect of CCL21 and/or tobacco on PD-L1 expression would be interesting to investigate in future studies. Interestingly, high PD-L1 expression was also correlated to a lower T stage ($P = 0.037$), and we found a significantly lower score of PD-L1-expressing tumor cells in the high-endothelial venule-positive T3/T4 compared to the T1/T2 tumors ($P = 0.010$). This may suggest that high-endothelial venule-positive large tumors have overcome PD-L1-mediated immunosuppression, which could contribute to the increased survival of patients with high-endothelial venule-positive T3/T4 tumors. PD-1/PD-L1 blockade is a new immunotherapeutic approach in the combat against cancer, and the clinical success of this treatment correlates to some extent with the tumors' PD-L1 expression [25–27]. It can be speculated that PD-L1-expressing T1/T2 tumors and high-endothelial venule-negative T3/T4 tumors with marked T-cell infiltration are good candidates for PD-1/PD-L1 checkpoint-targeting therapies.

In conclusion, we show that high-endothelial venules are markers of a favorable antitumor immune microenvironment in oral squamous cell carcinoma, and stronger prognosticators than other subsets of the immune infiltrate. As detection of these vessels is easy and reliable, high-endothelial venule status may serve as a valuable supplement to stratify oral squamous cell carcinoma patients for targeted therapeutic approaches. Oral squamous cell carcinomas are generally immunosuppressive tumors with poor patient outcome. Understanding the mechanisms that drive immune cell recruitment and generate effective antitumor responses may provide opportunities to develop new immunomodulatory targets and thereby increase the consistently low survival rates of patients with oral cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary information

Supplementary tables

Table S1. Spearman correlation coefficients for inter-observer agreement among EHO and AMW. Correlation coefficients were interpreted as follows: 0.7-0.9, good agreement; 0.9-1.0 very good agreement.

Category	Spearman correlation coefficient	Interpretation
CD3+ cells	0.878	Good
CD4+ cells	0.905	Very good
CD8+ cells	0.868	Good
CD20+ cells	0.872	Good
CD68+ cells	0.724	Good
Eosinophils	0.817	Good
Mast cells	0.813	Good

Table S2. Accession numbers and primer sequences of reference and target RNAs.

Gene	Accession no.	Full name	Primer sequence (5' to 3')	Size (bp)
Reference RNA				
eF1a	NM001402.5	Elongation factor 1 alpha	F: TATCCACCTTTGGGTCGCTTT R: TGATGACACCCACCGCAACT	63
RPL27	NM000988.3	Ribosomal protein L27	F: GCTGGACGCTACTCCGGAC R: CGATCTGAGGTGCCATCATCA	64
RPS13	NM001017.2	Ribosomal protein S13	F: AGAGAGCCGGATTCACCGTTT R: CAATTGGGAGGGAGGACTCG	62
Target RNA				
CCL19	NM006274.2	C-C motif chemokine ligand 19	F: CCGGAGTCCGAGTCAAGCA R: CCTTCCTTCTGGTCCTCGGTT	64
CCL20	NM001130046.1 NM004591.2	C-C motif chemokine ligand 20	F: TTTTCTGGAATGGAATTGGACA R: AAACCTCCAACCCCAGCAA	62
CCL21	NM002989.3 XM 011518004.2	C-C motif chemokine ligand 21	F: GCAGCTACCGGAAGCAGGA R: GGGCAAGAACAGGATAGCTGG	61
CXCL8	NM000584.3	C-C motif chemokine ligand 8	F: CTCCAAACCTTTCCACCCCA R: CGCAGTGTGGTCCACTCTCA	64
CXCL9	NM002416.2	C-C motif chemokine ligand 9	F: TGCTGGTTCTGATTGGAGTGC R: TGATGCAGGAACAGCGACC	62
CXCL12	NM000609.6 L36033.1 NM001277990.1	CXC motif chemokine ligand 12	F: GCCTGAGCTACAGATGCCCAT R: GCTTGACGTTGGCTCTGGC	62

	NM001033886.2			
	NM199168.3			
	U16752.1			
CXCL13	NM006419.2	CXC motif chemokine ligand 13	F: CCCTGATGCTGATATTTCCACTAAG	60
			R: AATCCAGAGCAGGGATAAGGGA	
IL1b	XM017003988.1	Interleukin 1 beta	F: AGTCTGCCCAGTTCCCCAAC	60
	NM000576.2		R: AAGACGGGCATGTTTTCTGCT	
LTb	NM009588.1	Lymphotoxin beta	F: GTCACCCCGATATGGTGGACT	78
	NM002341.1		R: GCACTCATATTCCCTCACCCC	
TNFa	NM000594.3	Tumor necrosis factor alpha	F: CACCACTTCGAAACCTGGGA	60
			R: TGGTTGCCAGCACTTCACTG	

Table S3. Comparison of clinicopathologic variables between oral squamous cell carcinoma patients* with low and high numbers of various immune cells using Pearson's Chi-square test.

	CD3*			**CD4**			**CD8**			**CD20**			**CD68**			**DC-lamp**			**Eosinophils**			**Mast cells**			**PD-L1+ cells**		
	low	high		low	high		low	high		low	high		low	high		low	high		low	high		low	high		low	high	
	no		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*
Gender																											
Male	11	31	0.632	27	14	0.645	24	17	0.558	19	24	0.758	26	17	0.089	19	19	0.504	27	15	0.217	23	19	0.501	17	10	0.619
Female	10	22		22	9		16	15		13	19		13	19		18	13		16	16		15	17		10	8	
Age at diagnosis, years																											
0-59	7	21	0.615	17	11	0.287	13	15	0.214	12	16	0.979	10	18	**0.029**	14	12	0.977	18	10	0.401	16	12	0.437	13	4	0.079
≥ 60	14	32		32	12		27	17		20	27		29	18		23	20		25	21		22	24		14	14	
Smoking history																											
Never smoker	2	15	0.178	12	5	0.306	9	8	0.946	3	14	0.053	5	12	0.127	7	9	0.376	9	8	0.771	6	11	0.480	7	3	**0.022**
Former smoker	3	7		7	3		5	5		5	5		5	5		3	5		6	4		5	5		1	3	
Current smoker	13	29		29	12		24	17		20	23		25	18		23	17		26	16		24	18		19	8	
Unknown	3	2		1	3		2	2		4	1		4	1		4	1		2	3		3	2		0	4	
Alcohol consumption																											
Never	2	9	0.541	10	1	0.108	8	3	**0.005**	4	8	0.567	5	7	0.168	5	5	0.548	7	4	0.793	5	6	**0.007**	4	2	0.079
≤ 1 times weekly	6	21		15	12		8	19		10	17		14	13		11	13		15	12		8	19		6	8	
> 1 times weekly or daily	7	13		15	4		15	4		9	11		8	12		10	9		13	7		16	4		10	1	
Unknown	6	10		9	6		9	6		9	7		12	4		11	5		8	8		9	7		7	7	
Tumor site																											
Mobile tongue	8	26	0.588	23	10	0.373	21	12	0.090	13	22	0.299	15	20	0.310	15	16	0.733	18	16	0.605	15	19	0.517	9	9	**0.017**
Floor of mouth	6	15		16	5		13	8		8	13		12	9		11	8		14	7		12	9		12	1	
All others**	7	12		10	8		6	12		11	8		12	7		11	8		11	8		11	8		6	8	
Tumor differentiation																											
Well	6	22	0.208	19	8	0.225	17	10	0.239	10	18	0.527	15	13	0.894	11	15	0.085	15	13	0.826	11	17	0.269	10	8	0.610
Moderate	12	29		25	15		19	21		19	23		21	21		22	17		25	16		24 (	17		13	9	
Poor	3	2		5	0		4	1		3	2		3	2		4	0		3	2		3	2		4	1	
T stage																											
T1/T2	11	41	0.062	34	18	0.632	28	24	0.416	20	33	0.215	22	31	**0.005**	25	22	0.644	26	26	0.084	25	27	0.330	15	15	**0.037**
T3/T4	10	11		14	5		12	7		12	9		17	4		11	10		16	5		13	8		12	2	
Unknown	0	1		1	0		0	1		0	1		0	1		1	0		1	0		0	1		0	1	
N stage																											
N0	15	35	**0.030**	31	18	0.424	26	23	0.679	21	30	0.457	26	25	0.483	25	22	0.428	27	23	0.371	25	25	0.736	16	12	0.853
N+	2	16		13)	4		11	6		7	11		11	7		8	9		11	7		9	9		8	4	
Unknown	4	2		5	1		3	3		4	2		2	4		4	1		5	1		4	2		3	2	
M stage																											
M0	18	50	0.226	44	22	0.652	38	28	0.397	28	41	0.353	38	31	0.183	32	31	0.291	38	30	0.398	34	34	0.564	24	16	0.659
M+	0	1		1	0		0	1		1	0		0	1		1	0		1	0		1	0		1	0	
Unknown	3	2		4	1		2	3		3	2		1	4		4	1		4	1		3	2		2	2	
HPV/p16																											
Negative	16	48	0.265	43	21	0.133	35	29	0.401	26	39	0.430	31	34	0.096	31	29	0.141	36	28	0.691	33	31	0.996	22	15	0.095
Positive	3	3		5	0		4	1		4	2		4	2		2	3		4	2		3	3		4	0	
Unknown	2	2		1	2		1	2		2	2		4	0		4	0		3	1		2	2		1	3	

* Analyses for CD3+ cells, eosinophils and mast cells were carried out in 74 patients, for CD4+ and CD8+ cells in 72 patients, for DC-lamp+ cells in 69 patients, and for PD-L1+ cells in 45 patients. All other analyses included 75 patients.

****** All other anatomical sites includes the following anatomical subsites: Alveolar ridge (n= 18); buccal mucosa (n= 10); unspecified oral cavity (n= 3).

Table S4. Infiltration of different immune cell subsets* and expression of various genes** in oral squamous cell carcinoma patients as predictors for 5-year disease-specific death in univariate Kaplan-Meier analysis.

	Patients (no. (%))	5-Year DSD (%)	*P*
CD3			
Low	21 (28.4)	42.9	0.200
High	53 (71.6)	28.3	
CD4			
Low	49 (68.1)	32.7	0.691
High	23 (31.9)	26.1	
CD8			
Low	40 (55.6)	25.0	0.304
High	32 (44.4)	37.5	
CD20			
Low	32 (42.7)	50.0	**0.002**
High	43 (57.3)	18.6	
CD68			
Low	39 (52.0)	43.6	**0.027**
High	36 (48.0)	19.4	
DC-lamp			
Low	37 (53.6)	37.8	0.639
High	32 (46.4)	31.2	
Eosinophils			
Low	43 (58.1)	37.2	0.187
High	31 (41.9)	25.8	
Mast cells			
Low	38 (51.4)	34.2	0.625
High	36 (48.6)	30.6	
PD-L1+ cells			
Low	27 (60.0)	37.0	0.207
High	18 (40.0)	22.2	
CCL19			
Low	20 (50.0)	25.0	0.592
High	20 (50.0)	40.0	
CCL20			
Low	13 (40.6)	23.1	0.198
High	19 (59.4)	47.4	
CCL21			
Low	24 (66.7)	29.2	0.876
High	12 (33.3)	33.3	
CXCL8			
Low	32 (76.2)	34.4	0.865
High	10 (23.8)	30.0	
CXCL9			
Low	19 (52.8)	26.3	0.724
High	17 (47.2)	35.3	
CXCL10			
Low	35 (89.7)	34.3	0.607
High	4 (10.3)	25.0	
CXCL12			
Low	27 (64.3)	40.7	0.120
High	15 (35.7)	20.0	
CXCL13			
Low	28 (70.0)	39.3	0.367
High	12 (30.0)	25.0	
IL1b			
Low	33 (78.6)	33.3	0.759
High	9 (21.4)	22.2	
LTb			
Low	30 (73.2)	40.0	0.157
High	11 (26.8)	18.2	
TNFa			
Low	20 (51.3)	40.0	0.349
High	19 (48.7)	31.6	

* Analyses for CD3+ cells, eosinophils and mast cells were carried out in 74 patients, for CD4+ and CD8+ cells in 72 patients, for DC-lamp+ cells in 69 patients and for PD-L1+ cells in 45 patients. All other analyses included 75 patients.

** The number of patients included in the different analyses varied dependent on availability of patient material.

Table S5. Comparison of clinicopathological variables between oral squamous cell carcinoma patients* with low and high expression of various genes using Pearson's Chi-square test.

	CCL19			**CCL20**			**CCL21**			**CXCL8**			**CXCL9**			**CXCL10**		
	low	high		low	high		low	high		low	high		low	high		low	high	
	no		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*
Gender																		
Male	10	16	**0.047**	8	11	0.837	14	11	**0.041**	21	6	0.746	11	12	0.429	22	3	0.632
Female	10	4		5	8		10	1		11	4		8	5		13	1	
Age at diagnosis, years																		
0-59	5	10	0.102	5	7	0.926	8	6	0.334	10	5	0.280	6	7	0.549	10	4	**0.005**
≥ 60	15	10		8	12		16	6		22	5		13	10		25	0	
Smoking history																		
Never smoker	4	4	0.764	2	6	0.727	6	2	0.392	8	1	0.391	5	3	0.090	7	2	0.550
Former smoker	1	1		1	1		2	0		2	0		0	2		2	0	
Current smoker	12	14		9	10		14	10		20	7		14	9		23	2	
Unknown	3	1		1	2		2	0		2	2		0	3		3	0	
Alcohol consumption																		
Never	2	1	0.582	0	3	0.480	3	1	0.968	3	1	0.172	2	1	0.849	4	0	0.197
≤ 1 times weekly	5	9		5	5		8	5		13	1		6	7		9	3	
> 1 times weekly or daily	6	4		4	5		7	3		6	5		5	3		10	1	
Unknown	7	6		4	6		7	3		10	3		6	6		12	0	
Tumor site																		
Mobile tongue	7	9	0.518	5	7	0.341	7	9	**0.024**	13	4	0.929	8	8	0.211	14	3	0.355
Floor of the mouth	7	8		6	5		12	3		11	4		9	4		13	1	
All others**	6	3		2	7		5	0		8	2		2	5		8	0	
Tumor differentiation																		
Well	10	10	0.329	8	8	0.450	11	6	0.589	16	5	0.658	11	8	0.519	19	1	0.399
Moderate	8	10		5	10		11	6		15	4		8	9		14	3	
Poor	2	0		0	1		2	0		1	1		0	0		2	0	
T stage																		
T1/T2	13	15	0.490	11	9	0.096	15	11	0.066	22	6	0.688	13	13	0.590	22	4	0.328
T3/T4	7	5		2	9		9	1		9	4		6	4		12	0	
Unknown	0	0		0	1		0	0		1	0		0	0		1	0	
N stage																		
N0	14	14	0.135	10	12	0.705	16	9	0.440	21	7	0.949	14	12	0.979	22	4	0.328
N+	3	6		2	5		5	3		8	2		4	4		9	0	
Unknown	3	0		1	2		3	0		3	1		1	1		4	0	
M stage																		
M0	17	20	0.198	12	17	0.787	22	11	0.223	29	9	0.793	17	16	0.631	31	4	0.775
M+	1	0		0	0		0	1		1	0		1	0		1	0	
Unknown	2	0		1	2		2	0		2	1		1	1		3	0	
HPV/p16																		
Negative	15	18	0.100	12	14	0.349	21	10	0.875	27	7	0.587	16	14	**0.049**	27	4	0.563
Positive	4	0		1	3		2	1		3	2		3	0		5	0	
Unknown	1	2		0	2		1	1		2	1		0	3		3	0	

	CXCL12			CXCL13			IL1b			LTb			TNFa		
	low	high		low	high		low	high		low	high		low	high	
	no		*P*	*no*		*P*	*no*		*P*	*no*		*P*	*no*		*P*
Gender															
Male	15	12	0.113	18	8	0.885	23	4	0.161	18	8	0.453	13	13	0.821
Female	12	3		10	4		10	5		12	3		7	6	
Age at diagnosis, years															
0-59	6	9	**0.014**	9	5	0.563	10	5	0.161	8	7	**0.029**	8	7	0.839
≥ 60	21	6		19	7		23	4		22	4		12	12	
Smoking history															
Never smoker	6	3	0.236	5	4	0.071	7	2	0.899	5	4	0.305	4	4	0.927
Former smoker	2	0		0	2		2	0		2	0		1	1	
Current smoker	15	12		21	5		21	6		19	7		14	12	
Unknown	4	0		2	1		3	1		4	0		1	2	
Alcohol consumption															
Never	2	2	0.416	2	2	0.660	2	2	0.412	2	2	0.559	2	1	0.924
≤ 1 times weekly	7	7		9	5		12	2		9	4		7	6	
> 1 times weekly or daily	8	3		8	2		8	3		8	3		5	6	
Unknown	10	3		9	3		11	2		11	2		6	6	
Tumor site															
Mobile tongue	9	8	0.356	11	6	0.251	12	5	0.538	10	7	0.208	7	9	0.150
Floor of the mouth	10	5		12	2		13	2		12	2		10	4	
All others**	8	2		5	4		8	2		8	2		3	6	
Tumor differentiation															
Well	13	8	0.556	14	5	0.496	15	6	0.229	16	5	0.544	10	9	0.987
Moderate	12	7		12	7		17	2		12	6		9	9	
Poor	2	0		2	0		1	1		2	0		1	1	
T stage															
T1/T2	16	12	0.092	19	8	0.290	23	5	0.142	18	9	0.057	14	11	0.494
T3/T4	11	2		9	3		10	3		12	1		6	7	
Unknown	0	1		0	1		0	1		0	1		0	1	
N stage															
N0	16	12	0.387	18	9	0.799	22	6	0.257	18	9	0.362	14	11	0.513
N+	8	2		7	2		9	1		9	1		5	5	
Unknown	3	1		3	1		2	2		3	1		1	3	
M stage															
M0	24	14	0.747	25	11	0.799	31	7	0.127	27	10	0.806	18	17	0.512
M+	1	0		1	0		1	0		1	0		1	0	
Unknown	2	1		2	1		1	2		2	1		1	2	
HPV/p16															
Negative	20	14	0.266	23	9	0.331	29	5	0.061	24	10	0.616	18	14	0.262
Positive	4	1		4	1		2	3		4	1		2	3	
Unknown	3	0		1	2		2	1		2	0		0	2	

* The number of patients included in the different analyses varied dependent on availability of patient material

** All other anatomical sites includes the following anatomical subsites: Alveolar ridge (n= 18); buccal mucosa (n= 10); unspecified oral cavity (n= 3).

Supplementary figures

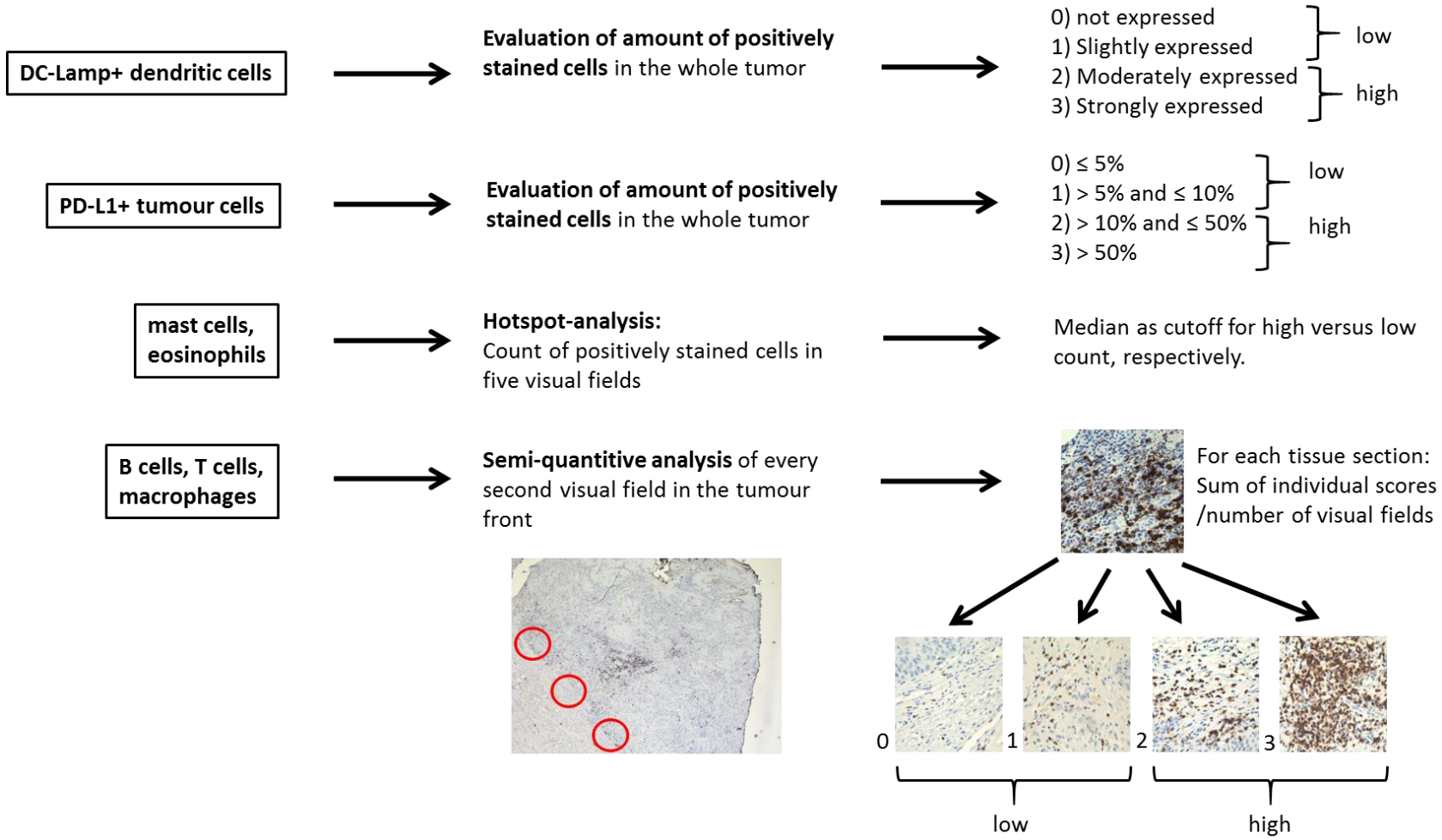

Figure S1. Flow chart of scoring evaluation of various components of the immune infiltrate.

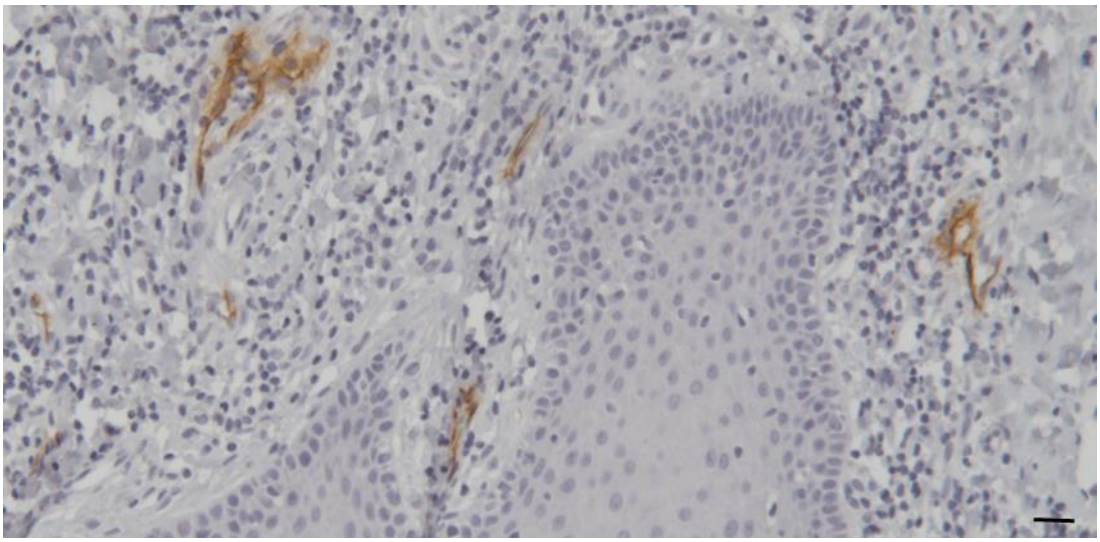

Figure S2. Representative immunohistochemical Peripheral node adressin staining for high-endothelial vessels in an oral squamous cell carcinoma tissue section. Peripheral node adressin staining is brown, and cell nuclei are stained blue by hematoxylin. Scale bars indicates 40 μm.

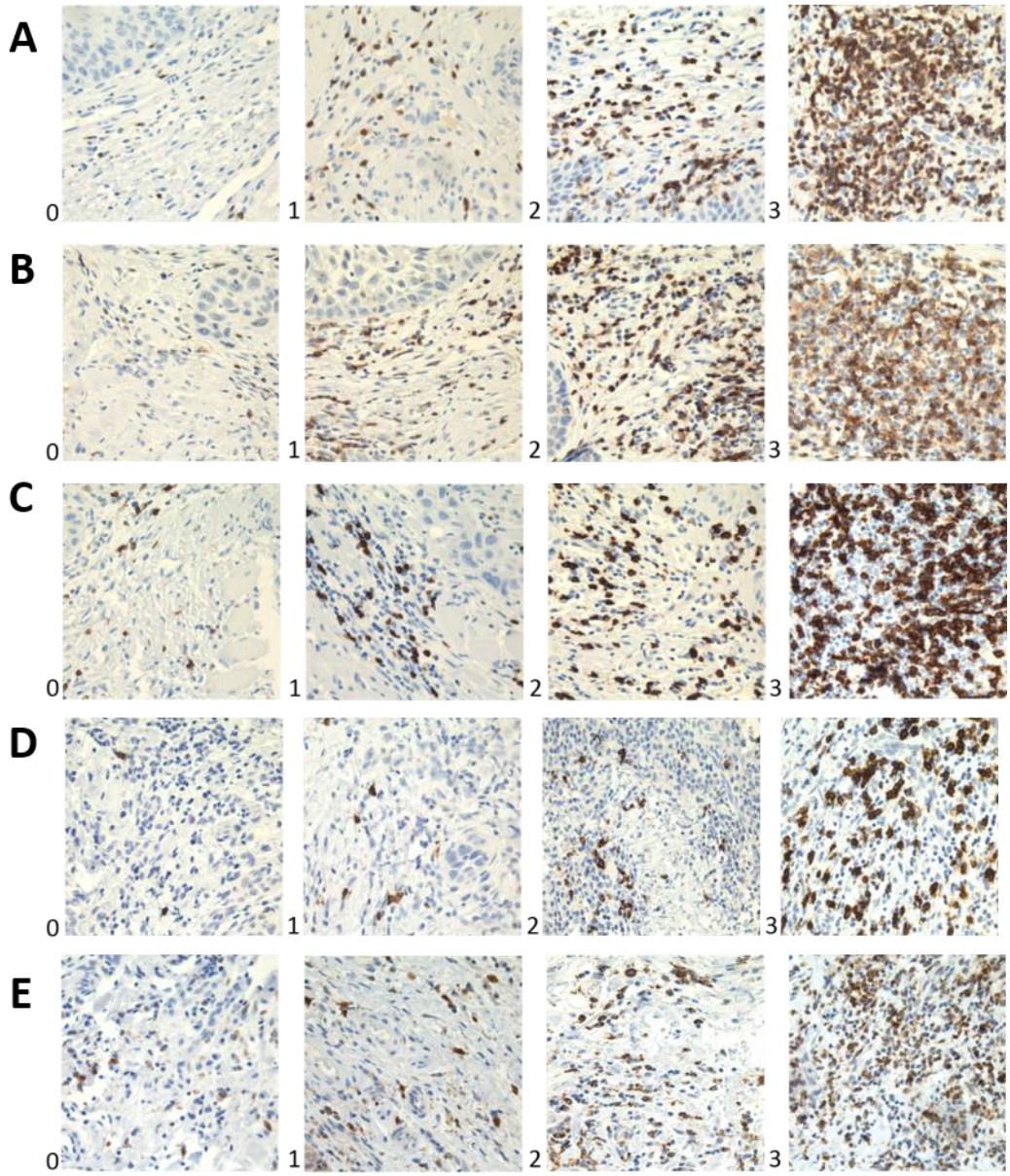

Figure S3. Four-degree scoring scale for semi-quantitative evaluation of different immunohistochemical stainings. A) CD3+ cells, B) CD4+ cells, C) CD8+ cells, D) CD20+ cells, E) CD68+ cells. Micrographs of the respective immunohistochemically stained cells were assigned to groups 0-3 of the respective scoring schemes. Groups 0-1 were evaluated as low and groups 2-3 as high count, respectively.

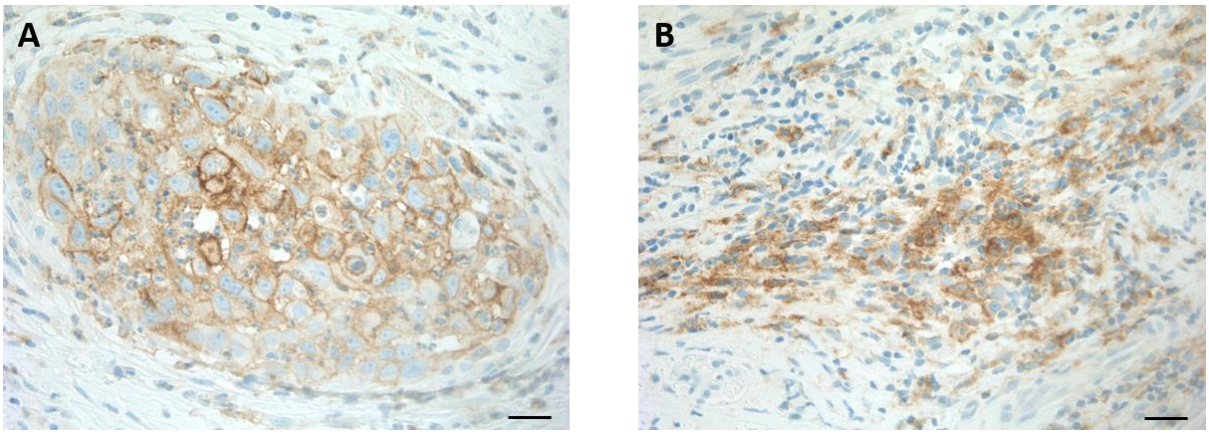

Figure S4. Representative immunohistochemical PD-L1 staining of A) tumor cells and B) stromal cells in an oral squamous cell carcinoma tissue section. PD-L1+ cells express membranous and/or cytoplasmatic brown staining, and cell nuclei are stained blue by hematoxylin. Scale bars indicate 40 μm.

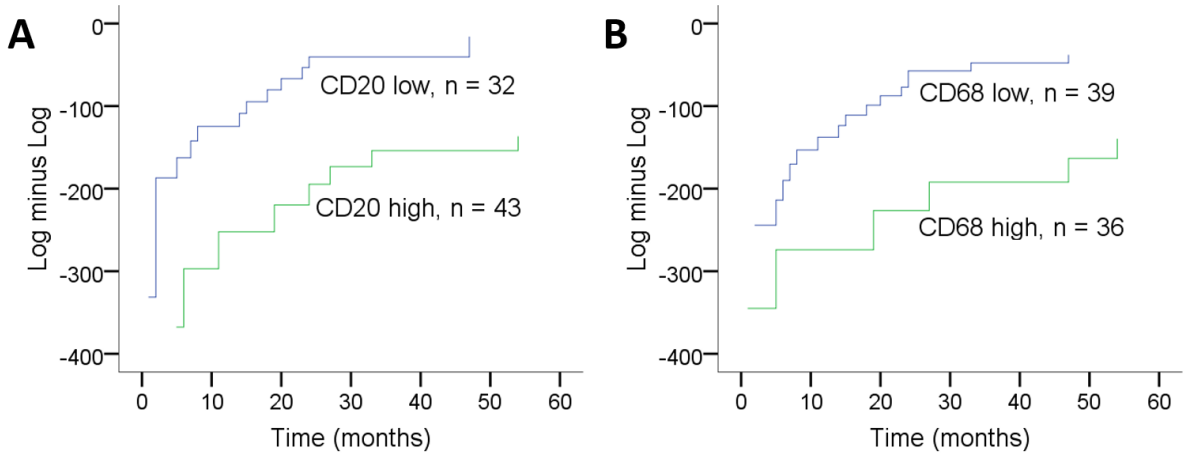

Figure S5. Log minus log plots for proportional hazards checking. A) CD20+ cells, B) CD68+ cells**.**

Paper IV

Tissue-infiltrating immune cells as prognostic markers in oral squamous cell carcinoma – a systematic review

Running title: Immune cells as prognostic markers in oral cancer

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Abstract

Background: A variety of immune cells have been suggested as prognostic markers for cancer patients. In this article, we present a systematic review of the existing literature on the prognostic value of tissue-infiltrating immune cells in oral cancer, and discuss the quality of the methodological reporting in the published studies.

Methods: We performed a systematic literature search and included all studies with 40 or more oral squamous cell carcinoma patients where the prognostic value of tumour-associated T cells, B cells, macrophages, dendritic cells, mast cells and natural killer cells were assessed by immunohistochemical staining and survival analyses. We also investigated to what extent the articles adhered to the reporting recommendations for tumour marker prognostic studies (REMARK).

Results: Of the 1960 articles identified, 33 passed our inclusion criteria. Among the immune cells analysed, CD163+ M2 macrophages, and CD57+ mature natural killer cells were the most promising predictors of survival in OSCC patients. The reporting of study design and results was heterogeneous among the published articles.

Conclusion: Despite the large amount of immune biomarker studies in oral cancer, the reliability and prognostic relevance of the suggested markers are debatable due to incomplete information on the conduct of most of the studies.

Background

Squamous cell carcinomas (SCC) account for the vast majority of oral (O) cancer[1]. Surgery is the standard treatment for these tumours, often accompanied by radiotherapy. The radiation causes severe, chronic side effects, including xerostomia and problems with speech, oral intake and dental health, making it important to avoid overtreatment[2]. Currently, the TNM classification system that stages cancers according to the tumour size and depth of invasion (T), the presence and extent of regional lymph node metastases (N), and the presence of distant metastases (M) is the most reliable prognostic and predictive factor for OSCC patients[3]. For analytical purposes, the individual TNM categories can be grouped into stages I-IV reflecting improved survival for patients with early- compared to advanced-stage tumours[3]. Nevertheless, tumours of the same stage are heterogeneous with respect to aggressiveness and therapy response. Thus, to better tailor the treatment to the patient's need, the TNM classification needs reinforcement with biomarkers that more reliably reflect the biological diversity of these tumours.

The promising results of immuno-modulating therapies, such as PD1/PD-L1 blocking antibodies demonstrate that the immune system is significantly involved in tumour progression [4, 5], and have boosted the interest in tumour immunology. A variety of infiltrating immune cells have been presented as putative prognostic markers in cancer. However, the immune system is a complex regulator of tumour development and progression, and infiltrating immune cells have been shown to exert variable roles in different types of cancer, as well as within tumours of the same type and at different sub locations of a single tumour[6, 7]. So far, none of the suggested OSCC immune-biomarkers have been generally accepted and implemented in clinical practice.

Recognizing the involvement of the immune system in cancer and the need of reliable prognostic markers for OSCC, we decided to conduct a systematic review of studies assessing the prognostic value of tissue-infiltrating immune cells in OSCC by survival analyses. We included studies employing immunohistochemistry to detect one or several of the following immune cells: T cells, B cells, macrophages, dendritic cells (DC), mast cells and natural killer (NK) cells. In addition to reviewing the prognostic value of the immune cells, we assessed to what extent clinical and pathological data as well as immunohistochemical staining and scoring procedures were adequately described, according to the reporting recommendations for tumour marker prognostic studies (REMARK)[8]. A critical evaluation of the reporting quality will help to assess the reliability of the suggested prognostic markers, and may guide research in the right direction.

Methods

Eligibility criteria

Included were original articles that fulfilled all of the following criteria:

- were written in English
- presented data from patients with SCC in the oral cavity proper that were not pooled with patients having cancer in other locations of the head- and neck area
- analysed tissue that had not been previously exposed to radiotherapy and/or chemotherapy
- used immunohistochemistry on tumour tissue sections to recognize 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
- employed some kind of survival as end-point in the survival analyses

Sources of information, search terms and screening

We searched Embase and Medline 14th of March 2018 using the Ovid interface with the search terms described in Figure 1. If applicable, our entry terms were defined based on Medical Subject Headings (MeSH) from Pubmed[9] to develop a controlled vocabulary. In addition to MeSH terms, we included relevant free-text entry terms. We defined three sets of entry terms describing 1. prognostic value, 2. selected immune cells, and 3. oral cancer, and combined the search terms in each set with the Boolean operator OR. The three sets were then combined with the Boolean operator AND. In addition, we searched the Cochrane Library and the reference lists of reviews on related topics[4, 10-14] to look for relevant papers missed in the Medline and Embase searches. In Medline and Embase, we automatically

discarded duplicate articles, all non-English literature and non-human studies. The two review authors independently read through the titles and abstracts of the remaining articles to extract those relevant. For all papers that appeared to meet the inclusion criteria, or where there were any uncertainty, the full text papers were screened. We designed a flow diagram with the excluded cases (Figure 2) adherent to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA)[15].

Data collection

For all studies included, we retrieved information about the name of the first author, year of publication, number of patients, the tumour sizes or stages included, the country and period during which the patient cohort was gathered, the tumour compartment analysed for immune cell infiltration, the primary antibody used and survival data. If the papers reported survival data over time, e.g. by Kaplan-Meier curves, we recorded whether tumour infiltration of the various immune cells were associated with longer or shorter survival, independent of the statistical significance of the presented results. We further assessed to what extent the papers reported immunohistochemical staining and scoring protocols with sufficient details, as recommended in the REMARK guidelines[8], as presented in an adapted checklist in the legend of Table 1. Due to lack of sufficient statistical data from many of the papers, along with variations in use of cellular markers and scoring protocols, meta-analyses were not performed.

Quantitative analyses

We assigned a quality score to each article based on the sum of scoring for reporting of immunohistochemical staining and scoring procedures that we retrieved from our checklist, with C/c=1, B/b=2, and A/a=3 (Table 1). We explored if there was an association between the quality score and publication year, and additionally, if studies that used IHC as the only method were associated with a

higher quality score than studies employing a broader repertoire of methods. The SPSS software version 22.0 for Windows (IBM, Armonk, NY) was used to perform bivariate correlation analyses, and the Pearson correlation coefficient as well as the P-value were reported. $P<0.05$ was determined significant.

Results

After screening the 1960 records identified in our search (excluding duplicates), we were left with 33 studies that passed our inclusion criteria (Figure 2). Table 1 summarizes the studies included in this review, and Tables 2-4 present the data for the different immune cell subsets.

Prognostic value of different immune cell subsets

Macrophages

We found 11 evaluating the prognostic value of tumour associated macrophages by staining for the pan-macrophage marker CD68[16-26] and seven using the M2 macrophage marker CD163[18, 19, 22, 23, 27-30] (Table 2). The majority of the studies reported lower survival for patients with a high number of macrophages in at least one of the tumour compartments analysed. The results were most consistent and more often statistically significant for studies using CD163 as the macrophage marker than for those using CD68, with four studies reporting CD163 as an independent negative prognostic marker. A few studies scored macrophages infiltrating the tumour islands and the tumour stroma separately, and found that the effect on survival differed between the compartments. However, there was no consistency in subsite specific effect of macrophage infiltration between these studies (Table 2).

Dendritic cells

Seven different studies assessed the prognostic value of DC, using seven different markers[24, 31-36] (Table 3). High numbers of both immature CD209 +DC and plasmacytoid CD123+ DC were associated with decreased survival in one study, whereas high levels of the other subsets of DC tended to be mostly associated with improved survival, although the results often lacked statistical significance. Studies

that included tumour subsite specific survival analyses of DC infiltration showed contradictory results (Table 3).

Mast cells

Only two studies of mast cells passed our inclusion criteria, both using mast cell tryptase as the cell marker[37, 38] (Table 3). One of these found a statistically significant negative effect of high mast cell numbers on survival in univariate analyses, the other found no statistically significant effect of mast cell number on survival, and did not report the direction of effect.

Lymphocytes

We found four studies evaluating the prognostic value of NK cells that passed our inclusion criteria[26, 34, 39, 40] (Table 3). The only study that used CD56 as a marker, found no effect on survival, whereas two of the three studies using CD57 as the NK cell marker reported a statistically significant, independent survival benefit for patients with a high number of such cells.

Thirteen different studies assessed various subsets of T cells [19, 24, 26, 29, 34, 40-47] (Table 4). High numbers of tumour infiltrating CD3+ T cells, CD4+ T cells or CD8+ T cells were usually associated with somewhat longer survival. However, statistically significant results were only reported for CD8+ T cells in three studies. High numbers of tumour associated T-regulatory cells on the other hand, showed significant association with decreased survival in two of the five studies addressing this cell type.

B cells were analysed in five studies using four different markers[19, 24, 44, 47, 48] (Table 4). B cells overall, recognized by the pan B-cell markers CD19 and CD20 were mostly associated with survival benefits, although statistically significant in only two of the four studies. The single study assessing plasma cells did not report the direction of effect on survival, but it was not statistically significant. B-regulatory

cells were significantly associated with decreased survival in a single study, but were not independent markers.

Reporting of immunohistochemical staining and scoring procedures

We evaluated the completeness of reporting of immunohistochemical staining and scoring procedures based on a checklist adapted from the REMARK guidelines[8] (Table 1). Of the 33 studies included in this review, 31 were published after the REMARK guidelines were first introduced in 2005[8]. We explored the hypothesis that more recent publications might adhere more to our checklist than older publications, as it usually takes years before new quality guidelines are implemented in research practice. However, we found no significant association between the year of publication and the quality of reporting using bivariate correlation analyses (Pearson's correlation coefficient = -0.001; P = 0.994). Only two of the studies included in this systematic review showed complete adherence to our checklist, and there was a huge variance in amount of information given and how it was presented. In general, there was more information lacking in the descriptions of the scoring protocols compared to the immunohistochemical procedures. The information that was most often missing was the inter- and/or intra-observer variability for the different scorings, and the use of positive and negative controls for immunohistochemical staining. Of the 33 studies included in this review, 21 used immunohistochemistry as the only method. We analysed whether these "pure" prognostic marker studies reported immunohistochemical and scoring procedures more in line with our quality checklist than studies including more complex methods, but found no association (Pearson's correlation coefficient = 0.114; P = 0.529).

Discussion

The rationale for seeking prognostic markers in cancer is obvious and rarely debated. OSCC are highly immunogenic tumours[4, 49], and in this study, we have reviewed the current literature on the prognostic potential of tumour infiltrating macrophages, DC, mast cells and lymphocytes in this cancer type. We have also assessed the completeness of reporting of immunohistochemical staining and scoring protocols in the studies included, to facilitate critical judgement of the presented results.

Macrophages were the most studied immune cells in our review. These are phagocytic and antigen-presenting cells that dependent on environmental cues can differentiate into classically activated M1 macrophages or alternatively activated M2 macrophages. M1 macrophages are pro-inflammatory and are thought to be tumour suppressive, whereas M2 macrophages are immunosuppressive and may promote tumour growth[50]. However, recent evidence suggests that there is a continuum of phenotypes between the pure M1 and M2 macrophages, where the cells can be redirected from one phenotype towards the other[51]. CD68 is a common pan macrophage marker, whereas CD163 more specifically labels M2 macrophages. Tumour-associated macrophages are often differentiated towards the tumour supporting M2 phenotype, which is indirectly supported by our results where both CD68+ and CD163+ cells were mostly associated with decreased survival. However, the results for CD163 were more consistent and more often statistically significant than for CD68 (Table 2), indicating that CD163 is a more reliable prognostic marker in OSCC than CD68. This suggests that there is a mixture of macrophage subtypes in the tumour, where some of those recognized by the CD68 antibody may have tumour-suppressive activities[52].

A high number of tumour infiltrating CD57+ NK cells was an independent positive prognostic factor in two of the three studies using this marker (Table 3). NK cells

are important effector cells in the innate immune system. They can kill cells lacking the self-recognition marker major histocompatibility complex 1 without further activation, and produce cytokines that activate other immune cells[53], supporting their role as tumour suppressors and putative prognostic markers. CD57+ NK cells and CD163+ macrophages seemed to have most prognostic potential of the cells addressed in this review, however, all but one of the studies for each of these cell types were performed on an East-Asian patient cohort (Table 2 and 3). Ethnical and cultural differences along with varying access to health care and diverse treatment regimens call for care when extrapolating results based on patients from one part of the world to others. Thus, well-controlled studies are needed to confirm the robustness and relevance of CD163+ and CD57+ cells as prognostic markers in OSCC patient cohorts outside of Asia.

For the other cells addressed in this review, their potential as prognostic markers was not convincing. Most of these immune cells can be divided into a number of different subclasses with distinct functional properties. This was most notable for DC and T cells analysed with seven and five different markers, respectively (Table 3 and Table 4). Subgrouping of immune cells seems rational with regard to biological differences, but can become very complex and extensive as the number of subgroups keeps evolving. Precise immunohistochemical recognition of a broad variety of immune cell subtypes in clinical routine is not only methodologically challenging, but also impractical in terms of costs and effort. Whereas this review addresses the prognostic significance of individual immune cell subsets, other studies have focused on combinations of immune cells [43, 46, 54], the organisation of the immune infiltrate [55], or the inflammatory infiltrate as a whole [56-58]. However, these approaches also show conflicting results or lack the evidence needed to be included in clinical practice.

In many studies, OSCC are grouped with SCC in the oropharynx, larynx, nasopharynx and nasal cavity under the term head and neck cancer. Due to

differences in anatomical features, etiological factors and aggressiveness[59], cancers from different sites of the head and neck region should be treated as separate entities. We therefore excluded studies that did not report specific survival data for patients with SCC in the oral cavity proper. In addition, the intraoral sub-site of the tumour as well as the localization of immune cells within a single tumour may affect their prognostic value. Most of the studies in this review included patients with cancers at various intraoral locations, but hardly any of them reported sub-site specific prognostic data. More often, various tumour compartments were analysed for immune cell infiltration, e.g. the invasive front, the tumour periphery or tumour nests (Tables 2-4). However, the definition of these compartments was often diffuse, making it difficult to compare the results. Furthermore, some studies used biopsy samples, some resection samples and others tissue microarrays, and these differences may affect how representative the results are for the complete tumour. For T-regulatory cells, some studies suggest that even the sub cellular localization of the marker FoxP3 may affect the functional properties of the cells[60], further emphasizing the importance of thorough reporting of how the scoring was performed.

To improve the quality and usefulness of prognostic marker studies, the REMARK guidelines were introduced in 2005 after recommendations from the US National Cancer Institute and the European Organization for Research and Treatment of Cancer[8]. They outline the minimum information and analyses to include in prognostic marker studies to ensure quality, reproducibility and opportunity to pool studies in meta-analyses. We have made a checklist adapted from the REMARK guidelines to evaluate the quality of reporting of immunohistochemical staining and scoring procedures, and noted that hardly any of the studies included in this review followed all of the criteria. Only two of the studies reported the inter- or intra observer variation in the immunhohistochemical scoring (marked as a in Table 1). Thus, it is difficult to estimate the reproducibility of the scoring, which is

essential if a marker is to be included in clinical practice. Importantly, some of the studies employed digital scoring, and specific quality criteria, different from the REMARK guidelines, may apply for these assessments. Many studies also lacked information on the use of positive and negative controls for immunohistochemical staining, which are important quality assessments to ensure sensitivity and specificity of the staining. The REMARK guidelines were intended to reach a broad spectrum of readers. However, our results demonstrate that the awareness of them does not seem to have increased significantly over time, and that they are yet to be implemented in research practice. Of note, we did systematic assessments of only a subset of the proposed REMARK guidelines that were of specific importance to ensure quality and reproducibility of the immunohistochemical staining and scoring, and this reporting may not reflect the overall quality of the studies. When retrieving data, we frequently noticed that other information such as inclusion and exclusion criteria, treatment regiment, inclusion period, TNM-stage, intraoral subsite and statistical information was missing in several studies, as reflected in Tables 2-4. When important information is missing or incomplete, comparison of studies is difficult, and conclusions derived from these studies will be hampered by uncertainty.

We chose to not do meta-analyses of the prognostic value of the different markers due to heterogeneity in data reporting and study design, along with incomplete reporting of statistical data in many studies. The most common measure of statistical significance is the p-value, and small differences in study design, such as the inclusion or exclusion of patients, can shift the p-value above or below the common 0.05 level of significance[61]. Most of the studies in this review encompassed less than 100 patients, with cancers of different stages and at various intraoral subsites. When controlling for important clinical parameters such as the tumour size, presence of metastases and intraoral site in such small patient cohorts, the group size may become critically small, and the prognostic value of a marker is

likely to be underestimated. Due to the widespread misinterpretation of p-values, evaluating the effect size and -direction on survival may be a more suitable approach to estimate the prognostic value of a marker [61]. Thus, when extracting data from the papers, we tried to interpret whether infiltration of the various cell types were associated with longer or shorter survival, irrespective of the statistical significance of the association, as indicated in Tables 2-4. However, several of the reviewed papers merely stated the statistical significance of a marker, but not its size and direction of the effect on survival, and thus missed essential information. Importantly, the effect on survival in many of the studies was minor, if any, and conclusions derived from these studies may have poor reliability. However, providing statistical data that more directly reflect the effect size of a marker, such as the hazard ratio, may help to increase the value even of small studies, as it opens the possibility to pool data in meta-analyses. Performing and reporting studies according to best-practice guidelines allows transparency and reliability of prognostic marker studies, and emphasizing this need in the research community may help to ensure good scientific practice.

Conclusion

The completeness of the data reporting in the papers reviewed in this study was variable, and important clinical information or reporting of immunohistochemical staining and scoring protocols as well as statistical data were often missing. Thus, there still seems to be a need for journal editors and reviewers to focus more on the quality of data reporting in prognostic marker studies to increase their impact and usefulness. With these methodological limitations in mind, we conclude that M2 macrophages identified by CD163 and mature NK cells identified by CD57 seem to be the most promising prognostic factors of the immune cells analysed in this review. The rest of the cells evaluated showed conflicting or not statistically significant results, and their potential as prognostic markers is far from being established.

Additional Information

Availability of data and materials

All data reported in this manuscript are found in the literature as cited in the text.

Conflict of interest

The authors declare that we have no competing interests, financial or non-financial, in relation to the work.

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Authorship

EHO and AMW both contributed in study planning, literature searches, literature review and data retrieval as well as writing of the manuscript.

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Figure legends

Figure 1:

Search strategy for this systematic review of studies reporting the prognostic value of selected immune cells in oral cancer.

Figure 2:

Flow chart demonstrating reasons for exclusion of studies identified in the searches.

Table legends

Table 1: Studies included and assessment of reporting of immunohistochemical staining and scoring procedures.

Abbreviations:
A= Includes information about antibody clone and/or product number, IHC procedures, positive and negative controls
B= Lacks/unclear information about one of the parameters in A
C= Lacks/unclear information about more than one of the parameters in A
a= Information about number of observers, clear scoring criteria, inter- and/or intra-observer variability
b= Lacks/unclear information about one of the parameters in a
c= Lacks/unclear information about more than one of the parameters in a
¥ quality score equals sum of scoring for reporting of immunohistochemical staining and scoring procedures with C/c=1, B/b=2, and A/a=3
* employed digital scoring methods in addition to/as alternative to manual scoring methods

Table 2: Studies assessing the prognostic value of macrophages in OSCC.

* denotes that information about chemotherapy / radiation exposure of tissue prior to surgery was missing or ambiguous. Abbreviations: OS = overall survival, if survival was not specified it was interpreted as overall survival, DSS = disease-specific survival, PFS= progression-free survival, RFS= recurrence-free survival, DFS = disease-free survival, HR = hazards ratio, RR = risk ratio, NA = Not applied

Table 3: Studies assessing the prognostic value of dendritic cells, mast cells and natural killer cells in OSCC.

* denotes that information about chemotherapy / radiation exposure of tissue prior to surgery was missing or ambiguous. Abbreviations: OS = overall survival, if survival was not specified it was interpreted as overall survival, DSS = disease-specific survival, PFS= progression-free survival, DFS = disease-free survival, HR = hazards ratio, NA = Not applied

Table 4. Studies assessing the prognostic value of T cells and B cells in OSCC.

* denotes that information about chemotherapy / radiation exposure of tissue prior to surgery was missing or ambiguous. Abbreviations: OS = overall survival, if survival was not specified it was interpreted as overall survival, DSS = disease-specific survival, PFS= progression-free survival, HR = hazards ratio, RR = risk ratio, NA = Not applied

Figure 1

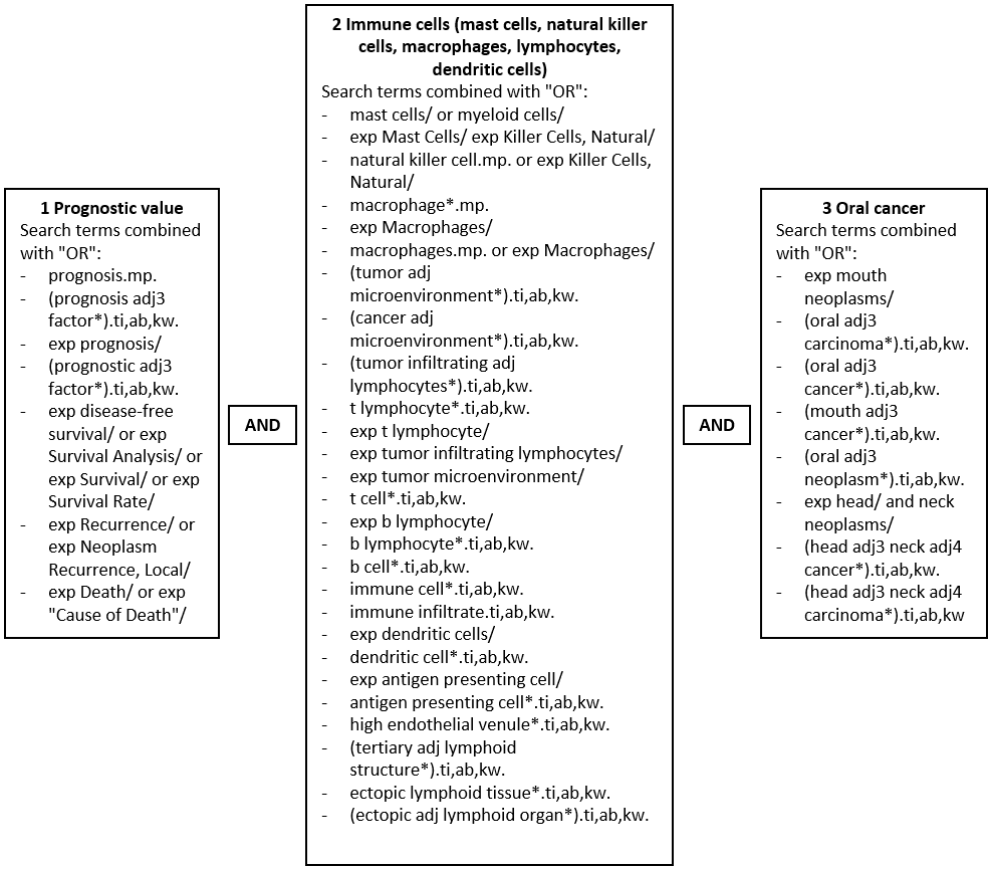
1 Prognostic value
Search terms combined with "OR":
- prognosis.mp.
- (prognosis adj3 factor*).ti,ab,kw.
- exp prognosis/
- (prognostic adj3 factor*).ti,ab,kw.
- exp disease-free survival/ or exp Survival Analysis/ or exp Survival/ or exp Survival Rate/
- exp Recurrence/ or exp Neoplasm Recurrence, Local/
- exp Death/ or exp "Cause of Death"/
AND
2 Immune cells (mast cells, natural killer cells, macrophages, lymphocytes, dendritic cells)
Search terms combined with "OR":
- mast cells/ or myeloid cells/
- exp Mast Cells/ exp Killer Cells, Natural/
- natural killer cell.mp. or exp Killer Cells, Natural/
- macrophage*.mp.
- exp Macrophages/
- macrophages.mp. or exp Macrophages/
- (tumor adj microenvironment*).ti,ab,kw.
- (cancer adj microenvironment*).ti,ab,kw.
- (tumor infiltrating adj lymphocytes*).ti,ab,kw.
- t lymphocyte*.ti,ab,kw.
- exp t lymphocyte/
- exp tumor infiltrating lymphocytes/
- exp tumor microenvironment/
- t cell*.ti,ab,kw.
- exp b lymphocyte/
- b lymphocyte*.ti,ab,kw.
- b cell*.ti,ab,kw.
- immune cell*.ti,ab,kw.
- immune infiltrate.ti,ab,kw.
- exp dendritic cells/
- dendritic cell*.ti,ab,kw.
- exp antigen presenting cell/
- antigen presenting cell*.ti,ab,kw.
- high endothelial venule*.ti,ab,kw.
- (tertiary adj lymphoid structure*).ti,ab,kw.
- ectopic lymphoid tissue*.ti,ab,kw.
- (ectopic adj lymphoid organ*).ti,ab,kw.
AND
3 Oral cancer
Search terms combined with "OR":
- exp mouth neoplasms/
- (oral adj3 carcinoma*).ti,ab,kw.
- (oral adj3 cancer*).ti,ab,kw.
- (mouth adj3 cancer*).ti,ab,kw.
- (oral adj3 neoplasm*).ti,ab,kw.
- exp head/ and neck neoplasms/
- (head adj3 neck adj4 cancer*).ti,ab,kw.
- (head adj3 neck adj4 carcinoma*).ti,ab,kw

Figure 2

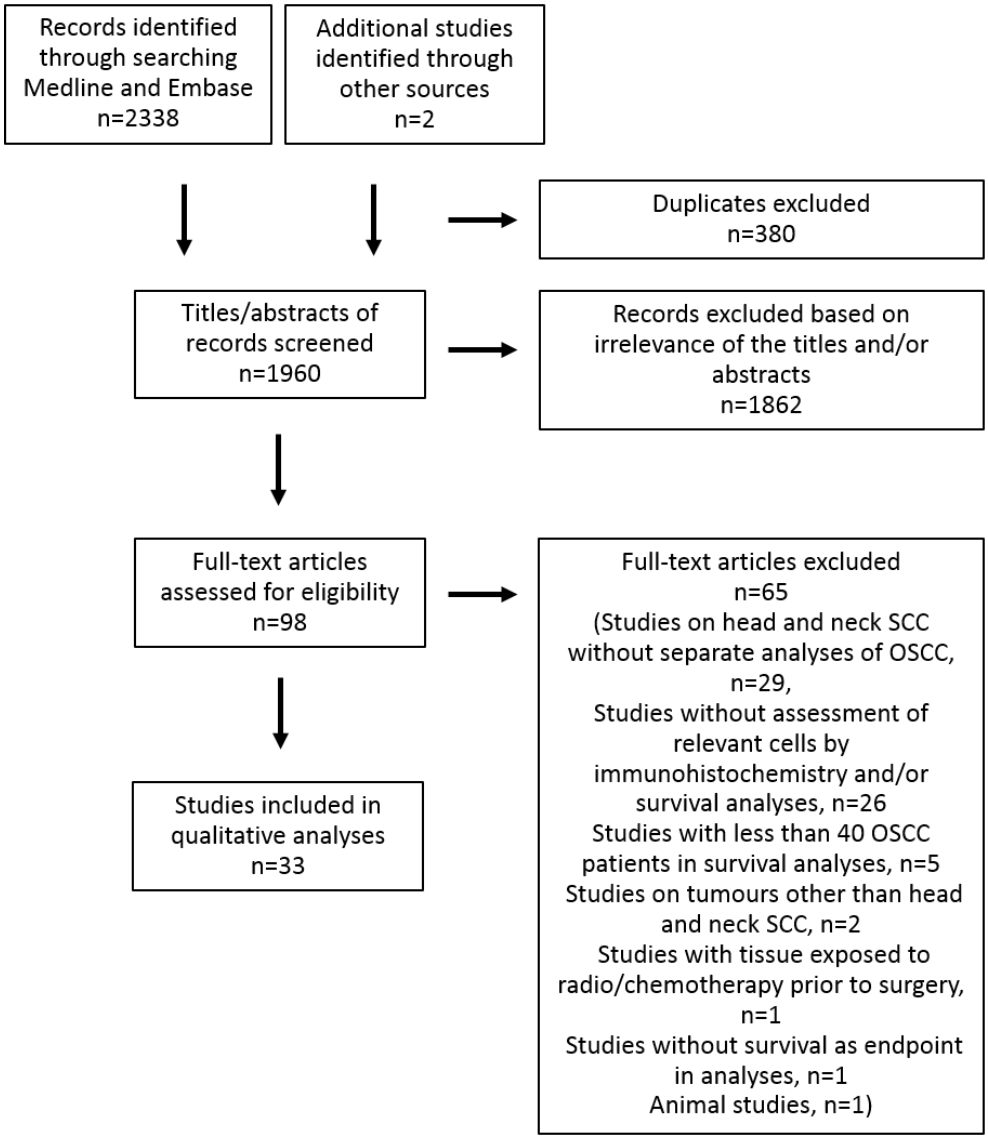
Records identified through searching Medline and Embase n=2338
Additional studies identified through other sources n=2
Duplicates excluded n=380
Titles/abstracts of records screened n=1960
Records excluded based on irrelevance of the titles and/or abstracts n=1862
Full-text articles assessed for eligibility n=98
Full-text articles excluded n=65 (Studies on head and neck SCC without separate analyses of OSCC, n=29, Studies without assessment of relevant cells by immunohistochemistry and/or survival analyses, n=26 Studies with less than 40 OSCC patients in survival analyses, n=5 Studies on tumours other than head and neck SCC, n=2 Studies with tissue exposed to radio/chemotherapy prior to surgery, n=1 Studies without survival as endpoint in analyses, n=1 Animal studies, n=1)
Studies included in qualitative analyses n=33

Table 1

Authors, year	Relevant markers	Reporting of immunohistochemical			IHC only method used
		Staining procedures	Scoring procedures	Quality score ¥	
Jardim *et al,* 2018	CD1a, CD83	B	c*	3	yes
Sun *et al,* 2018	CD68	C	c	2	no
Wirsing *et al,* 2018	CD68, CD208, CD3, CD4, CD8, CD20	A	a (b CD208)	6 (5 CD208)	no
Ahn *et al,* 2017	CD3, FoxP3, CD8, CD20	A	b	5	no
Fang *et al,* 2017	CD68, CD57, T-bet, CD4, CD8	B	b*	4	yes
Kogashiwa *et al,* 2017	CD8	B	c	3	yes
Kubota *et al,* 2017	CD163	B	c	3	no
Mattox *et al,* 2017	CD4, CD8	C	c	2	no
Wu *et al,* 2017	CD8	C	c*	2	yes
Akbarzadeh *et al,* 2016	Mast cell tryptase	B	c	3	yes
Hu *et al,* 2016	CD68, CD163	C	b	3	no
Lao *et al,* 2016	CD19	B	c	3	yes
Sakakura *et al,* 2016	CD68, CD163	B	b	4	no
Taghavi *et al, 2*016	CD57	A	b	5	yes
Zhou *et al,* 2016	FoxP3 +/- CCR4, IL19/CD19	C	c	2	no
Matsuoka *et al,* 2015	CD163	C	b	3	yes
Ni *et al,* 2015	CD68	B	b	4	no
Sakakura *et al,* 2015	CD1a, CD56, CD3	B	b	4	yes
Fujita *et al,* 2014	CD163, FoxP3 +/- CCR4	C	c	2	no
Ishikawa *et al,* 2014	Mast cell tryptase	B	b	4	yes
Ni *et al,* 2014	CD208	C	c	2	yes
Wang *et al,* 2014	CD163	A	b*	5	yes
Costa *et al,* 2013	CD68	A	c	4	no
Dayan *et al,* 2012	CD68, CD163, CD3, CD4, FoxP3, CD8, CD20, CD138	B	c	3	no
Fuji *et al,* 2012	CD68, CD163	B	c	3	yes
Cho *et al,* 2011	CD4, CD8	A	b	5	yes

Lu *et al,* 2010	CD68	A	a	6	yes
Watanabe *et al,* 2010	CD4, FoxP3 +/- CCR4, CD8	A (B CD4)	b	5 (4 CD4)	yes
Zancope *et al,* 2010	CD8, CD57	A	b	5	yes
Liu *et al,* 2008	CD68	C	c	2	yes
O'Donnell *et al,* 2007	CD123, CD209	B	c	3	yes
Reichert *et al,* 2001	S100, P55	B	b	4	yes
Goldman *et al,* 1998	CD1a, S100	C	c	2	yes

Table 2

Authors, year	Number of patients, Tumor size/ stage, Inclusion period, Country	Tumour compartment analysed	Direction of effect on survival high cell count	Statistical significance	
				UV	MV
Marker: CD68, pan macrophage marker					
Sun *et al*, 2018	72*, Stage I-IV, 2009-2011, China	No info	Negative	**OS:P=.034**	**OS:P=.015**
Wirsing *et al*, 2018	75, T1-T4, 1986 – 2002, Norway	Stroma at tumour front	Positive	**DSS: P=.027**	DSS: P>.05
Fang *et al*, 2017	78*, Stage I-IV, 2007-2009, China	Stroma	Positive	OS: P=.293, HR= 0.733	OS: P=.177, HR= 1.533
Hu *et al*, 2016	127, Stage I-IV, 2007 – 2013, China	Nest Stroma	Negative Positive	**OS: P= .01, RR=3.08** OS: P=.3, RR=0.69	NA NA
Sakakura *et al,* 2016	74, Stage I-IV, 2000-2012, Japan	Stroma	Negative	**OS: P=.035**	OS: P>.05
Ni *et al*, 2015	91, T1 - T4, 2003-2011, China	Stroma Nest	Negative Positive	**OS: P=.033, HR=1.947** DFS: P= .435 OS:P=.802 , HR=.904 DFS: P=.562	OS: P=.736, HR=1.55 NA NA NA
Costa *et al*, 2013	45, T1-T4, Period missing, Brazil	Peritumour	Negative	OS: P=.08	NA
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Fujii *et al*, 2012	108, Stage I-IV, 1990 –2005, Japan	Stroma at invasive front	No info.	OS: P= .16	NA
Lu *et al*, 2010	92, Stage I-IV, 1995-2003, Taiwan	Stroma	Negative	**OS:P< .001,** **DFS: P= .001**	**OS: P=.015** **DFS: P=.005**
Liu *et al,* 2008	112*, T1 -T4, Period missing, Taiwan	No info	Negative	**DFS: P=.001**	NA

Marker: CD163, M2 macrophages					
Kubota *et al*, 2017	46, Stage I-IV, 2005-2015, Japan	Nest	Negative	PFS: P=.21, HR=1.53 DSS: P=0.58, HR=1.22	PFS: P=.64, HR=1.18 DSS: P=.61, HR=1.20
Hu *et al*, 2016	127, Stage I-IV, 2007–2013, China	Nest Stroma	Negative Positive	**OS: P=.02, RR=2.83** OS: P=.48, RR=.78	NA NA
Sakakura *et al,* 2016	74, Stage I-IV, 2000-2012, Japan	Stroma	Negative	**OS: P=.025** **PFS: P=.011**	**OS: P=.034** **PFS: P=.023**
Matsuoka *et al*, 2015	60, Stage I-IV, 2003-2009, Japan	Stroma at invasive front	Negative	**OS: P=.003** **DFS: P=.007**	OS: P=.195, HR=2.299 DFS: P=.258, HR=1.749
Fujita *et al*, 2014	50*, Stage I-IV, 2006 - 2010, Japan	Invasive front Intratumour	Negative No info.	**OS: P=.006** **DFS:P=.002** OS: P>.05 DFS: P>.05	OS:NA **DFS:P=.006, RR=2.625** NA NA
Wang *et al,* 2014	240, Stage I-IV, M0, 1982-2005, China	Stroma	Negative	**OS: P<.001, HR=4.411**	**OS: P=.001, HR=3.561**
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Fujii *et al,* 2012	108, Stage I-IV, 1990-2005, Japan	Stroma at invasive front	Negative	**OS: P=.007**	**OS:P=.045, HR=2.636**

Table 3

Authors, year	Number of patients, Tumor size/ stage, Inclusion period, Country	Tumour compartment analysed	Direction of effect on survival high cell count	Statistical significance	
				UV	MV
DENDRITIC CELLS					
Marker: CD1a, immature DC					
Jardim et al, 2018	53, Stage I-IV, 2002-2010, Brazil	Intratumour Peritumour	Positive Positive	OS:P=.148 DFS:P=.089 **OS:P=.03** **DFS:P=.007**	NA NA **OS: P=.001, HR=3,61** **DFS: P=.001, HR=4.23**
Sakakura *et al*, 2015	74, Stage I-IV, 2000- 2012, Japan	Tumour periphery	No info	OS:P=1.000 PFS:P=1.000	NA NA
Goldman *et al*, 1998	43, T1-T4, 1987 – 1998, US	Peritumour Intratumour	Positive Negative	DSS: P=.05 DSS: P=.21	DSS: P=.23 **DSS: P=.04**
Marker: S100, pan DC marker					
Reichert *et al*, 2001	132, Stage I-IV, 1980 – 1993, Germany	Stroma	Positive	**OS**: **P<.001**	**OS: P<=.001, HR=.422**
Goldman *et al*, 1998	43, T1-T4, 1987 – 1998, US	Peritumour Intratumour	Positive Negative	DSS: P=.30 DSS: P=.24	DSS: P=.07 DSS: P=.80
Marker: CD83, mature DC					
Jardim et al, 2018	53, Stage I-IV, 2002-2010, Brazil	Intratumour Peritumour	Positive Positive	OS:P=.274 DFS:P=.346 OS:P=.276 DFS:P=.392	NA NA NA NA
Marker: P55, fascin-expressing DC					
Reichert *et al*, 2001	129, Stage I-IV, 1980 – 1993, Germany	Stroma	Positive	**OS: P<.001**	OS: P>.05
Marker: CD208/DClamp, mature DC					
		Stroma	Positive	DSS: P=.639	NA

Wirsing *et al*, 2018	69, T1-T4, 1986 – 2002, Norway				
Ni *et al*, 2014	79, Stage I-IV, 2011-2012, China	Nest Stroma	Positive Negative	OS:P>.05 OS: P>.05	NA NA
Marker: CD123, plasmacytoid DC					
O'Donnel *et al*, 2007	63*, T1-T4, Period missing, US	Extranestal Intratumour	Negative/no info Negative/no info.	**OS: P<.0001, Compartment unclear**	NA NA
Marker: CD209/DCsign, immature DC					
O'Donnel *et al*, 2007	63*, T1-T4, Period missing, US	Extranestal Intratumour	Negative/ no info Negative/no info.	**OS: P<.0001, Compartment unclear**	NA NA
MAST CELLS					
Marker: Mast cell tryptase					
Akbarzadeh Baghban *et al,* 2016	57*, Stage missing, Period missing, Iran	Peritumour	No info	NA	OS:P=.719, HR=1.117
Ishikawa *et al,* 2014	81*, Stage I-IV, 1982-2007, Japan	Stroma	Negative	**DFS: P=.038**	NA
NATURAL KILLER CELLS					
Marker: CD56, pan NK cell marker					
Sakakura *et al,* 2015	74, Stage I-IV, 2000-2012, Japan	Tumour periphery	No info	OS: P=1.000 PFS: P=1.000	NA
Marker: CD57, mature/activated NK cell marker					
Fang *et al.* 2017	78*, Stage I-IV, 2007-2009, China	Stroma	Positive	**OS: P<.001, R=7.718**	**OS:P<.001, HR=6.576**
Taghavi *et al,* 2016	57*, Stage missing, Period missing, Iran	Intratumour	Positive	NA	**OS:P<.001, HR=17.34**
Zancope et *al*, 2010	40, Stage I-IV,Period missing, Brazil	Peritumour Intratumour	No info No info	OS: P=.70 OS: P=.69	NA

Table 4

Authors, year	Number of patients, Tumor size/ stage, Inclusion period, Country	Tumour compartment analysed	Direction of effect on survival high cell count	Statistical significance	
				UV	MV
T CELLS					
Marker: CD3, pan T cell marker					
Wirsing *et al*, 2018	74, T1-T4, 1986 – 2002, Norway	Stroma at tumour front	Positive	DSS: P=.200	NA
Ahn *et al*, 2017	68*, Stage I-IV, 2003-2011, South Korea	Stroma	Positive No effect	OS: P=.142, HR=.99 DSS: P=.552, HR=1.00	NA NA
Sakakura *et al*, 2015	74, Stage I-IV, 2000 – 2012, Japan	Tumour periphery	No info	OS:P=.856, PFS:P=.981	NA
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Marker: CD4, various T cell subsets					
Wirsing *et al*, 2018	72, T1-T4, 1986 – 2002, Norway	Stroma at tumour front	Positive	DSS:P=.691	NA
Fang *et al*, 2017	78*, Stage I-IV, 2007 – 2009, China	Stroma	Positive	OS:P=.207, HR= .686	OS:P=.909, R=1.038
Mattox *et al*, 2017	47*, T1/T2, N0-N2, Period missing, US	No info	Positive	OS:P=.18	NA
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Cho *et al*, 2011	45, Stage I-IV, Period missing, South Korea	Peritumour	Positive	OS:P=.571	NA
Watanabe *et al*, 2010	87, Stage I-IV (M0), 1994-2003, Japan	Stroma Nest	No info No info	OS:P=.072 No info	NA NA
Marker: T-bet, Th1 cell marker					
Fang *et al*, 2017	78*, Stage I-IV, 2007 – 2009, China	Stroma	Negative	OS:P=.639 HR= .871	OS:P=.836, HR=1.066
Marker: FoxP3 +/- CCR4, Treg marker					

Ahn *et al*, 2017	68*, Stage I-IV, 2003-2011, South Korea	Stroma	Positive Negative	OS: P=.374, HR=.98 DSS: P=.754, HR=1.01	NA NA
Zhou *et al*, 2016	46, Stage I-IV, 2006-2011, China	Stroma	Negative	**OS:P= .001**	**OS:P=.021, RR=0.066**
Fujita *et al*, 2014	50*, Stage I-IV, 2006-2010, Japan	Invasive front Intratumour	No info No info	OS: P>.05, DSS: P>.05 OS: P>.05, DSS: P>.05	NA NA
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Watanabe *et al*, 2010	87,Stage I-IV (M0), 1994-2003, Japan	Stroma	Negative	OS:P=.31 (FoxP3) **OS**: **P=.001** (FoxP3/CCR4+)	No info No info
Marker: CD8, cytotoxic T cell marker					
Wirsing *et al*, 2018	72, T1-T4, 1986 – 2002, Norway	Stroma at tumour front	Negative	DSS: P=.304	NA
Ahn *et al*, 2017	68*, Stage I-IV, 2003-2011, South Korea	Stroma	Positive Positive	OS: P=.181, HR=.99 DSS: P=.282, HR=.99	NA NA
Fang et al, 2017	78*, Stage I-IV, 2007 – 2009, China	Stroma	Positive	**OS: P<.001, HR= 3.808**	**OS:P=.030, HR= 2.174**
Kogashiwa *et al*, 2017	84, Stage III-IVA, 2007 – 2014, Japan	No info	Positive	OS:P=.058 PFS:P=.35	NA
Mattox *et al*, 2017	48*, T1/T2, N0-N2, period missing, US	No info	No info	OS: P=.41	NA
Wu *et al*, 2017	165, T1-T4, 2008-2010 and 2012-2015, China	No info (TMA)	Positive	**OS:P=.0498**	OS:P=.052, HR=0.579
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Cho et al, 2011	44, Stage I-IV, period missing, South Korea	Intratumour Peritumour	No info Positive	No info OS:P=.178	NA NA
Watanabe *et al*, 2010	87, Stage I-IV, M0, 1994-2003, Japan	Stroma Nest	Positive Positive	**OS: P=.001** **OS**: **P=.001**	NA NA

Zancope *et al*, 2010	40, T1-T4, period missing, Brazil	Peritumour Intratumour	Positive No info	OS: P=.40 OS:P=.9	NA NA
B CELLS					
Marker: CD20, pan B cell marker					
Wirsing *et al*, 2018	75, T1-T4, 1986 – 2002, Norway	Stroma at invasive front	Positive	**DSS:P=.002**	DSS:P>,05
Ahn *et al*, 2017	68*, Stage I-IV, 2003-2011, South Korea	Stroma	Positive Positive	OS: P=.186, HR=.98 DSS:P=.496, HR=.99	NA NA
Dayan *et al*, 2012	54, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Marker: CD19, pan B cell marker					
Lao *et al*, 2016	93*, Stage I-IV, period missing, China	Stroma	Positive	**OS: P=.008**	OS: Significant, but P-value and HR missing
Marker: CD138, plasmacell marker					
Dayan *et al*, 2012	64, Stage I-IV, 1990-2006, Israel	Tumour-stroma interface	No info	OS: P>.05	NA
Marker: IL19/CD19, Breg marker					
Zhou *et al*, 2016	46, Stage I-IV, 2006-2011, China	Stroma	Negative	**OS:P=.001**	OS:P=.528, RR=.635