Role of Interleukin-17F in Oral Tongue Squamous Cell Carcinoma

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TABLE OF CONTENTS

1. LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following publications:

I. **Almahmoudi R**, Salem A, Sieviläinen M, Sundquist E, Almangush A, Toppila-Salmi S, Paavonen T, Salo T, Al-Samadi A. Extracellular interleukin-17F has a protective effect in oral tongue squamous cell carcinoma. *Head & Neck*. 2018; 40:2155-2165.

II. **Almahmoudi R**, Salem A, Murshid S, Dourado MR, Apu EH, Salo T, Al-Samadi A. Interleukin-17F Has Anti-Tumor Effects in Oral Tongue Cancer. *Cancers (Basel)*. 2019; 11:650.

III. **Almahmoudi R**, Salem A, Hadler-Olsen E, Svineng G, Salo T, Al-Samadi A. The Effect of Interleukin-17F on Vasculogenic Mimicry in Oral Tongue Squamous Cell Carcinoma. *Cancer Sci*. 2021; 112:2223-2232.

IV. **Almahmoudi R**, Kasanen M, Sieviläinen M, Salem A, Pirinen M, Salo T, Al-Samadi A. Prognostic value of blood and lymphatic vessel markers in tongue cancer: A systematic review. *Cancer Sci*. 2019; 110:3424-3433.

These publications are cited in the text according to their respective Roman numerals (**I–IV**).

2. ABBREVIATIONS

CAF: cancer-associated fibroblasts
CD: cluster of differentiation
CI: confidence interval
CK: pan-cytokeratin
DAB: 3,3'-diaminobenzidine
DAPI: 4',6-diamidino-2- phenylindole
DFS: disease-free survival
DMEM: Dulbecco's modified Eagle's medium
DSS: disease-specific survival
EC: endothelial cell
END: elective neck dissection
FACS: fluorescence-activated cell sorting
FBS: fetal bovine serum
FFPE: formalin-fixed & paraffin-embedded
FOXP3: forkhead box protein P3
FVIII: factor VIII
HCC: hepatocellular carcinoma
HNSCC: head and neck squamous cell carcinoma
HOK: human oral keratinocyte
IF: Immunofluorescence staining
IFNγ: interferon-gamma
IL-17: interleukin 17
LVD: lymphatic vessel density
LYVE-1: lymphatic vessel endothelial hyaluronan receptor-1
MAStARI: meta-analysis of statistics assessment and review instrument tool
MC: mast cell
MVD: microvessel density
OED: oral epithelial dysplasia
OLP: oral lichen planus
OPMD: oral potentially malignant disorders
OPSCC: oropharyngeal squamous cell carcinoma
OS: overall survival
OSCC: oral squamous cell carcinoma
OTSCC: oral tongue squamous cell carcinoma
PAS: periodic acid-Schiff
PBS: Phosphate-buffered saline
PDX: patient-derived xenograft
PFA: paraformaldehyde
PFS: progression-free survival
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-analyses
RBC: red blood cell
RDLV: the relative density of lymphatic vessels
REMARK: reporting recommendations for tumour marker prognostic studies
RFS: recurrence-free survival

RR: risk ratio
RT: room temperature
TGF-β: transforming growth factor beta
Th: T helper cell
TME: tumour microenvironment
TMEM: tumour microenvironment matrix
TNF: tumour necrosis factor
Treg: T regulatory cell
TSCC: tongue SCC
VC: vessel count
VEGF: vascular endothelial growth factor
VM: vasculogenic mimicry
VWF: Von Willebrand Factor
WHO: world health organization

3. ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a group of common and aggressive malignant tumours that arise in the oral cavity, oropharynx, hypopharynx and larynx. The majority of HNSCC tumours are encountered in the oral cavity, where the tongue is the most frequently affected site intraorally (oral tongue SCC; OTSCC). The multimodality treatment includes surgery and radiotherapy with or without chemotherapy. In addition, some immunotherapeutic agents have been recently approved to treat HNSCC patients. However, patients with HNSCC in general, and those with OTSCC in particular, have a relatively unfavourable prognosis. Unfortunately, five-year overall survival rates in the Nordic countries including Finland remain between 50-60%. Therefore, there is an urgent need to identify new prognostic and therapeutic targets to improve the survival outcomes of OTSCC patients. Interleukin-17F (IL-17F) is the latest identified member of the IL-17 cytokine family. IL-17F was recently shown to induce antitumorigenic effects in several cancer types through different mechanisms. The serum level of IL-17F was lower in OSCC patients compared with leukoplakia patients and healthy individuals. Furthermore, IL-17F inhibited tumour angiogenesis and the level of IL-17F was significantly lower in tumour tissues such as in colon and hepatocellular carcinoma (HCC) compared with the healthy control samples. Overall, these reports suggest an inverse relationship between IL-17F levels and tumorigenesis.

The aims of the present doctoral thesis were set as follows: 1) to investigate the expression levels and cellular sources of IL-17F in OTSCC tissues and its potential association with patients' prognosis and mortality outcomes; 2) to assess the effects of IL-17F on several tumorigenic features including cell proliferation, migration, invasion and angiogenesis; 3) to test the possible effect of IL-17F on the formation of tumour-derived *de novo* vessel-like structures (vasculogenic mimicry; VM); 4) based on the antiangiogenic effect of IL-17F, we also aimed to systematically review the prognostic value of blood microvessel density (MVD) and lymphatic vessel density (LVD) in patients with tongue SCC (TSCC).

The studies included clinical samples obtained from OTSCC patients who were treated surgically at Oulu and Tampere University Hospitals during the period from 1990-2016. The study was approved by the relevant Ethics Committees. In addition, different OTSCC cell lines, human umbilical vein endothelial cells (HUVEC), cancer-associated fibroblasts (CAF), and human oral keratinocytes (HOKs) were used for the *in vitro* assays. Gene analysis assays were performed using RT-PCR and droplet-digital PCR. Immunostaining and blinded-scoring were used to map IL-17F expression in patient samples. The IncuCyte system, tumour spheroids, FACS analysis, and tube-formation assays were also used. Survival curves were constructed according to the Kaplan-Meier method. We also tested different patterns of VM structures in patient samples and in an orthotopic mouse model of OTSCC. The systematic review search was conducted in the following databases: Ovid Medline, Scopus, and Cochrane libraries.

Briefly, we first showed that mast cells are the main source of IL-17F in OTSCC. Using multivariate analysis, the extracellular mast cell-derived IL-17F at the tumour invasion front was associated with better disease-specific survival in all-stages and early-stages OTSCC patients. We also showed that OTSCC cells had lower levels of IL-17F, IL-17RA, and IL-17RC mRNA compared with normal HOKs. Importantly, IL-17F inhibited tumour cell proliferation, random migration, and CAF-mediated tumour cell invasion. The tube formation by HUVEC was inhibited by IL-17F in a dose-dependent manner. Additionally, we showed that OTSCC cells contained CD31 at both mRNA and protein levels, and formed tube-like VM on Matrigel similar to those formed by HUVEC. These VM structures were also identified in a mouse orthotopic model of human OTSCC. Furthermore, IL-17F supressed the formation of VM *in vitro*, which mimics the effect produced by the anti-angiogenic drug Sorafenib. Finally, our systematic review results revealed that higher expression of MVD/LVD markers were associated with worse survival outcomes in TSCC patients in most of the relevant studies.

Taken together, our findings support an anticancerous role of IL-17F in OTSCC. Based on levels of extracellular mast cell-derived IL-17F at the tumour invasive front, patients with OTSCC were categorized into high- and low-risk groups. The *in vitro* assays revealed inhibitory effects of IL-17F on tumour cell proliferation, migration, invasion and VM formation, and thus further preclinical studies are warranted. We also concluded that more clinical studies with larger patient cohorts are needed before the MVD/LVD markers can be recommended for clinical prognostication. Development of IL-17F-based drugs, or targeting one of its regulatory pathways, may have prognostic implications as well as serve as a promising therapeutic approach in patients with OTSCC.

4. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is among the most common cancers worldwide. HNSCC comprises a group of malignant tumours that collectively account for more than 500,000 new cases each year globally (Sung et al., 2021). HNSCC tumours develop in the epithelial lining of the oral cavity, oropharynx, hypopharynx and larynx. Intraorally, oral tongue squamous cell carcinoma (OTSCC) is the most frequently diagnosed tumour. The risk factors of developing HNSCC include sustained exposure to chemical carcinogens such as tobacco and tobacco-like products, alcohol abuse, and oncogenic viruses, mainly the human papilloma virus (HPV). However, while the incidence of HPV-positive HNSCC has increased significantly worldwide, patients with HPV-positive HNSCC have a substantially better prognosis than HPV-negative patients. The development of HNSCC has also been shown to associate with mutations of several genes such as *TP53*, *EGFR*, *PIK3CA*, *NOTCH1* and many others (Jiang et al., 2019; Johnson et al., 2020).

The management standard of early-stage OTSCC patients typically involves surgery with or without radiotherapy. In more advanced stages of the disease, the management includes a multimodality treatment approach of surgery and radiotherapy combined with adjuvant chemotherapy. Molecular targeted therapy including inhibitors of the epidermal growth factor receptor (EGFR) such as cetuximab, which remains the only targeted drug approved for HNSCC to date, can also be administered to the patients. Recently, immunotherapy has been approved to treat some cisplatin resistant and recurrent HNSCC (Johnson et al., 2020). In spite of the extensive advancement and technical improvement in cancer research and treatment, patients with OTSCC continue to have a relatively poor prognosis, with five-year survival rates in the Nordic countries, including Finland, of around 50-60% (Hakulinen et al., 2010; Chi et al., 2015). Therefore, there is an urgent need to identify and develop better therapeutic molecular targets to improve the survival outcomes of the patients.

The tumour microenvironment (TME) consists of an extremely heterogenous population of cancer cells, immune cells, stromal factors, secreted inflammatory proteins and extracellular molecules. The TME can modify the therapeutic outcomes, mediate drug resistance and constitute a crucial target for cancer treatment research (Whiteside 2008; Jin and Jin, 2020). Intratumoural immune cell infiltrate including T helper (TH) cells and their cytokines represent key components of the TME. Indeed, immune cell-derived cytokines can induce antitumour effects and inhibit tumour growth and development. However, their significance is still not well understood (Hirata and Sahai 2017). Recently, a new distinct lineage of TH cells has been described (i.e. TH-17), which was named after their signature proinflammatory cytokine family, interleukin 17 (IL-17). Of these cytokines, IL-17F is the newest characterized cytokine of the IL-17 family, and it shares the greatest structural homology with IL-17A (Gu et al., 2013; Raphael et al., 2015).

Importantly, IL-17A and IL-17F have been shown to be involved in tumour growth and angiogenesis in a wide range of cancers, including colorectal carcinoma,

hepatocellular carcinoma (HCC), lung cancer, and skin cancer (Wang et al., 2014; Lee et al., 2014; Pan et al., 2015; Wang et al., 2010). Targeting IL-17A was shown to inhibit tumour cell metastasis and enhanced the therapeutic sensitivity of chemo- and radiotherapy in preclinical cancer models (Coffelt et al., 2015; Lotti et al., 2013). Furthermore, a comprehensive systematic review by Punt et al. concluded that elevated levels of serum IL-17A were associated with worse survival outcomes for several types of solid tumours (Punt et al., 2015). Additionally, more recent studies showed that IL-17A polymorphisms resulted in an increased cancer susceptibility (Al Obeed et al., 2018; Bedoui et al., 2018; Elshazli et al., 2018; Samiei et al., 2018). On the other hand, IL-17F seems to produce more anticarcinogenic effects. For example, IL-17F was shown to produce anti-angiogenic effects in HCC and therefore may reduce the vascular supply in TME and inhibit tumour cell growth and metastasis (Xie et al., 2010)

Overall, the identification of IL-17F and its anticancer effects, along with the pharmaceutical success of IL-17 family-based drugs in targeting chronic inflammatory diseases, could pave the way for developing new therapeutic targets in cancer patients. Therefore, the aim of this doctoral dissertation was to explore the possible prognostic role of IL-17F in OTSCC and its potential contribution to oral tumorigenesis.

5. LITERATURE REVIEW

5.1. Oral tongue squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is a malignant tumour that develops from the stratified squamous epithelial lining of the oral cavity, which represents one of the most common forms of head and neck cancers **(Figure 1).** In fact, OSCC constitutes more than 90% of all oral cancers (Coletta et al., 2020). Although OSCC can develop anywhere in the oral cavity proper, it is often encountered in the floor of the mouth and in the anterior two-thirds of tongue (i.e. oral tongue squamous cell carcinoma; OTSCC). Due to the anatomical proximity of "OTSCC" with other oral cancers, this term is usually used interchangeably with "OSCC" and "oral cancer" (Markopoulos 2012; Coletta et al., 2020). Therefore, although this literature review aims to focus on OTSCC whenever possible, the context may also include "OSCC" and "oral cancer" particularly when the tumour subtype is not specified in the respective literature.

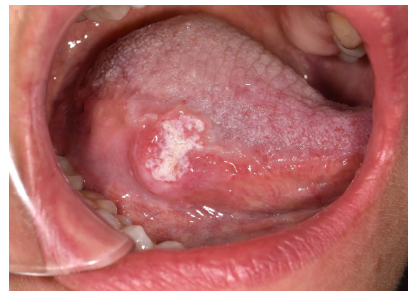

Figure 1. Clinical case of OTSCC. Modified from (Olmos et al., 2021)

The incidence of OTSCC has increased in recent years and is more frequently diagnosed in women and in individuals under the age of 45-years (Marur and Forastiere, 2016; Ng et al., 2017). Furthermore, it has been noted that OTSCC tends to have more aggressive clinical behaviour and worse outcomes in younger patients with no previous history of smoking (Durr et al., 2013). Despite the recent advancement in the diagnosis and treatment of cancer, the treatment outcomes of OSCC patients remain limited and the five-year survival rate is around 50-60% (Hakulinen et al., 2010; Chi et al., 2015; Sim et al., 2019). The poor prognosis of OTSCC has been attributed to several factors including, among others, the swift locoregional metastasis to regional lymph nodes (Sano and Myers, 2007; Brinkman and Wong, 2006).

5.1.1. Incidence

Specific data regarding the incidence of OTSCC are limited and can be affected by definition heterogeneity (as mentioned above), or due to the pooling of different subtypes of HNSCC together to increase the reported sample size (Radoï and Luce 2013). According to the Finnish Cancer Registry, the incidence of OTSCC in Finland has been increasing during the last three decades with 180 new cases (105 males, incidence rate 3.89; 75 females, incidence rate 2.35) in 2019 alone (Pitkäniemi et al., 2021). Globally, a recent study analysed data from 22 cancer registries and 89 212 cases diagnosed with OTSCC and found an increased incidence rate ranging from 0.4% to 3.3% per year. Interestingly, out of these 22 registries, 14 showed a higher incidence among younger (i.e. < 45-year-old) patients (Ng et al., 2017).

Collectively, cancers of the lip and oral cavity, including OTSCC, rank as the 16th most common neoplasm worldwide with an estimated 377 713 new cases and over 177 000 deaths in 2020 (Sung et al.2021; Miranda-Filho et al., 2020). There are marked differences in incidence between countries and over time periods. On a global scale, lip and oral cavity cancers constituted an estimate of 2.9% of all cancer cases in males and 1.0% in females in 2020 (Miranda-Filho et al., 2020). Geographically, the incidence of lip and oral cavity neoplasms is considerably more frequent in South Central Asia including India, Sri Lanka, and Pakistan. Additionally, the incidence of these neoplasms was noticeably high for both sexes in Melanesia, which might be linked to the habitual use of tobacco and betel nut among the inhabitants of these areas (Gupta and Johnson 2014). In 2020, cancers of the lip and oral cavity were also the leading cause of cancer death in Indian males (Sung et al. 2021; Miranda-Filho et al., 2020).

Although global patterns of lip and oral cavity cancers are widely variable, the incidence of lip, oral cavity, and pharyngeal cancers has increased among females and people from low/middle-income countries over the period of 1990 - 2017. In addition, another alarming observation is the increase in the incidence of these cancers in the younger age (≤ 45 years-old) populations (Du et al., 2020; Ng et al., 2017). It is logical to assume that the consumption of tobacco and alcohol contributes to this increasing pattern in these areas of the globe where the majority of world's smokers live in the low- and middle-income countries. Also, females are currently more exposed to tobacco and alcohol consumption than before (Coletta et al., 2020). However, it is not yet clear why there is an increasing incidence pattern of these cancers among younger age populations, as they are often lacking the classical risk factors, and hence suggesting other predisposing factors such as genetic alterations and pollutants (Coletta et al., 2020).

5.1.2. Aetiology and risk factors

Although OTSCC accounts for more than 90% of oral malignancies, the actual aetiology and the exact mechanisms behind the development of the disease still need to be further explored. In addition, the substantial variation in the pattern of the tumour incidence indicates the diversity and overlapping of many risk factors. However, some critical aetiological factors have been attributed to oral tumorigenesis as follows:

a) Tobacco:

Tobacco consumption is the most commonly cited risk factor for head and neck cancers including OTSCC. According to the International Agency for Research on Cancer, smoked tobacco is categorized as a group 1 carcinogen for the oropharynx, whereas smokeless-tobacco as a group 1 carcinogen for the oral cavity (International Agency for Research on Cancer; 2015; Chi et al., 2015). Indeed, a large body of evidence supports the role of tobacco smoking in the development of oral cancer both in a dose- and time-dependent manner, where tobacco smokers have been reported to exhibit a 5- to 25-fold increased risk of

developing cancer compared to non-smokers (Lambert et al., 2011). In a meta-analysis study, Gandini et al. showed a significant increase of the risk ratio (RR) for developing oral cancer in current smokers (RR = 3.43; 95% confidence interval CI = 2.37 – 4.94), which was much higher than that observed for ex-smokers (RR = 1.40; 95% CI = 0.99 – 2.00) who had a smoking-free period of at least one year (Gandini et al., 2008). The risk of oral cancer development is also proportional to the smoking duration period (Hashibe et al., 2007).

Long-term use of other forms of tobacco (i.e. smokeless-tobacco) such as chewing tobacco (loose-leaf) and snuff (finely cut tobacco) are likewise considered as potent carcinogenic products and among the risk factors for developing OTSCC (Regezi et al., 2017). The betel quid (or paan) with tobacco is one of the most common form of smokeless-tobacco, which is consumed as a cultural practice by more than 500 million people in many Asian countries such as India, Sri Lanka, Thailand and China (Gupta and Warnakulasuriya 2002; Merchant et al., 2000). A comprehensive meta-analysis study comprising 4648 patients has concluded that betel quid chewing is an independent risk factor for the development of oral cancer (Gupta and Johnson 2014).

In Finland, snuff (snus) use and sale have been banned since 1995 (Hamari et al., 2013). A Finnish study conducted on young male military recruits (n = 1151, mean age 19.4 years) found that 15.6% of the participants reported daily snus use, of which half were dual consumers of both cigarettes and snus, while 66.3% were occasional snus users (Hamari et al., 2013). Although smokeless-tobacco is an important risk factor for OSCC, the association between Swedish moist snus and oral carcinogenesis is still unclear. Recently, Araghi et al. used pooled data of 418 369 male participants from the Swedish Collaboration on Health Effects of Snus Use to assess the association between snus use and the development of oral cancer. The authors found that, compared to never-snus use, ever-snus use was not associated with oral cancer (adjusted HR 0.90, 95% CI: 0.74, 1.09), and it was concluded that Swedish snus use does not seem to be involved in the development of oral cancer in men (Araghi et al., 2020).

b) Alcohol consumption:

Alcohol consumption and tobacco use can work synergistically to induce mucosal hyperproliferation and thus increase the risk of oral cancers by several folds (Hashibe et al., 2007; Scoccianti et al., 2016). Moreover, alcohol consumption seems to induce oral carcinogenesis in a dose-dependent manner. Turati et al. found that the risk of developing oral neoplasms increases in heavy drinkers (RR = 4.64; 95% CI = 3.78 – 5.70) compared with light drinkers (RR = 1.17; 95% CI = 1.01 – 1.35) (Turati et al., 2010). The association between the use of alcohol-containing mouthwashes and increasing the risk of oral cancer is still a controversial issue. In their review, McCullough et al. argued that there is a strong indication that oral cancer can be induced, at least partly, by alcohol-based mouthwashes (McCullough et al., 2008). In contrast, a recently published systematic review by Ustrell-

Borràs et al. concluded that there is a lack of evidence to support the role of alcohol mouthwashes in oral carcinogenesis (Ustrell-Borràs et al. 2020).

c) Oral potentially malignant lesions:

It has been documented that OSCC can be preceded by mucosal premalignant changes or lesions that are collectively termed oral potentially malignant disorders (OPMD). Among the common OPMD are leukoplakia, erythroplakia, erythro-leukoplakia (speckled erythroplakia), oral lichen planus (OLP), oral submucous fibrosis, and the proliferative verrucous leukoplakia (Warnakulasuriya et al., 2007; Iocca et al., 2020). Histopathologically, these lesions may reveal cellular and morphological alterations named oral epithelial dysplasia (OED), which can be mild, moderate or severe. Importantly, the presence of OED in OPMD has been considered as a risk for cancer progression (Weijers et al. 2002). However, these dysplastic changes are not necessarily found in association with the OPMD lesions, but they are most commonly found in erythroplakia lesions. More importantly, the majority of erythroplakia lesions reveal severe dysplasia or carcinoma in situ (Speight 2007; Speight et al., 2018).

In a recently published meta-analysis, the highest malignant transformation rate of OPMD was found in the proliferative verrucous leukoplakia as 49.5% (99% CI 26.7%-72.4%) and erythroplakia lesions as 33.1% (99% CI 13.6%-56.1%) and thus they should always be biopsied. The overall malignant transformation rate of all the OPMD combined was found to be 7.9% (99% CI 4.9%-11.5%) (Iocca et al., 2020). Another meta-analysis using data from 20095 patients found that the malignant transformation rate of OLP ranges from 0.9 - 1.1%. Noteworthy, the malignant transformation risk was significantly increased among smokers, alcoholics and in hepatitis C virus-infected patients (Aghbari et al., 2017).

d) Fungal and viral infections:

Candida albicans (*C. albicans*) is one of the most prevalent Candida species in the oral cavity. The association between *C. albicans* infections and carcinogenesis has been widely investigated because candidiasis benefits from the immunosuppressed state resulting from cancer treatment with intensive chemotherapy (Ramirez-Garcia et al., 2016). In addition, there are some studies that have linked the presence of *C. albicans* to the development of oral cancer by producing potent carcinogens such as nitrosamine (O'Grady, et al., 1992; Sankari et al., 2015). Furthermore, it has been suggested that *C. albicans* can promote carcinogenesis by mediating production of strong proinflammatory cytokines (Ramirez-Garcia et al., 2016).

Human papillomavirus (HPV) infection, mainly by HPV-16 or -18 types, has been increasingly linked to HNSCC, with significantly improved prognostic outcomes in HPV-positive patients compared to the HPV-negative counterpart group (Marur et al., 2010; Isayeva et al., 2012). HPV-positive carcinogenesis is linked most predominantly with

oropharyngeal SCC (OPSCC) but its role in the OTSCC remains uncertain (Isayeva et al., 2012). In fact, DNA from HPV has been identified in the oral cavity of 1680 healthy adults with HPV16 as the most frequently detected subtype (Kreimer et al., 2011). A meta-analysis study comprising patients with OSCC (n = 1885) and OPMD (n = 956) concluded a significant association between HPV and OSCC (OR = 3.86; 95% CI: 2.16-6.86); and OPMD (OR = 3.87; 95% CI: 2.87-5.21), particularly oral leukoplakia, OLP, and OED (Syrjänen et al., 2011). In another systematic review, the weighted prevalence of HPV DNA detection in 4 195 oral cavity cancer patients was 20.2 % of which HPV16 was the most commonly detected subtype (Isayeva et al., 2012). In contrast with these findings, no HPV activity was identified in a Norwegian cohort of 146 OTSCC samples (Søland et al., 2021). Furthermore, no tumours were positive for HPV by DNA in situ hybridization, and only two cases demonstrated consistently intense p16-staining in >70% of cancer cells (Søland et al., 2021). Recently, Sahovaler et al. analysed data from 24 clinical studies representing 24 854 patients (Sahovaler et al., 2020). The authors found that patients with HPV-positive non-oropharyngeal SCC tumours had worse disease-free survival (DFS; HR, 1.81; 95% CI, 1.12-2.91; I2 = 47%) but these had no effect on the overall survival (OS). On the contrary, laryngeal and hypopharyngeal HPV-positive tumours were associated with improved OS (HR, 0.71; 95% CI, 0.54-0.92; I2 = 38% and HR, 0.60; 95% CI, 0.47-0.76; I2 = 0%), respectively (Sahovaler et al., 2020).

e) Other risk factors

Other potential risk factors for OTSCC may include oral hygiene and diet. Poor oral hygiene has been reported as a potential contributor to oral carcinogenesis, where poor condition of the oral cavity, lack of tooth brushing, and less frequent dental visits were associated with a greater incidence of oral cancer cases independently of tobacco and alcohol consumption (Guha et al., 2007; Marques et al., 2008). Also, it seems clear that chronic traumatic lesions of the oral mucosa, such as those arising from sharp teeth or restorations, may contribute to oral cancer risk in the presence of other confounding factors such as tobacco use and alcohol drinking (Piemonte et al., 2010).

Poor dietary intake has also been suggested as a possible risk factor of many cancers including OTSCC, while the consumption of more fruit and vegetables is associated with a reduced risk of oral cancer. Interestingly, Pavia et al. concluded that each portion of fruit consumed per day can significantly reduce the risk of oral cancer by 49%. Moreover, their meta-analysis showed a significant reduction in the overall risk of oral cancer of 50% for vegetable consumption (Pavia et al., 2006).

5.1.3. Clinical manifestations

OTSCC has a general predilection for older men (> 50 years-old age), with an increasing incidence pattern in women and younger age groups in recent years (Ng et al., 2017). The early stages of OTSCC are typically asymptomatic. However, as the lesion develops and

invades locally, patients usually experience pain that ranges from mild discomfort to severe pain, particularly when the intraoral tumour reaches a significant size. Patients may also complain from bleeding, mobility of teeth, dyspnea, speech difficulties, trismus, paraesthesia, dysphagia, and enlargement of cervical lymph nodes (Selvamani et al., 2015; Regezi et al., 2017).

The lesions can be variable in colour and size, and they are observed clinically as well-demarcated, indurated, non-healing ulcerative lesions, red lesions, white lesions, or as a speckled red-and-white lesion on the tongue. Leukoplakia and erythroplakia patches may represent carcinoma in situ or invasive OSCC when biopsied. OSCC lesions present with variable sizes which usually ranges from a few millimetres to several centimetres (≥ 4 cm) in the more advanced cases (Regezi et al., 2017). The most frequently involved sites of oral cancer are the tongue and the floor of the mouth. OTSCC commonly involves the lateral borders of the tongue. Less commonly, OTSCC lesions may also develop on the dorsum surface or the tip of the tongue. Neoplasms that develop in the posterior third of the tongue pose a particular challenge as they are more difficult to observe and usually discovered in advanced stages when the tumour has metastasized locoregionally (Haya-Fernández et al., 2004; Bagan et al., 2010; Selvamani et al., 2015; Regezi et al., 2017).

5.1.4. Histopathological features

OSCC tissue exhibits several histopathological features with varying degrees of differentiation (i.e. its resemblance to the normal oral epithelium). Lesions are graded as well differentiated, moderately differentiated, and poorly differentiated (Barnes et al., 2005) **(Figure 2)**.

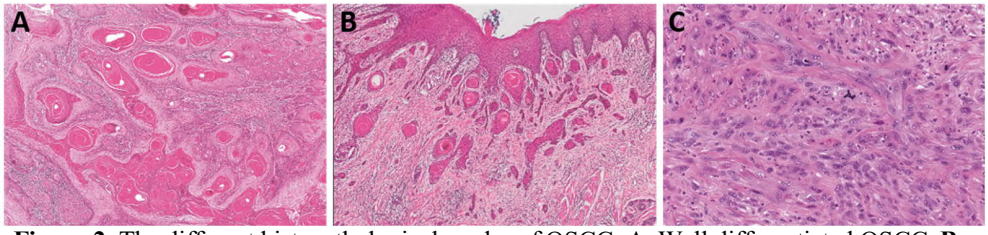

Figure 2. The different histopathological grades of OSCC. **A.** Well differentiated OSCC. **B.** Moderately differentiated OSCC. **C.** Poorly differentiated OSCC. Figure is reproduced with permission from Springer Nature (Speight and Farthing 2018).

In the initial stages of carcinogenesis, the majority of OTSCC lesions show moderately or well differentiated tumour cells with evident foci of keratin pearls. In most cases, OTSCC lesions develop gradually from dysplastic epithelial changes (i.e. mild, moderate or severe dysplasia) to an in situ carcinoma, which can further develop to an invasive tumour. The different histopathological grades of OSCC are defined in **Table 1**. These histological features include cellular pleomorphism, disorganized cellular growth,

dyskeratosis (i.e. pre-mature keratinization in single cells), loss of cellular polarity, higher nuclear-to-cytoplasmic ratio, irregular nuclear chromatin, distinct eosinophilic nucleoli, and increased mitotic activity. Tumour cells are often seen in small invading nests into the subjacent connective tissue, which may extend to involve salivary excretory ducts (Woolgar and Triantafyllou 2009; Regezi et al., 2017; Ahmed et al., 2019).

Table 1. Classification of oral cancer tumours based on their histopathological differentiation

Degree of differentiation	Features
Well differentiated OSCC	The individual tumour cells have a close resemblance to the squamous epithelial cells of the normal oral mucosa. The tumour contains foci of evident keratin pearls. The cells have well-defined outlines with low mitotic rate. Cellular pleomorphism, nuclear pleomorphism and atypical mitoses are rarely seen.
Moderately differentiated OSCC	Tumour cells show less keratinization with more mitotic figures. Tumour tissue shows more cellular and nuclear pleomorphism. A few atypical mitotic figures may present in the tissue.
Poorly differentiated OSCC	OSCC tissue shows nests of atypical tumour cells that lack keratinization with high mitotic figures. Tumour cells show more frequent variation in cell size and shape. Nuclear pleomorphism and atypical mitoses are common.

According to the World Health Organization (WHO), six microscopic variants of SCC have been described in the oral cavity (Thompson 2006; Barnes et al. 2005; Garcia and Crowson 2011; Regezi et al., 2017), including:

Verrucous carcinoma: is characterized by very well-differentiated SCC cells with evident keratin pearls and rare mitoses. Verrucous carcinoma has a key feature of a broad pushing invasive front, which is commonly surrounded by a lymphocytic inflammatory cell response.

Papillary SCC: resembles the verrucous variant but shows fewer differentiated cells with a papillary growth pattern, low keratinization, and foci of necrosis. Papillary SCC has a dense lymphocytic inflammatory cell response at the tumour-stroma interface.

Basaloid SCC: this subset comprises two tumour cell components (biphasic): basaloid cells and SCC cells. Basaloid SCC is characterized by the presence of periodic acid-Schiff (PAS)-positive and Alcian blue-positive material within evident cystic spaces.

Spindle cell carcinoma: biphasic, consists of two tumour cell compartments: spindle cells, which forms the bulk of the tumour, and SCC cells.

Adenosquamous carcinoma: biphasic, consists of SCC and gland-like, tubular, mucin-producing adenocarcinoma cells.
Acantholytic SCC: this variant is also called adenoacanthoma, or pseudoglandular SCC because of the presence of foci of acantholysis in tumour nests and cords, mimicking a glandular structure.

5.1.5. Diagnosis

Early inspection by a specialist of mucosal changes in the tongue that persist for more than two weeks is important. Indeed, early diagnosis of OTSCC is considered as a key factor to reduce the risk of metastasis and therefore improve cancer-specific mortality rates (van der Waal et al. 2011). In spite of the clear clinical characteristics of oral cancers, particularly at advanced stages which can facilitate a clinical diagnosis, biopsy remains the "gold standard" to confirm the diagnosis (Wolff et al., 2012; Macey et al., 2015). However, detecting OSCC in the early stages with standard visualisation techniques can be difficult because the lesions are often asymptomatic with only small epithelial changes (Muto et al., 2004; Bagan et al., 2010). Therefore, other visualization techniques such as vital tissue staining, fluorescence and narrow band imaging can be applied for the early detection of OSCC and OPMD-related changes. Exfoliative cytology (aka oral brush biopsy) and fine needle aspiration have also been employed in diagnosing a spectrum of different lesions in the oral cavity including OSCC. However, it has been concluded that none of the adjunctive diagnostic tests can be recommended as a substitute for the currently employed standard of biopsy and histological assessment (Macey et al., 2015; Carreras-Torras and Gay-Escoda 2015).

Despite the convenient clinical accessibility to the oral tongue for physical examination, the majority of OTSCC patients present with advanced diseases locoregionally at the time of diagnosis. Furthermore, the incidence of synchronous (i.e. second or multiple) tumours is not uncommon in OTSCC patients, particularly in the advanced stages, and the incidence has been estimated between 4 – 33% (Haughey et al., 1992; Cianfriglia et al., 1999). The 5-year survival rate of those patients who developed synchronous tumours was significantly decreased. Thus, it has been recommended that during the primary diagnosis of OSCC, patients should also be screened by a multidisciplinary team including ear, nose, and throat (ENT) specialists to exclude the presence of such tumours (Jang et al., 2008; Wolff et al., 2012). Along with biopsy and histopathological assessment, several imaging modalities are utilized for the diagnosis of OSCC and potential synchronous tumours. These include magnetic resonance imaging (MRI), ultrasonography, computed tomography and positron emission tomography. The use of these imaging techniques can also improve the overall assessment of tumour stage, vascularity, and dissemination (both regional and distant). However, these modalities may not be sufficient for detecting occult metastases. The risk for occult neck metastasis is considerably high in OSCC patients (estimated between 20 – 40% of cases) and thus elective neck dissection (END) should be considered especially for patients with advanced diseases (Haughey et al., 1992; Cianfriglia et al., 1999;

Jang et al., 2008; Wolff et al., 2012; Thiagarajan et al. 2014; Chi et al., 2015; Pałasz et al., 2017).

5.1.6. Staging

Cancer staging is a crucial part of the diagnosis process that is necessary to guide clinical care and decision making in addition to assessment of prognosis and treatment outcomes (Brierley et al., 2016). The TNM classification of malignant tumours was first developed by the oncologist Pierre Denoix in the period 1943-1952. This system is used for categorizing malignant neoplasms based on the anatomical extent of the lesions by assessing the primary tumour size or extent (T), status of the regional lymph nodes (N), and the presence/absence of distant metastasis (M). Following the assessment of TNM scores, the data can be grouped together into stages (O'Sullivan et al., 2017). **Tables 2** and **3**.

Table 2. The TNM classification of cancers of lip and oral cavity (the 8th edition)*

T: Primary Tumour	
TX	Primary tumour cannot be measured (no information about the primary tumour)
T0	Primary tumour cannot be found (no evidence for a primary tumour)
Tis	Carcinoma in situ
T1	Tumour is ≤ 2 cm and depth of invasion is ≤ 5 mm
T2	Tumour is ≤ 2 cm and depth of invasion is 5 to 10 mm; or Tumour is 2 to 4 cm and depth of invasion is ≤ 10 mm
T3	Tumour is > 4 cm or depth of invasion is > 10 mm
T4a	Tumour has invaded the cortical bone of the mandible or maxillary sinus, or the skin of the face
T4b	Tumour has invaded muscles, pterygoid plates, or skull base, or internal carotid artery
cN: Clinical Regional Lymph Nodes	
cNX	Regional Lymph nodes cannot be measured
cN0	No evidence of regional lymph node metastasis
cN1	Metastasis in a single ipsilateral lymph node (≤ 3 cm; no ENE**)
cN2a	Metastasis in a single ipsilateral lymph node (> 3 cm but ≤ 6 cm; no ENE)
cN2b	Metastasis in multiple ipsilateral lymph nodes (≤ 6 cm; no ENE)
cN2c	Metastasis in bilateral or contralateral lymph nodes (≤ 6 cm; no ENE)
cN3a	Metastasis in a single lymph node (> 6 cm; no ENE)
cN3b	Metastasis in a single or multiple lymph nodes with evident ENE
M: Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis
pN: Pathological Regional Lymph Nodes	
pNX	Regional Lymph nodes cannot be measured
pN0	No evidence of regional lymph node metastasis
pN1	Metastasis in a single ipsilateral lymph node (≤ 3 cm; no ENE)

pN2a	Metastasis in a single ipsilateral lymph node (< 3 with ENE; or ≤ 6 cm but without ENE)
pN2b	Metastasis in multiple ipsilateral lymph nodes (≤ 6 cm; no ENE)
pN2c	Metastasis in bilateral or contralateral lymph nodes (≤ 6 cm; no ENE)
pN3a	Metastasis in a single lymph node (> 6 cm; no ENE)
pN3b	Metastasis in a single lymph node (> 3 cm with ENE) or multiple ipsilateral, contralateral or bilateral node(s) with ENE

*Modified from Brierley et al., 2017. ENE, extranodal extension. **ENE indicates the presence of skin or soft tissue invasion with deep fixation to underlying structures or clinical signs of nerve involvement.

Cancer is categorized into four distinct stages (0 to IV), where the value (0) indicates the score of Tis (i.e. carcinoma in situ), in which the lesion is separated from the tissue parenchyma by basement membrane. As the tumour grows and invades locoregionally, the staging numbers also increase accordingly (T1 to T4, N1 to N3, and M1) with the most advanced status designated as stage IV. When there is no evidence of a primary tumour, it can be designated (T0); or (TX) when it cannot be assessed (Brierley et al., 2016; O'Sullivan et al., 2017; Rosen and Sapra 2021).

There are different types of TNM classifications described for malignant tumours including, among others, the clinical and pathological classification. In the clinical classification (cTNM), the scores are obtained before initiating the treatment, and it is based on pre-surgical diagnostic approaches such as physical examination, imaging, endoscopy etc. The pathological classification (pTNM) is a histopathological scoring that is conducted post-surgically to provide prognostic estimates and guide adjuvant therapy if needed (Brierley et al., 2017).

Table 3. The TNM staging system of cancers of lip and oral cavity (the 8th edition)*

OTSCC stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1-T3	N1	M0
IVA	T4a	N0-N1	M0
	T1-T4a	N2	M0
IVB	any T	N3	M0
	T4b	any N	M0
IVC	any T	any N	M1

*Modified from Brierley et al., 2017. Tis: carcinoma in situ.

5.1.7. Prognosis and Management

In spite of the notable improvement in the treatment of cancers over the past decades, the 5-year survival rate of OSCC patients has remained at around 50-65%, and the involvement of

cervical lymph nodes can significantly decrease the patient's survival. The principal treatment modality of OSCC is determined based on the stage of the disease, which therefore necessitates an accurate staging obtained from the clinical and imaging evaluation. Importantly, the chosen modality should also aim, as much as possible, to preserve the vital functions of the oral cavity to reduce the post-treatment morbidity. Surgical resection is the mainstay treatment approach for OSCC. However, the multidisciplinary team may opt for a multimodality management plan for very large tumours or for patients with a tumour spreading locoregionally. This approach may comprise surgery in addition to radiotherapy or chemo-radiation (Sutton et al., 2003; Omura 2014; Chi et al., 2015; Johnson et al., 2020).

The END has been indicated for the management and the detection of possible occult metastasis, since occult metastases in the draining cervical lymph nodes might be present even in OSCC patients with small tumours. Noteworthy, the overall incidence of occult lymph node metastasis in early-stage OSCC patients is about 23% and mainly in patients with T2 rather than T1 tumours (Massey et al., 2019). The main benefit of establishing END as a routine practice is the early detection of occult metastasis, which can improve the management plan and prognosis. However, the END can be associated with a significant morbidity and dysfunction. Minimally, the sentinel node biopsy should be used as a reliable approach for early-stage patients with a radiologically N0 neck to detect occult metastases with a sensitivity that reaches up to 94% (Sutton et al., 2003; Omura 2014; Liu et al., 2017; Schilling et al., 2017; Yang et al., 2020; Jain et al., 2020; Johnson et al., 2020). However, if the sentinel node biopsy gives even a single micro metastasis, END or radiation therapy for the neck is needed (Stoeckli 2007).

Inadequate removal of the tumour tissue can increase the risks of developing recurrent tumours and reduce the survival expectancy. Thus, the surgical resection should aim to remove the neoplasm with a safe, tumour-free, margin of normal tissue (i.e. clear or negative margins) while optimizing preservation of functionality and aesthetic. Microscopically positive (tumour cells are seen at the biopsy edges) and close (tumour cells are close to the edge) surgical margins may require more surgical resections. However, there is a lack of consensus regarding the standard "close" margin width, although it has been suggested to be between 1-5 mm. Oral cancer patients with positive surgical margins (< 1 mm) were found to have shorter DFS and OS compared to those with close margins (1-5 mm). It was also found that invasive OSCC cells within 1 mm of the surgical margin were associated with a significantly higher local recurrence rate (Tasche et al., 2017). In another study on patients with OTSCC, the local recurrence-free survival was significantly affected only with surgical margins of ≤ 2.2 mm (Omura 2014; Zanoni et al., 2017; Jain et al., 2020).

5.2. Tumour microenvironment (TME)

The relationship between the role of the tissue surrounding the tumour in the process of tumorigenesis dates back to 1863 when Rudolf Virchow proposed a link between chronic inflammation and cancer development. The tumour microenvironment (TME) is a term that

describes the environment surrounding the tumour that results from the interaction of tumour cells with the host's tissue during the course of tumour progression. The TME landscape consists of numerous heterogeneous cell types including actively growing tumour cells, infiltrating immune cells (e.g. T lymphocytes, dendritic cells, mast cells (MCs), and macrophages), and stromal cells (e.g. cancer-associated fibroblasts (CAF) and vascular endothelial cells (EC)). Additionally, the TME consists of the tumour-associated acellular part including a wide variety of molecules such as cytokines, growth factors, etc. Importantly, intracellular components (e.g. vimentin and actin) and extracellular components (e.g. collagen, fibrin and laminin) play a key role in TME and can mediate cancer growth and progression. There is accumulating evidence that supports the role of the TME in orchestrating the different aspects of tumorigenesis including tumour initiation, invasion, metastasis, and resistance to cancer therapy. Recently, there has been an increasing interest in the therapeutic potential of the TME. In fact, targeting the TME could improve the efficacy of existing anticancer therapies and may introduce new therapeutic opportunities (Whiteside 2007; Whiteside 2008; Jin and Jin, 2020; Sahai et al., 2020; Hirata et al., 2017).

Relevant and well-designed preclinical models that mimic the three-dimensional (3D) tumour microenvironment matrix (TMEM) are required for the development of novel therapies against cancer. However, the translational cancer research field has long been lacking in vitro models that closely resemble the human TMEM, since the majority of the commercially available matrices (e.g. Matrigel®) are derived from animal tumours (Roma-Rodrigues et al., 2019). Despite the important contribution of animal-based models to cancer research, these models remain inadequate in mimicking human TMEM, which can affect, for example, the screening of cancer cell sensitivity to the potential drugs. Therefore, human-based 3D models, such as organoids, have become popular in cancer research since they represent more a physiologically relevant environment to tumour cells and thereby enable a better prediction of their response to the tested drugs (Drost and Clevers, 2019). The development of these in vitro tumour models not only enhanced the predictability of anticancer drugs but also has a positive environmental impact by reducing the need for future animal testing (Drost and Clevers, 2019). In this regard, our research group has developed a novel matrix derived from human benign leiomyoma "Myogel" (Salo et al., 2015). Interestingly, Myogel has shown an excellent reproducibility of human TMEM for studying different cancer-related properties of HNSCC cells in vitro (Salo et al., 2015). Furthermore, HNSCC cells grown on Myogel showed more relevant responses to those reported in the clinical trials of epidermal growth factor receptor (EGFR)-targeted monotherapy compared with other culturing conditions (Tuomainen et al., 2019). Currently, our group is developing another novel TMEM designed to assess the properties of cancer cells in metastatic tumours. The new matrix, named Lymphogel, is produced from regional lymph nodes of HNSCC patients.

5.2.1. TME-related angiogenesis and non-angiogenic mechanisms

During the early stages of epithelial tumours (e.g. carcinoma in situ), the underlying vascularized stroma is separated from the tumour by a basement membrane, which makes the carcinoma less accessible to blood sources. Therefore, in order to grow and progress, tumour tissue requires induction of an angiogenic switch with the subsequent development of tumour-related vascular networks. Indeed, sustained tumour-related angiogenesis is considered one of the main hallmarks of cancer. However, the patterns of tumour vascularization vary according to the tumour type, stage, site, and whether it is primary or metastatic. The TME contains ECs and they form the tissue blood vessels that are vital for delivering oxygen and nutrients to the tumour. In addition, the tumour-related vasculature removes the metabolic waste products and provides an escape route for metastatic tumour cells. Hypoxia is a key driver of tumour angiogenesis (Hanahan and Weinberg 2000; De Palma et al., 2017).

At the initial stages of tumorigenesis, tumour cells may adopt the pre-existing vessels in the host tissue. However, as the tumour grows in size, these vessels become unable to meet its nutrient needs. Therefore, hypoxic tumour cells modulate their microenvironment by secreting a plethora of molecules and paracrine signals (e.g. vascular endothelial growth factor; VEGF) to the EC for the induction of angiogenesis. A tumour cell-derived VEGFA, for instance, can bind VEGF receptor 2 expressed on the EC membrane to initiate angiogenesis. Other tumour-associated stromal cells, such as CAFs, have also been implicated in the formation of tumour vasculatures by releasing angiogenic growth factors and proteases (e.g. matrix-metalloproteinase-9, MMP-9). MMP-9 can break down the basement membranes and facilitate angiogenesis and tumour invasion (Stuelten et al., 2005; Orimo et al. 2005; Watnick 2012; De Palma et al., 2017).

A novel concept of tumour-related non-angiogenic mechanisms was first introduced by Maniotis et al. in 1999. In this new phenomenon, which has been termed "vasculogenic, or vascular, mimicry" (VM), the authors showed that aggressive melanoma cells are able to form *de novo*, matrix-rich, vessel-like networks on a 3D matrix in vitro (Maniotis et al., 1999). These vessel-like channels are believed to be used by the aggressive tumours to secure additional sources of nutrition and also to provide an escape route for metastasis **(Figure 3)**.

The VM channels are traditionally characterized in HNSCC samples as red blood cell (RBC)-containing lumens with PAS-positive basement membrane and negative EC-specific markers such as CD31/CD34. Also, the pan-cytokeratin (CK)-positive and CD31- or CD34-negative pattern was used to characterize VM structures in OSCC specimens (Shieh et al., 2004; Liu et al., 2008; Valdivia et al., 2019). Importantly, a positive expression of VM lumens was significantly associated with worse OS rates (HR = 0.50; 95% confidence interval: 0.38–0.64) in patients with HNSCC or oesophageal SCC (Hujanen et al., 2020; Salem and Salo, 2021a).

Recently, our group has suggested a potential novel mechanism of lymphatic metastasis in HNSCC, which was named lymphatic mimicry (Karinen et al., 2021). It is a

process whereby tumour cells form a mosaic pattern of lumens positive for both CK and lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1). These endothelial-like vessels were observed both in vitro and in vivo and were negative for other lymphatic cell markers other than LYVE-1. Interestingly, LYVE-1-silenced cancer cells had less capacity to generate these vessels in vitro and to metastasize in vivo. These important developments in the field of tumour-related angiogenesis and lymphangiogenesis could provide avenues for developing promising anti-cancer drugs in future.

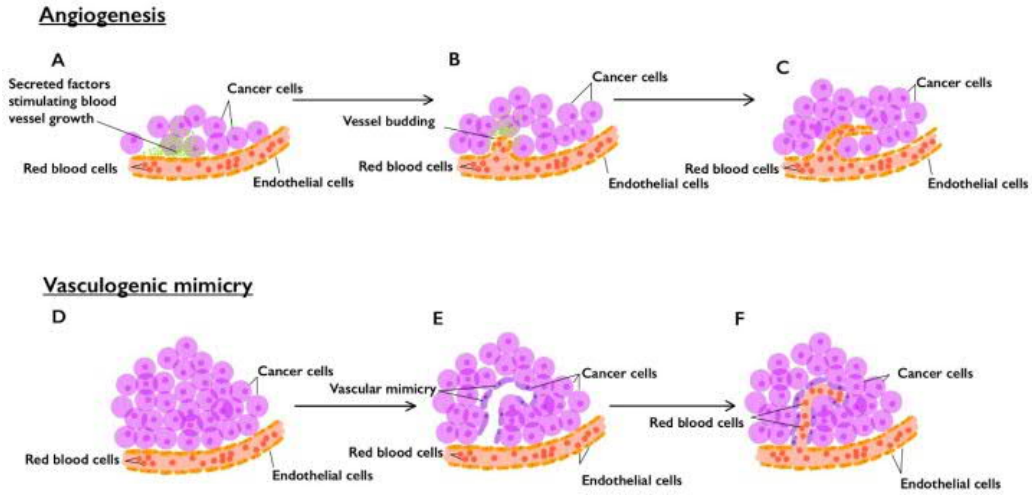

Figure 3. Angiogenesis versus vasculogenic mimicry (VM) in the tumorigenesis of solid tumors (Hujanen et al., 2020).

5.2.2. Immune cells in the TME

A critical compartment of the TME is the infiltrating immune cells, which include cells involved in innate immunity such as macrophages and polymorphonuclear leukocytes. Although they were initially thought of as allergy mediators, MCs have been recently, and increasingly, studied as a powerful regulator of tumorigenesis. Interestingly, our group showed that degranulated MCs were significantly increased in OTSCC patients, and that MC-derived mediators were able to regulate the expression of several genes critical for oral carcinogenesis (Salem et al., 2017; Salem and Salo, 2021b). Furthermore, MCs are implicated in tumour-related angiogenesis, and they were significantly associated with the microvessel density (MVD), depth of wall invasion, lymph node metastasis, and tumour progression in patients diagnosed with SCC of the oesophagus (Elpek et al., 2001). However, the exact role of MCs in the TME is not yet fully understood, since they tend to have a dual role by stimulating tumour growth in some cases and suppressing it in others (Varricchi et al., 2017; Ribatti et al., 2018).

In addition, the TME contains immune cells specified for adaptive immunity, primarily the tumour-infiltrating T lymphocytes which also serve as an independent prognostic factor in patients with HNSCC. In this regard, recent reports concluded that higher levels of T lymphocytes within the TME have a beneficial role and associate with better survival outcomes in HNSCC patients. These findings suggest that an immunophenotype-based prognostic score, also referred to as the immunoscore, may have a better prognostic value than traditional cancer staging (Whiteside, 2007; Balermpas et al., 2014; Nguyen et al., 2016; Spector et al., 2019; Galli et al., 2020).

The effector T cells include CD8+ cytotoxic T cells and CD4+ helper (Th) cells, which constitute the most efficient mediators of the adaptive anti-tumour immunity. CD4+ T cells play key functional roles in orchestrating the adaptive immune system through influencing and maintaining the CD8+ T-cell responses; helping B cells to generate antibodies; regulating macrophage activity; enhancing the immunologic memory; and maintaining self-tolerance by controlling/suppressing the immune response. After interacting with antigen-presenting cells (e.g. dendritic cells), naive CD4+ T cells became activated and may differentiate into one of several effector subsets, with distinct cytokine expression profiles and functions, such as Th1, Th2, T regulatory (Treg) and Th17. In fact, a successfully functioning CD4-mediated immune response relies on an adequate and complex equilibrium among these different subsets (Zhu et al., 2010; Gajewski et al., 2013; Galli et al., 2020).

Th1 and Th2 clones could be discerned based on their cytokine signatures. Of these, interferon gamma (IFNγ) and IL-2 are the main cytokines of Th1, which are crucial for eliminating intracellular pathogens and activating macrophages. Additionally, Th1 cells produce other important cytokines such as tumour necrosis factor (TNF), lymphotoxin, and granulocyte-macrophage colony-stimulating factor. On the other hand, Th2 cells are best known for their role in allergic/atopic disorders and mediating the immune response against parasites by releasing potent cytokines including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. In general, Th2 cells tend to promote anti-inflammatory responses based on their ability to counteract the Th1-mediated reactions. It is noteworthy that evidence for a shift toward the Th2-cytokine response has been reported in patients with HNSCC showing elevated levels of IL-4, IL-10 and TGFβ accompanied by a decrease in the Th1 mediator, IFNγ (Raphael et al., 2015; Maggioni et al., 2017; Knochelmann et al., 2018; Galli et al., 2020).

The highly immunosuppressive Treg cells represent another subset of CD4+ T cells, which can hinder the anti-tumour immunity by suppressing the CD8-driven cytotoxic immune response in the TME. Treg cells express the Forkhead box protein P3 (FOXP3) and CD25 molecules, and they become activated by transforming growth factor beta (TGF-β) and IL-2 to induce a peripheral immune tolerance. In the course of tumorigenesis, Tregs can suppress the immune surveillance which may result in tumour immune escape. However, due to the substantial diversity of Treg functional pathways, their

contribution in carcinogenesis is not yet fully understood (Sakaguchi et al., 2008; Knochelmann et al., 2018; Zhu et al., 2010; Galli et al., 2020).

5.3. T helper 17 cells and their cytokines

Th17 cells are a more recently recognized subset of CD4+ T cells, which show a significant degree of plasticity in their cytokine profile and functions. Th17 cells can be found in normal mucosal tissues (e.g. in the colon); however, their number is highly elevated in inflammation and infection. Primarily, the Th17-derived cytokines serve as potent proinflammatory mediators against eukaryotic pathogens such as extracellular bacteria and fungi. In addition to IL-17, Th17 cells were shown to produce other cytokines such as IL-21 and IL-22. The IL-17 family currently consists of six subsets of cytokines that mediate both acute and chronic inflammatory reactions: IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Although Th17 has long been considered the major source of IL-17 cytokines, other cell types were shown to express IL-17, including cells of the innate immunity system such as neutrophils (Korn et al., 2009; Gu et al., 2013; Raphael et al., 2015; Stockinger and Omenetti, 2017).

IL-17A (or IL-17) is the most widely studied member of the IL-17 family. The IL-17 receptor (IL-17R) constitutes a family of 5 receptors that share sequence homology, including: IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. Although IL-17RA is widely expressed in many tissues and cell types, it cannot mediate the IL-17 response alone, but rather through a heterodimeric receptor complex composed of both IL-17RA and IL-17RC. Functionally, IL-17A is a potent cytokine that mediates the host's proinflammatory response against invading pathogens by stimulating the secretion of a plethora of molecules including anti-microbial peptides and MMPs. However, the dysregulated production of this cytokine in tissue may result in excessive tissue damage, which has been implicated in different disorders including autoimmune diseases (e.g. multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus) and cancer (discussed in the next section). Interestingly, these pathogenic effects of IL-17 had led to the development of clinically approved anti-IL17 targeted therapies. Currently, IL-17 inhibitors (i.e. secukinumab and ixekizumab) are used for the treatment of three chronic immune disorders: psoriasis, psoriatic arthritis, and ankylosing spondylitis. For example, secukinumab produced a >80% response rate in patients with moderate-to-severe psoriasis (Gu et al., 2013; Raphael et al., 2015; Chang 2019; Miossec 2021).

The newest member of this cytokine family, IL17F, exhibits the highest sequencing homology with IL-17A (about 56% homology), and they both use the same receptor complex to mediate IL-17 signalling. However, IL-17F shows a ~100 to 1000 times lower binding affinity than 17A to IL-17RA. But both cytokines bind IL-17RC with a closely similar affinity. IL-17F and IL-17A are both major mediators of the inflammatory response against extracellular bacterial and fungal infections by producing anti-microbial peptides and recruiting neutrophils (Gu et al., 2013). Thus, persistent mucosal infections, such as

chronic mucocutaneous candidiasis, were reported in patients with inborn autosomal dominant IL-17F deficiency and mutations that inhibit IL-17 immunity (Puel et al., 2011). In spite of the similarities between them, it has been shown that IL-17A and F are produced by different cell types and may mediate disparate roles in the host tissue. While IL-17A was secreted mainly by CD4+ T cells, IL-17F was found to be produced by CD4+ T cells, neutrophils, mast cells, and epithelial cells (Ishigame et al., 2009). Furthermore, Yang et al. showed that IL-17A, but not IL-17F, was able to induce an experimental autoimmune encephalomyelitis. In an asthma model, IL-17F-Knockout mice promoted Th2 cytokines and eosinophil function, whereas IL-17A deficiency reduced these responses (Yang et al., 2008).

5.3.1. The role of interleukin 17 in cancer

In addition to a role in immune disorders, pathological production of IL-17 has recently emerged as an important player in different stages of carcinogenesis. Th17 cells are found within a wide variety of tumours such as melanoma, ovarian, liver and colorectal cancer. These cells can sustain chronic, unresolved, inflammation and tissue damage that could possibly lead to tumour initiation and progression (Chang 2019; Zhao et al., 2020; Kuen et al., 2020). Of all the IL-17 family members, IL-17A was the most investigated cytokine in cancer.

Extensive pulmonary inflammation has been linked to lung carcinogenesis. In a mouse model of lung cancer, Th17 and Treg cells, but not Th1, were significantly increased in the tumour tissues. In addition, the loss of IL-17A, but not IL-17F, caused a significant reduction in the frequency of lung tumours in mice challenged with a procancerous pathogen (NTH-influenza). Conversely, loss of IL-17A not only diminished tumour cell proliferation and angiogenesis, but also reduced the expression of key proinflammatory mediators as well as the recruitment of myeloid cells (Chang et al., 2014). In agreement with these findings, IL-17A-deficient mice had a significantly lower incidence of chemically induced HCC (Sun et al., 2016). Zhang et al. showed that when pancreatic tumour cells were exposed to IL17A and injected into immunocompetent mice, they formed tumours significantly faster than controls (Zhang et al., 2018). It has also been demonstrated that IL-17A stimulation in vitro promoted the proliferation of human nasopharyngeal carcinoma (Cai et al., 2017).

In colon cancer, IL-17A was shown to target epithelial cells and promote tumorigenesis in a mouse model of microbe-induced tumorigenesis (Chung et al., 2018). Furthermore, the use of exogenous IL-17A increased the self-renewal and invasion capacity of cancer-initiating cells in human colorectal cancers. Additionally, chemotherapy-treated colorectal CAFs had augmented expression of IL-17A and promoted cancer cell growth in vitro which suggests a link to chemotherapy resistance (Lotti et al., 2013). Wei et al. reported a significant increase in IL-17A levels in serum and tumour samples from OTSCC patients, which also positively correlated with tumour metastasis and clinical stage. Additionally, IL-17A increased OSCC cell migration and invasion in vitro (Wei et al., 2017). In support of

these interesting studies, a systematic review found that increased levels of serum IL-17 were associated with poor patient survival in different types of solid tumours. However, in the same study, higher Th17 cell frequency was found to be associated with improved prognosis (Punt et al., 2015).

Studies investigating the role of IL-17F in cancer are far fewer than those of IL-17A, which could be a result of the recent identification of IL-17F. Moreover, IL-17A signalling is about twenty times more potent than IL-17F, which may explain the widespread involvement of IL-17A in many tissues compared with IL-17F which is implicated mainly in mucosal immunity (Vitiello and Miller 2020). However, unlike IL-17A which is best known for promoting carcinogenesis, IL-17F seems to play both anti- and protumorigenic roles in different neoplasms. Therefore, more studies are still needed before its actual role in cancer can be determined. Importantly, IL-17F has been recognized for its inhibition of angiogenesis, which could make it a potential anti-angiogenic therapy for cancer. In HCC, IL-17F-transfected human cancer cells exhibited a significant decrease in tumour size and MVD when xenografted into nude mice. Interestingly, the diminished tumour growth in vivo was associated with evident inhibition of vascular EC and as well as the key proangiogenic cytokines IL-6, IL-8, and VEGF (Xie et al., 2010).

Several studies reported a reduced expression of IL-17F in the tumour tissue from patients with colorectal cancers compared with the healthy controls (Liu et al., 2018; Al-Samadi et al., 2016; Tong et al., 2012). Furthermore, Tong et al. showed a decreased tumour growth pattern of IL-17F-transfected cancer cells when xenografted into nude mice. In contrast, following tumour induction, IL-17F(-/-) mice showed more colonic tumours and tumour areas compared with the wild-type controls. The authors concluded that IL-17F tends to play an inhibitory role in colon tumorigenesis (Tong et al., 2012). In OSCC patients, the serum levels of IL-17F were assessed and found to be significantly downregulated in cancer patients compared with the levels found in the healthy group (82.96 pg/ml; 394.3 pg/ml, respectively) (Ding et al., 2015). In contrast to these findings, Chen et al. showed recently that IL-17F expression was induced in tumour mucosa of colorectal cancer compared with the paired non-tumour tissue. Moreover, recombinant human IL-17F protein promoted cancer cell migration and invasion in vitro (Chen et al., 2019). In another study on colorectal cancer patients, the advanced stage IV was associated with elevated tumour levels of Th17-derived cytokines including IL-17F (Sharp et al., 2017). However, Lereclus et al. did not find any association between IL-17F levels and survival parameters (e.g. OS or progression-free survival, PFS) among colorectal cancer patients (Lereclus et al., 2017).

Overall, despite the well-recognized involvement of IL-17A and IL-17F in carcinogenesis, the exact role of endogenous IL-17F in tumour immunity remains undefined. Therefore, more functional studies using in vitro and in vivo models are needed to better understand the underlying mechanisms that control the role of IL-17F in cancer.

6. STUDY OBJECTIVES

In spite of the growing evidence of IL-17F involvement in the tumorigenesis of several cancers, its potential role and prognostic value in OTSCC are not known. Therefore, we set the following objectives:

1) To investigate the expression levels and cellular sources of IL-17F in tissue samples from OTSCC patients and to evaluate its potential association with the patients' prognosis.
2) To assess the effects of IL-17F on several tumorigenic features of OTSCC including cell proliferation, migration, invasion and angiogenesis.
3) To test the possible effect of IL-17F on the formation of tumour cell-derived VM.
4) Finally, and based on the anti-angiogenesis role of IL-17F, to systematically review the prognostic value of MVD and LVD in patients with tongue cancer.

7. PATIENTS, MATERIALS AND METHODS

7.1. Patient samples and ethical permission (studies I, III)

In this research project, we used formalin-fixed & paraffin-embedded (FFPE) samples obtained from OTSCC patients (study **I**, n = 83; study **III**, n = 30). These patients were treated surgically at Oulu and Tampere University Hospitals during the period of 1990-2010. All patients provided their written informed consent before participating in the research study. The data inquiry was approved by the National Supervisory Authority for Welfare and Health (VALVIRA) and the Ethics Committee of the Northern Ostrobothnia Hospital District (statement #8/2006 and amendment 19/10/2006). The clinicopathological features of the cohort used in study I are presented in **Table 4**.

Table 4. The clinicopathological features of the OTSCC cohort used in study I

Patient group	No. of patients
All patients	83
Age (years)	
Average	64.23
More than 60	29
Less than 60	54
Gender	
Male	48
Female	35
Tumour Grade	
I	21
II	53
III	9
Tumour Stage	
I-II	49
III-IV	34
Cervical Lymph Nodes	
Positive	33
Negative	50
Recurrence	
No	47
Yes	36

7.2. Orthotopic mouse model of tongue cancer (study III)

A mouse model of OTSCC was prepared as described earlier (Åström et al., 2017). Briefly, the OTSCC-derived HSC-3 cells were detached and mixed with cold Matrigel 1:1 to make 8×10^6 cells/ml. To form a patient-derived xenograft (PDX) mouse model of OTSCC, a suspension of 2×10^5 cells was injected into the lateral border of the tongue of 7-week-old BALB/c nude male mice (Charles River, Germany). Tongues and draining lymph nodes were collected on day 13, fixed in 4% PFA and embedded in paraffin for further

immunostaining. All experiments complied with the ARRIVE guidelines and in accordance with the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes guidelines on accommodation and care of animals. The experiments were also approved by the Animal Welfare Committee at UiT-The Arctic University of Norway.

7.3. Immunohistochemistry IHC (studies I, III)

In study I, The IHC was performed on 5-µm thick FFPE sections using BioCare's Goat on Rodent HRP-polymer kit (Biocare Medical, Pacheco, CA, US). Antigens were retrieved using Dako citrate buffer (Dako Corp., Carpinteria, CA, US) for 15 min in a microwave and then cooled down for 20 min at room temperature (RT). Dako Peroxidase blocking solution was used to block endogenous peroxidase activity. Samples were incubated with 1µg/ml polyclonal goat anti-human IL-17F primary antibody (R&D Systems, Inc., Minneapolis, MN, US) first for 30 min at 37°C then overnight at 4°C. Next, the slides were incubated with the goat probe followed by goat on rodent HRP-polymer each for 15 min at RT. The slides were then incubated with 3,3'-diaminobenzidine (Pierce™ DAB Substrate Kit, Thermo Fisher Scientific) for 5 minutes and washed with distilled water. Finally, the slides were stained with Mayer's haematoxylin solution and mounted in Mountex (HistoLab, Gothenburg, Sweden).

In study III, the slides were incubated with 1:100 diluted polyclonal rabbit anti-human CD31 antibody (Abcam) for 1 hour at RT, followed by horseradish peroxidase (HRP) treatment for 30 minutes at RT. After incubation with DAB as above, the slides were incubated with 0.5% freshly prepared periodic acid for 10 minutes at RT and washed twice with dH2O. Schiff stain was added for 15 minutes, followed by washing under running tap water for 15 minutes. The slides were stained with Cole's haematoxylin for 6 minutes and mounted as above.

7.4. Immunofluorescence staining IF (studies I, IV)

In study I, after deparaffinization and antigen retrieval as mentioned earlier, the slides were incubated in 10% normal serum for 1 hour at RT. Next, the following primary antibodies were used: 1:100 goat anti human, polyclonal, IL-17F (R&D); 1:500 rabbit anti human, monoclonal, mast cell tryptase (Abcam); 1:100 mouse anti human, monoclonal, CD8 (DAKO); 1:80 mouse anti human, monoclonal, CD4 (DAKO); 1:100 mouse anti human, monoclonal, CD56 (DAKO); 1:200 mouse anti human, monoclonal, CD20 (DAKO); 1:200 mouse anti human, monoclonal, CD163 (DAKO). Then, the slides were incubated with fluorescein-conjugated secondary antibodies (Alexa Fluor, Molecular Probes, Leiden, Netherlands) for one hour, followed by nuclei staining with 4',6-diamidino-2- phenylindole (DAPI; Sigma-Aldrich) for 10 min at RT. The slides were mounted in Vectashield® mounting medium (Vector Laboratories, Burlingame, CA). The specificity of each staining was confirmed using proper staining controls.

In study III, FFPE sections from both patients and mouse models were used. After blocking with 10% donkey normal serum for 1 hour at RT, the slides were incubated in 1:200 diluted monoclonal mouse anti-human pan-cytokeratin (M351501-2; Agilent Technologies) combined with either 1:50 diluted polyclonal rabbit anti-human CD31 antibody (ab28364; Abcam); 1:200 diluted polyclonal rabbit anti-human VE-Cadherin (CD144, ab232880; Abcam); or 1:1000 diluted polyclonal rabbit anti-human Von Willebrand Factor (VWF, ab6994; Abcam) overnight at 4°C. Then the secondary antibody incubation and staining steps were followed as above.

7.5. Scoring of IL-17F immunoexpression (study I)

Staining of IL-17F was analysed independently by two authors who were blinded to the clinical data and sample information. Scoring criteria are listed in **Table 5**.

Table 5. Scoring criteria of OTSCC samples

Inflammatory cell distribution pattern	
Score	
0	Inflammatory cells are mainly in tumour area
1	Inflammatory cells are mainly in the invasion front
2	Inflammatory cells are even in the two areas
IL-17F expression by tumour cells	
Score	
0	Negative
1	5-75% of cancer cells are IL-17F^{+}
2	>75% of cancer cells are IL-17F^{+}
Inflammatory cell type (in tumour area and invasive front)	
Score	
1	Predominance of mast cells over inflammatory cells
2	Predominance of inflammatory cells over mast cells
IL-17F localization within mast cells	
Score	
1	IL-17F is expressed both intracellularly and at the extracellular matrix
2	IL-17F is expressed only intracellularly

7.6. Cell lines and culture conditions (studies II, III)

Human OTSCC cell lines

The following OTSCC cell lines were used: HSC-3 (Japan Health Sciences Foundation), SCC-25 (ATCC® CRL-1628™, Rockville MD, USA), and SAS (Japanese Collection of Research Bioresources, Tokyo, Japan) cells. The cell lines were cultured in Dulbecco's

modified Eagle's medium (DMEM)-12 (Gibco). The culture medium was supplied with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml fungizone, 50 µg/ml ascorbic acid, and 0.1% hydrocortisone (all from Sigma-Aldrich, MO, USA). Confluent cells were detached, counted and plated in cell culture well-plates for further stimulations **(Table 6)**.

Table 6. Cell-culture stimulants

Stimulant	Source	Working concentrations
IL-17A	R&D Systems, MN, USA	1,10, 50, 100 ng/ml
IL-17F	R&D Systems, MN, USA	1,10, 50, 100 ng/ml
Sorafenib	LC Laboratories, MA, USA	1, 2.5, 5 µM

Cancer-associated fibroblasts (CAFs)

CAFs were isolated from histologically well-differentiated OSCC and maintained as reported previously (Dourado et al., 2019). Briefly, fragments from normal and cancerous tissues were washed in DMEM containing antibiotics and Mycozap™ 2 µl/ml (Lonza, Basel, Swiss). Next, fragments were cut and incubated in DMEM containing 10% FBS and antibiotics. The media was changed weekly until cells detached and became confluent. CAFs were cultured maximally up to passage 7.

Human umbilical vein endothelial cells (HUVEC)

HUVEC were cultured in 75-cm^2 tissue-culture flasks using 200PRF medium supplemented with low-serum growth supplement (all from Thermo Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Sigma-Aldrich).

Primary human oral keratinocytes (HOKs)

Primary HOKs were purchased from ScienCell (Uppsala, Sweden). The cells were cultured in a 75ml T-flask containing oral keratinocyte culture medium (ScienCell, Uppsala, Sweden) until 80-90% confluence had been reached.

7.7. Polymerase chain reaction PCR

Two PCR techniques were used in this study. GAPDH was used as the housekeeping gene. Primer sequences are listed in **Table 7.**

Quantitative real-time (qRT) PCR

In study II, total RNA was collected from HOKs, HSC-3 and SCC-25 cell lines using an RNeasy Mini-Kit (Qiagen, Düsseldorf, Germany). The complementary DNA (cDNA) synthesis was performed using 200 ng of pure RNA and an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The PCR reaction contained 10 µL iQ SYBR green, 7 µL water, 1 µL of 250 nM primer and 2 µL of first-strand cDNA.

Droplet-digital PCR (DDPCR)

DDPCR was used in study III to verify the expression of the CD31 gene in OTSCC cells. DDPCR is an advanced technology that permits the absolute quantification of even rare DNA targets without the need for standard curves through a water-oil emulsion droplet system (Salem et al., 2019). Total RNA was purified as above. The PCR reaction mix contained QX200™ EvaGreen® ddPCR™ Supermix (Bio-Rad Laboratories), cDNA and CD31 primers. The droplet generator cartridge was loaded with the samples and EvaGreen droplet generation oil. The droplets were then pipetted into a ddPCR™ 96-well plate, sealed and loaded into a T100 thermal cycler with annealing temperature set to 60°C. The plate was then transferred into the droplet reader using the QX200™ Droplet Digital™ PCR Systems. The data were analysed using QuantaSoft software version 1.7.4.0917 (all from Bio-Rad Laboratories).

Table 7. Human primer sequences used in this study

Primer name	Primer sequences	Study
CD-31	Forward: 5′-AACAGTGTTGACATGAAGAGCC-3′ Reverse: 5′-TGTAAAACAGCACGTCATCCTT-3′	II, III
IL-17F	Forward: 5′-CCCTGGAATTACACTGTCACTTGG -3′ Reverse: 5′-GAAAGAAACAGAGCAGCCTTGGTG-3′	II
IL-17RA	Forward: 5′-CTGGTTCATCACGGGCATCTCC-3′ Reverse: 5′-GGTGGTCGGCTGAGT AGATGATC-3′	II
IL-17RC	Forward: 5′-CGTCACTGTGGACAAGGTTCTCG-3′ Reverse: 5′-TCGTGGAGGCTTTGCTGGGT AG-3′	II
GAPDH	Forward: 5′-AAGGTCATCCCTGAGCTG-3′ Reverse: 5′-TGCTGTAGCCAAATTCGTTG-3′	II, III

7.8. Proliferation and random migration assays (study II)

The wells of a 96-well plate were coated with 300 µg/mL Myogel (Salo et al., 2015) and incubated overnight at 37 °C. HSC-3 and SCC-25 cells were added at a density of 2 × 10^3 cells/well and left to adhere overnight. The next day, 100 µL of fresh media with or without IL-17F or IL-17A (at 10, 50, or 100 ng/mL) was added. The plate was placed in an IncuCyte Live-Cell analysis instrument (Essen Bioscience, Ann Arbor, MI, USA) and images were taken every hour for 3 subsequent days. The images were used to measure the proliferation rate (by IncuCyte software, Essen Bioscience) as well as the migration velocity of cancer cells and their accumulated distance.

7.9. The apoptosis assay (study II)

The OTSCC cells were cultured in wells of a 96-well plate (1 × 10^3 cells/well) at 37 °C. On the next day, the media was replaced with 100 µl of fresh media with or without IL-17F at concentrations of 10, 50, or 100 ng/mL combined with the IncuCyte™ Caspase-3/7 Reagent at a concentration of 0.5 µM (Essen Bioscience). Images were taken at a magnification of

10× daily for 3 subsequent days using an EVOS XL Core Cell Imaging System (Thermo Fisher) and the percentages of apoptotic OTSCC cells were calculated.

7.10. Scratch-wound cell migration and invasion assays (study II)

The wells of a 96-well Image-lock plate (Essen Bioscience) were coated with Myogel (300 µg/ml) and incubated overnight at 37 °C. The next day, HSC-3 and SCC-25 cells were seeded on top of the Myogel at a density of 30×10^3 cells/well and incubated overnight. The WoundMaker™ tool from Essen Bioscience was used to make homogeneous scratches in each well. For the migration assay, the cells were stimulated with IL-17F at 10, 50, or 100 ng/ml. For the invasion assay, the wounds were filled with 50 µl of Myogel (2.4 mg/ml)/type 1 rat-tail collagen (0.8 mg/ml). NaOH was used to control the pH of the gel. The gel was allowed to solidify for 30 min before stimulation with IL-17F at 10, 50, or 100 ng/ml as above. The IncuCyte Live-Cell Imaging System was used to monitor the wound confluence, and images were taken hourly for 24 hours.

7.11. Generation of tumour spheroids (study II)

The OTSCC cells (SCC-25 or HSC-3), with or without CAFs, were subcultured and re-suspended in a complete growth medium at 1×10^4 cells/mL and 5×10^3 cells/mL for OTSCC and CAFs, respectively. Cell suspensions were then dispensed at 200 µl/well into ultra-low attachment 96-well round bottom plates, incubated at 37 °C and observed for 4 days. The formation of 300-500 µm diameter spheroids was confirmed under an Eclipse TS100 inverted microscope (Nikon DS-Fi2 camera; Nikon, Tokyo, Japan).

7.12. Spheroid invasion assay (study II)

One hundred µL of 0.5 mg/mL Myogel with type 1 rat-tail Collagen mix was gently pipetted into the spheroid-containing ultra-low attachment plates and incubated at 37°C. HSC-3 or SCC-25 alone or in combination with CAFs were exposed to the same concentration range of IL-17F in the Myogel/Collagen mixture for 5 subsequent days and imaged as above. Cancer cell invasion is calculated as the distance from the centre of the spheroid to the farthest cancer cell.

7.13. Tube-formation assay (study II, III)

One hundred µL of Matrigel® was pipetted into 24-well plates and incubated for 30 minutes at 37°C. Cells were seeded at a density of 8×10^4 cells/well for HUVEC and 12×10^4 cells/well for OTSCC cells. The cells were exposed to the same concentrations of IL-17F. In addition, to test the possible therapeutic effect of the IL-17F, HSC-3 cells were also treated with the clinically approved anti-angiogenic drug (Sorafenib) at 1, 2.5, or 5 µM. The cells were incubated for 8 hours (HUVEC) or 24 hours (OTSCC cells) at 37°C. For the IF assay, cells on Matrigel were cultured on coverslips. Next, the coverslips were washed with PBS and fixed using 4% PFA for 20 minutes at RT. Staining was performed using the same

protocol described above. HUVEC were used as a positive control for CD31 staining. The primary antibodies were replaced with an antibody isotype control for negative control staining.

7.14. Flow cytometry (study IV)

Cultured HUVEC and OTSCC cell lines were fixed with 4% PFA and permeabilized with 0.1% Triton-X for 10 minutes at RT. To perform the fluorescence-activated cell sorting (FACS) analysis, the cultured cells were incubated with 4 µl Alexa Fluor® 488 monoclonal mouse anti-human CD31 (ab187594; Abcam) for 30 minutes at RT, and then centrifuged at 400 g for 5 minutes, resuspended in 1 ml of ice-cold PBS, and run on a BD influx sorter (BD Biosciences, NJ, USA).

7.15. Systematic review protocol and search methods (study IV)

Study IV was registered at the international prospective register of systematic reviews (PROSPERO) with the registration number: CRD42019115141. The literature search was conducted without language restrictions in Ovid Medline, Scopus, and Cochrane Library until the date 28 June 2018. Search terms included: ("tongue") AND ("cancer" OR "neoplasm" OR "carcinoma" OR "squamous cell carcinoma" OR "tumor*") AND ("angiogenesis" OR "blood vessel" OR "lymphangiogenesis" OR "lymphatic vessel" OR "lymph vessel" OR "cd31*" OR "cd34*" OR "cd45*" OR "icam-1*" OR "cd54*" OR "lyve-1*" OR "tie-2*" OR "tek*" OR "vcam-1*" OR "cd106*" OR "ve cadherin" OR "vegf-r2" OR "vascular endothelial growth factor receptor 2" OR "FVIII-RA" OR "FVIII" OR "factor 8" OR "von willebrand factor" OR "vwf" OR "erg" OR "vegf" OR "d2-40" OR "podoplanin" OR "prospero-related homeobox-1" OR "vegf-r3" OR "peripheral node addressin antibody"). The abbreviated and full name of each vessel marker were both used.

Inclusion criteria included: studies using human tissue samples of TSCC patients; studies that investigated the association between expression of blood/lymphatic vessel markers and the clinical features and/or the survival outcomes. Excluded articles included non-original or non-English research studies; studies on other cancer types; non-human samples or lack of prognostic/survival data. Article screening, data extraction and quality assessment were done independently by two authors and the relevant information was extracted from each study. The reporting quality was evaluated according to six-items selected from the reporting recommendations for tumour marker prognostic studies (REMARK) guidelines (McShane et al., 2005). The risk of bias was assessed using the MAStARI evaluation form (Hujanen et al., 2020).

7.16. Microscopy and ImageJ analysis

A Nikon Eclipse TS100 inverted microscope (Nikon Instruments) was used to monitor and image cell culture. A fully automated Leica DM6000 microscope (Leica Microsystems, Wetzlar, Germany) and Leica TCS SP8 confocal microscope (Leica Microsystems) were

used for imaging samples stained with IF and IHC using different magnification powers 10×, 20×, 40×, and 63×. The ImageJ software (Wayne Rasband, National Institute of Health, Bethesda, MD, USA) was used to analyse the images obtained from migration, spheroids, and tube-formation assays. Parameters for tube-formation analysis included: (1) Nodes (represented as pixels with three neighbours as a circular dot, (2) Junctions (corresponding to nodes or a group of fusing nodes), (3) Segments (elements delimited by two junctions), and (4) Meshes (areas enclosed by segments or master segments). The Chemotaxis and Migration Tool (Ibidi, Martinsried, Germany) was employed to measure random cell velocity and accumulated distances.

7.17. Statistical analysis

Independent experiments were repeated at least three times, each in duplicate or triplicate. Data are presented as means ± standard deviations. Data were analysed using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA). Kruskal–Wallis and Friedman tests (study II) or Mann-Whitney *U* test and Friedman tests (study III) were used to determine the statistical significance. Significance was indicated as *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Life tables were calculated according to the Kaplan–Meier method. The survival curves were compared with the log-rank test. Uni- and multivariate survival analysis was conducted with the Cox´s proportional hazards model. P≤ 0.05 was regarded as statistically significant. Statistical significance, which was set to P ≤ 0.05. Significance was indicated as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001.

8. RESULTS

8.1. Mapping inflammatory cells, IL-17F and IL-17F receptors in OTSCC (study I, II)

First, we examined the distribution of inflammatory cells in OTSCC samples using the scoring criteria in **Table 4**. The majority of OTSCC samples showed an even distribution of inflammatory cell infiltrate in tumour and invasive front areas (56.6%; **Table 8**). We next assessed the immunoexpression of IL-17F in both tumour and inflammatory cells. Although some samples had IL-17F-positive tumour cells (24.1%; **Figure 4A**), tumour cells were negative for IL-17F in the majority of the studied OTSCC specimens (75.9%; **Figure 4B**). As expected, inflammatory cell infiltrate showed strong IL-17F expression (**Figure 4C**).

Table 8. Inflammatory cell scoring in OTSCC specimens

Inflammatory cell distribution pattern	
Score	**Number of specimens (%)**
0	14 (16.9%)
1	22 (26.5%)
2	47 (56.6%)
IL-17F expression by tumour cells	
0	63 (75.9%)
1	15 (18.1%)
2	5 (6%)
Inflammatory cell type (invasive front)	
1	50 (60.2%)
2	33 (39.8%)
Inflammatory cell type (tumour area)	
1	14 (16.9%)
2	67 (80.7%)
IL-17F localization within mast cells (invasive front)	
1	54 (65.1 %)
2	29 (34.9 %)
IL-17F localization within mast cells (tumour area)	
1	62 (74.7 %)
2	21 (25.3%)

Interestingly, the majority of MCs expressed IL-17F, which was observed both intracellularly and extracellularly, but the extracellular pattern was the most predominant form in OTSCC samples (**Figure 4D**). Furthermore, the number of these IL-17F-positive MCs was significantly higher in the invasive front area (60.2%; $P<0.0001$; **Figure 4E**). However, CD4+ cells showed less IL-17F staining, and these cells were more common in the tumour area than in the invasive front (80.7%; **Figure 4F**). Other inflammatory cells including CD20+ B cells, CD8+ T cells, and CD163+ macrophages were negative for IL-17F. The scoring results can be found in **Table 8**.

Lastly, we evaluated the gene expression of IL-17F and its receptors in OTSCC cells compared with HOKs. The mRNA level of IL-17F was lower in the invasive HSC-3 compared with the less invasive cells (i.e. SCC-25) and normal HOKs (**Figure 4G**). In addition, IL-17RA and IL-17RC levels were both reduced in OTSCC cell lines compared with normal HOKs (**Figure 4H, I**).

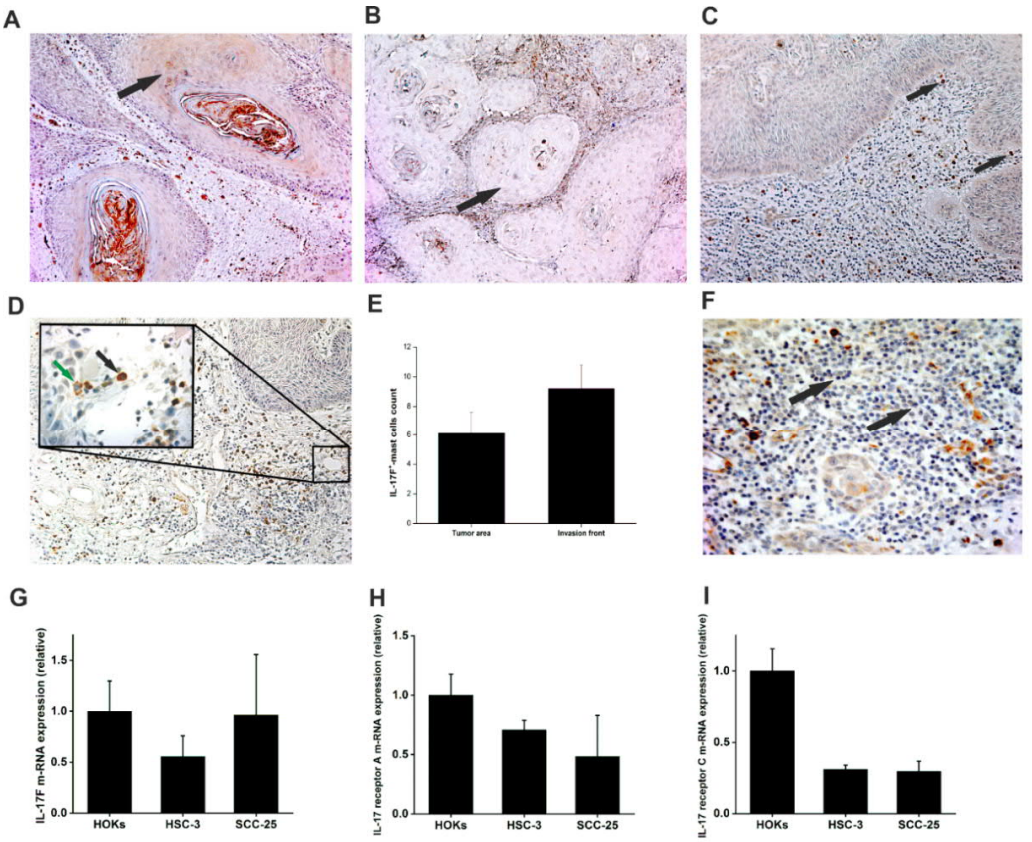

Figure 4. A, OSCC cells positive for IL-17F (black arrow). **B**, OSCC cells negative for IL-17F (black arrows). **C**, Inflammatory cells (black arrows) showed strong IL-17F staining in OSCC. **D**, IL-17F was detected in MCs intracellularly (black arrow) and extracellularly (green arrow) in OSCC. Magnification 10x (inset, 63x). **E**, the count of interleukin IL- 17F-positive MCs was significantly higher in the invasion front compared to the tumour areas. **F**, the inflammatory cells were more frequent in the tumour areas adjacent to the epithelial tissue. Magnification 40x. **G**, compared with HOKs, IL-17F mRNA was downregulated in the highly invasive HSC-3 cells but not in the weaker SCC-25. **H-I,** the gene expressions of both IL-17F receptor A and C were decreased in OTSCC cell lines compared to normal controls.

8.2. Extracellular MC-derived IL-17F has a prognostic utility in OTSCC (study I)

The univariate analyses showed that OTSCC patients with extracellular MC-derived IL-17F in the invasion front had better disease-specific survival (DSS) than the intracellular IL-17F group. This was shown when all stages were combined (HR = 3.79, 95% CI = 1.64-8.80, P = 0.001; **Figure 5A**), as well as in early stages (HR = 4.99, 95% CI = 1.28-19.38, P =0.01; **Figure 5B**) and late stages (HR 3.34, 95% CI = 1.11-10.06, P = 0.03; **Figure 5C**) of OTSCC. Interestingly, this positive effect of IL-17F on DSS was further confirmed using multivariate Cox's proportional hazard regression model analysis. It showed that MC-derived extracellular IL-17F was significantly associated with longer DSS in all stages (HR = 3.24, 95% CI = 1.35-7.79, P = 0.008) and early stages (HR = 4.18, 95% CI = 1.01-17.26, P = 0.04) of OTSCC. However, the difference was not statistically significant in the late stages of the disease (HR = 2.28, 95% CI = 0.69-7.48, P = 0.17).

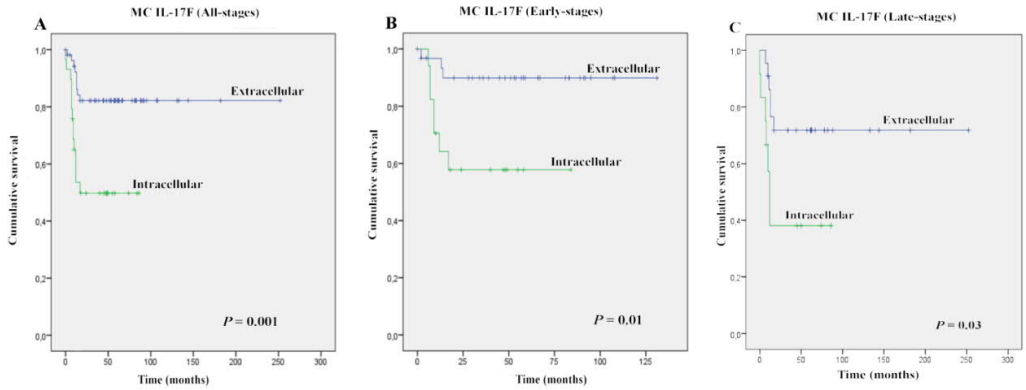

Figure 5. Survival curves of the invasion front mast cell interleukin IL-17F location in all stages, early-stage and late-stage OTSCC. **A**, Kaplan-Meier curves representing the mast cell IL-17F location in all stages, **B**, early-stage, and **C**, late-stage of patients with OTSCC.

8.3. IL-17F diminished proliferation and induced apoptosis in OTSCC cells (study II)

Using the label-free proliferation assay on HSC-3 and SCC-25 cells, IL-17F was able at 50 and 100 ng/mL to inhibit the cell proliferation of the highly invasive HSC-3 cell line (P= 0.04 and P = 0.008, respectively; **figure 6A**). The less invasive SCC-25 cells showed a similar response; however, the difference was not significant **(Figure 6B)**. Furthermore, 100 ng/mL IL-17F induced tumour cell apoptosis, mainly in HSC-3 cells, on day 1 (P = 0.04; **Figure 6C**). A similar trend of cell death was observed on the subsequent days (2 and 3), although the differences were not statistically significant. This effect was not found in SCC-25 (**Figure 6D**). Interestingly, although IL-17A shares the highest sequence homology with IL17F, it produced the opposite effect and slightly induced the cell proliferation of both cell lines **(Figure 6E, F)**.

8.4. IL-17F inhibited the random migration of HSC-3 cells (study II)

Tumour cell migration is crucial for invasion and metastasis during the course of carcinogenesis (Yamaguchi et al., 2005). IL-17F at 100 ng/mL decreased the random migration velocity and the accumulated distance, but not the directed migration, of the highly invasive HSC-3 cells (**Figure 7A-C**). However, it had no effect on SCC-25 cells.

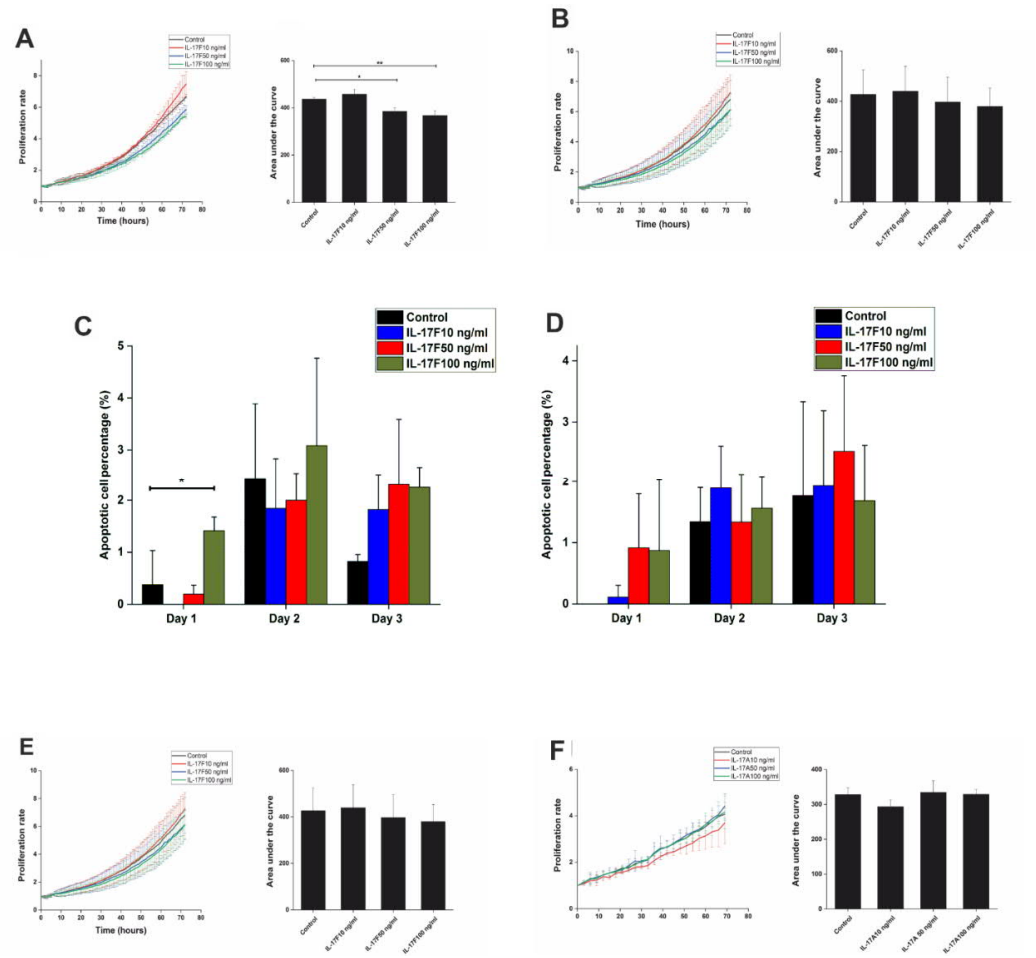

Figure 6. A, IL-17F inhibited HSC-3 cell proliferation at concentrations of 50 and 100 ng/mL compared with non-stimulated control samples. **B**, IL-17F-treated SCC-25 cells revealed a similar but non-significant difference. **C**, on day 1, 100 ng/mL of IL-17F induced apoptosis in the highly invasive HSC-3 cell line compared with the non-stimulated controls. A similar trend was also observed on the following days, but the differences were not statistically significant. **D,** on the other hand, IL-17F seems to slightly increase apoptosis signals in the less-invasive SCC-25 cell line; however, the response was not statistically significant. **E-F**, IL-17A produced an effect opposite to that of IL-17F and showed a slight induction of HSC-3 (E) and SCC-25 (F) proliferation, although no statistical significance was reached.

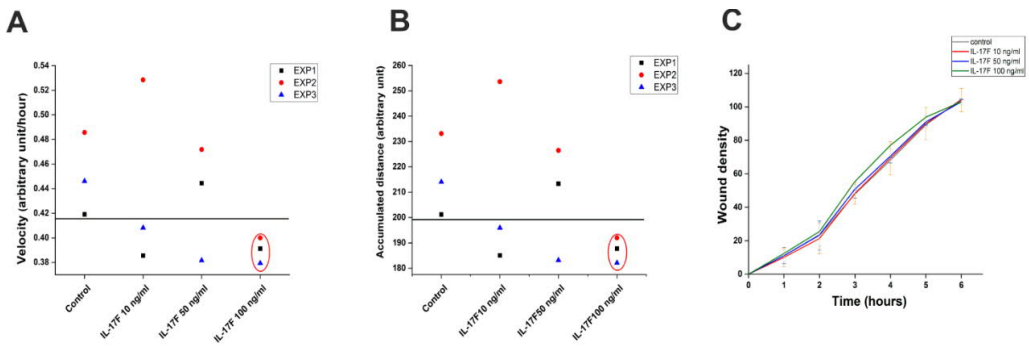

Figure 7. A, 100 ng/mL of IL-17F reduced the random migration velocity of the highly invasive HSC-3 cell line compared with the non-stimulated cells. **B,** 100 ng/mL of IL-17F reduced the accumulated distance of the highly invasive HSC-3 cell line compared with the non-stimulated cells. **C,** IL-17F had no observable effects on the directed migration (wound density) of HSC-3 cells.

8.5. IL-17F counteracts CAF-mediated HSC-3 cell invasion (study II)

Next, we assessed the role of IL-17F on OTSCC cell invasion in vitro. When the scratch-wound assay was used, IL-17F treatment had no effect on the invasiveness of OTSCC cells seeded on Myogel/Collagen matrix. Thus, to simulate the TMEM, we used the 3D spheroid model containing HSC-3, SCC-25 or a combination of one of these cells with CAFs. Spheroids were exposed to IL-17F in a Myogel/Collagen matrix for 5 subsequent days. CAF-HSC-3 spheroids showed increased invasion rates in all experiments compared with spheroids of HSC-3 alone. Importantly, 100 ng/mL IL-17F inhibited this CAF-mediated effect (**Figure 8**).

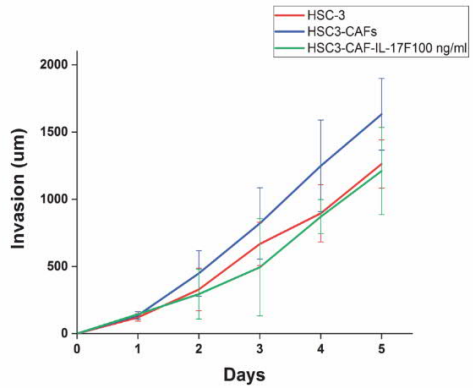

Figure 8. The invasion capacity of HSC-3 was enhanced when cells were combined with CAFs in spheroids (HSC-3/CAFs) compared to that observed with HSC-3 alone. This effect was abolished with the addition of 100 ng/mL IL-17F.

8.6. IL-17F exhibits potent anti-angiogenic effects in vitro (study II)

The anti-angiogenic potential of IL-17F was evaluated in vitro using a HUVEC tube formation assay. HUVEC cells formed interconnected tubes when seeded on Matrigel® (**Figure 9A**). Interestingly, IL-17F inhibited HUVEC tube formation in a dose-dependent manner (**Figure 9B-D**). Using the ImageJ angiogenesis analyser, only IL-17F, but not IL-17A, reduced all the aspects of angiogenesis including total mesh area (P = 0.009), number of junctions (P = 0.04), and number of segments (P = 0.02 (**Figure 9E-G**). The IL-17F serum level in healthy individuals is around 0.4 ng/mL. Thus, we tested the effect of a more physiologically relevant concentration using 1 ng/mL of both IL-17A and IL-17F. Although the effect was minimal due to the very low concentration, IL-17F gave significantly lower tube formation parameters compared with IL-17A (**Figure 9H-J**).

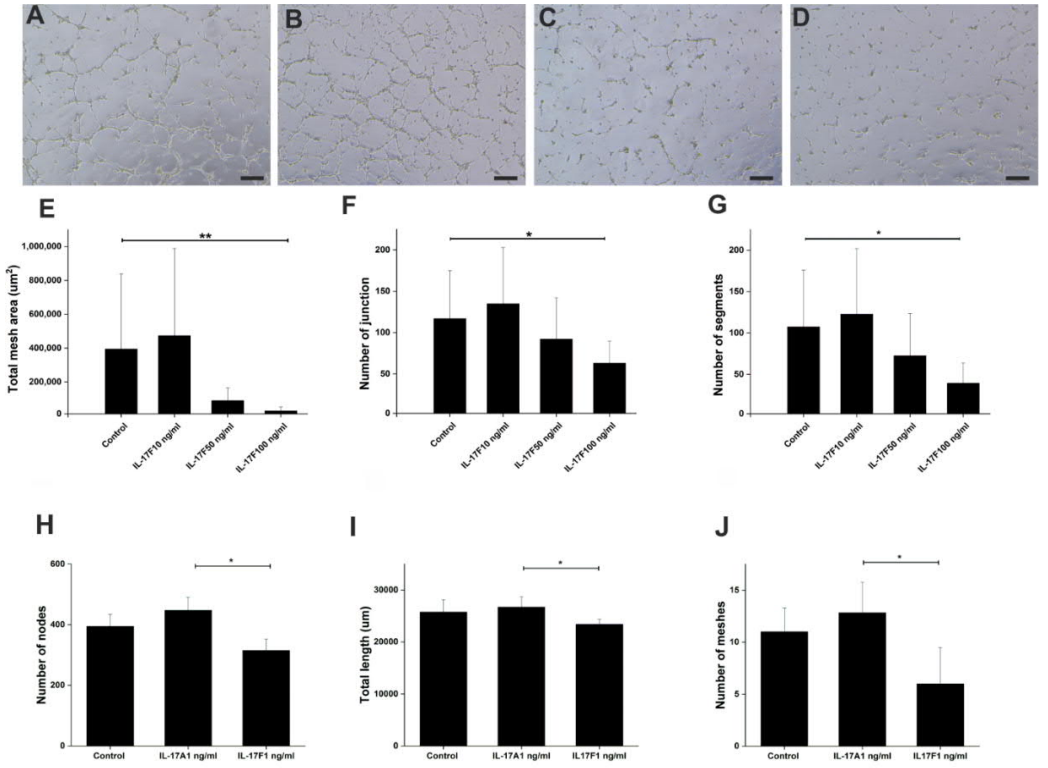

Figure 9. IL-17F inhibits endothelial cell tube formation in a dose-dependent manner. **A,** Non-treated HUVEC form clear interlaced tubes when cultured on Matrigel. Scale bar = 100 µm. **B,** 10 ng/mL of IL-17F partially suppressed HUVEC tube formation. **C,** a higher level of IL-17F (50 ng/mL) induced more inhibition of tube formation. **D,** complete inhibition of tube formation in HUVEC was achieved with 100 ng/mL IL-17F. **E-G,** HUVEC morphometric analysis demonstrated that IL-17F reduced the total mesh area, junction and segment numbers. **H-I,** interestingly, a smaller concentration of IL-17F (1 ng/ml) reduced HUVEC tube-formation, while IL-17A, at the same concentration, showed the opposite effect.

8.7. Tumour cells formed VM structures and expressed EC-specific markers (study III)
Tumour-related angiogenesis is one of the main hallmarks of cancer. However, anti-angiogenic drugs showed limited efficacy in HNSCC patients, suggesting a role of non-angiogenic pathways such as VM (Vassilakopoulou et al., 2015; Ribatti et al., 2019: Salem and Salo 2021a). Therefore, after showing the anti-angiogenic role of IL-17F on HUVECs, we studied the VM phenomenon in OTSCC. We first assessed whether all OTSCC cells can make VM networks on Matrigel. Interestingly, only HSC-3 and SAS, but not SCC-25, cell lines formed clear tube-like structures that were similar to the tubes formed by HUVEC (**Figure 10**).

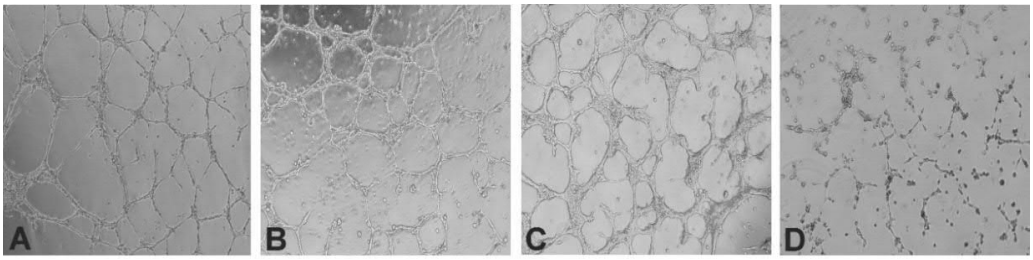

Figure 10. Cancer cells form tube-like structures on Matrigel. **A,** HUVECs formed a tube-like network on Matrigel, **B-C,** similarly, the two high-invasive OTSCC cell lines (HSC-3 and SAS) formed interconnected tube-like structures resembling those formed by HUVEC. **D,** the less-invasive SCC-25 did not form tube-like structures when cultured under the same conditions.

As HUVECs express the EC-specific CD31, we next determined if OTSCC cells also express CD31 in vitro using DDPCR, immunostaining and FACS analysis. Interestingly, all OTSCC cell lines expressed CD31. In DDPCR, the average absolute quantification level was 10 copies/µl (**Figure 11A**).

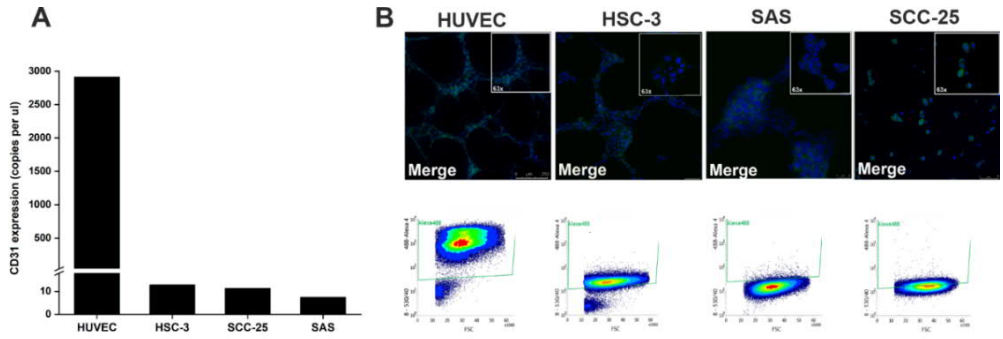

Figure 11. A, absolute quantification of CD31 by droplet-digital PCR revealed a positive expression of the endothelial receptor in OTSCC cells (HSC-3, SCC-25, and SAS) in vitro (approximately 10 copies/µl), which was less than the control (HUVEC). **B,** cultured OTSCC cell lines showed clear positive immunostaining of CD31. FACS also revealed a positive CD31 expression in HUVEC (96%), HSC-3 (90%), SCC-25 (44%), and SAS (1.8%).

The double IF staining showed positive immunoreactivity for CD31 in OTSCC cells, which was further confirmed by FACS analysis that revealed the CD31-positivity was very close in both HSC-3 (90%) and HUVEC (96%) cell lines. However, SCC-25 and SAS cell lines showed less CD31-positivity (44% and 1.8%, respectively; **Figure 11B**).

To confirm the capacity of tumour cells to form VM *in vivo*, surgical samples from the PDX model were collected from tongues and stained by IF using anti-human antibodies to CK, CD31, VE-cadherin, and VWF. Interestingly, CD31-expressing and RBC-containing VM vessels were observed within and adjacent to the tumour area, which were absent in the adjacent tumour-free mouse tissue (**Figure 12A-C**). Furthermore, HSC-3 cells expressed the endothelial marker VE-cadherin but were negative for VWF, despite the VM status (**Figure 12D, E**).

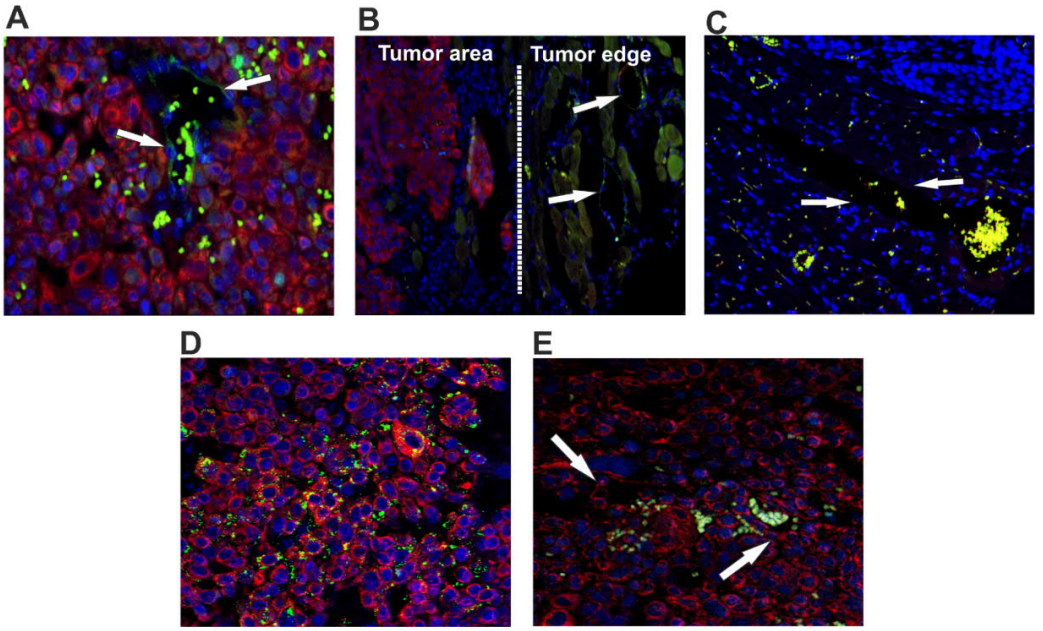

Figure 12. Identification of VM structures in vivo. **A-B,** development of CD31-positive mimicry vessels (arrows; pan-cytokeratin: red; CD31: green) in samples obtained from an orthotopic mouse model of OTSCC. **C,** these human CD31-positive mimicry vessels were not detected in the adjacent mouse tissue (arrows). **D,** human tumour cells showed strong staining intensity of CD144 (VE-cadherin). **E,** Von Willebrand factor was not detected in the orthotopic mouse model of OTSCC (green: red blood cell autofluorescence).

8.8. Different VM patterns are recognized in patients with OTSCC (study III)

To confirm the presence of similar VM structures in the samples from OTSCC patients, the PAS-CD31 IHC assay as well as the double-label IF of the mosaic pattern were used. Interestingly, the two different VM patterns (i.e. PAS+/CD31- and CK+/CD31+) were observed in the tissue samples from OTSCC patients (**Figure 13**).

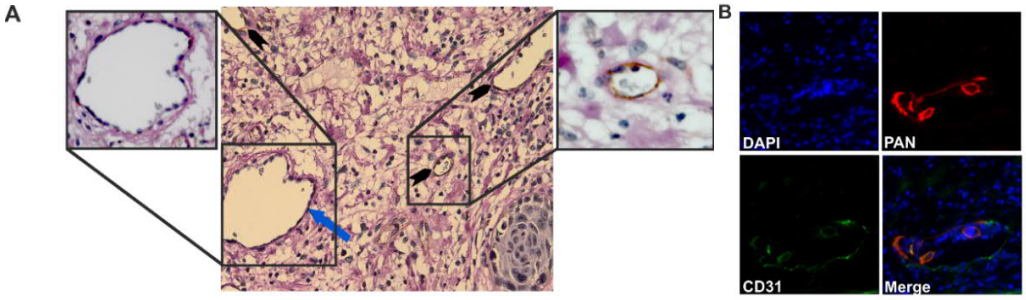

Figure 13. Identification of the vasculogenic mimicry (VM) structures in OTSCC patients. **A,** the PAS+/CD31- VM structures (blue arrow) and PAS+/CD31+ blood vessels (black arrow heads) were observed in OTSCC patients. Magnification: 40×. **B,** cytokeratin+/CD31+ mosaic pattern of VM was observed in OTSCC patients (pan-cytokeratin: red; CD31: green).

8.9. IL-17F inhibits the formation of HSC-3 cell-derived VM in vitro (study III)

Based on the anti-angiogenic role of IL-17F, we next determined if it has an inhibitory effect on VM formation in vitro. Different concentrations of IL-17F and the anti-angiogenic drug, sorafenib, were used to challenge HSC-3 cells seeded on Matrigel. Following the treatment with sorafenib, at a concentration range of 1-5 μM for 24 hours, a significant reduction in the tube formation was observed (**Figure 14**). Importantly, IL-17F produced similar inhibitory effects on the formation of VM structures in HSC-3 cells with the best effect seen at a concentration of 100 ng/ml.

8.10. Systematic review results (study IV)

After confirming the inhibitory effect of IL-17F on both angiogenesis and VM in vitro, we performed a systematic review of the published literature to evaluate the association between the expression of vessel-related markers (i.e. MVD/LVD) and the survival of TSCC patients.

8.7.1. Literature search and risk of bias results

On the initial search, we retrieved a total of 516 studies, of which only 13 articles met the inclusion criteria and were eligible for our study. In these reports, the expression of the following MVD and LVD markers were assessed in TSCC patients: CD34, CD31, CD105, factor VIII (FVIII), D2-40, and LYVE-1. The end-point analyses included OS, PFS, DFS, DSS, recurrence-free survival (RFS), as well as tumour-specific survival. Most of the patients were not exposed to any preoperative treatments. However, this information was not disclosed in 5 studies, while in one study patients were treated by surgery and/or radiotherapy. According to the MAStARI tool, the risk of bias in the eligible studies ranged from low to moderate.

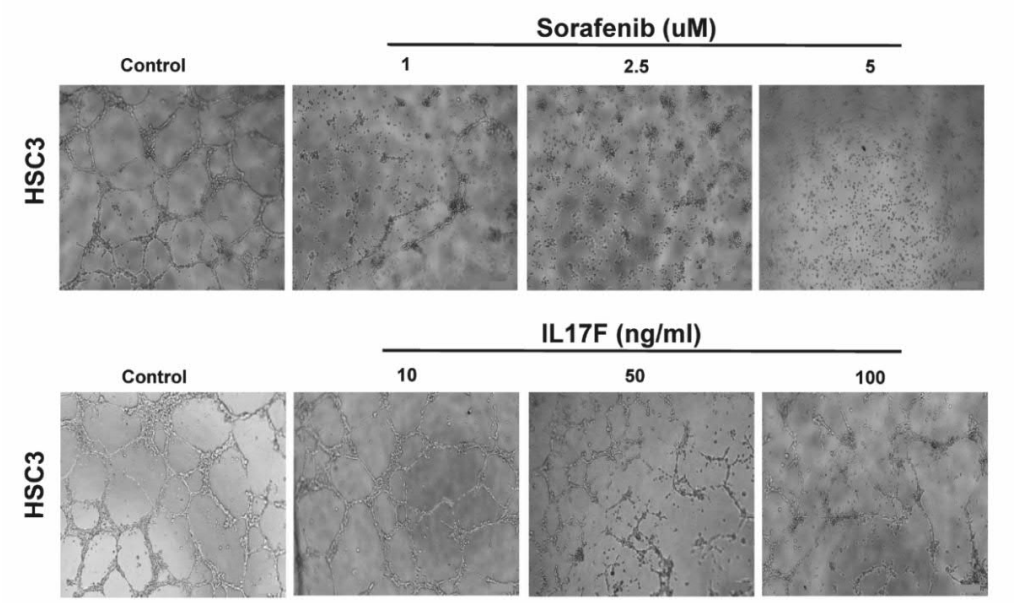

Figure 14. The inhibitory effect of IL-17F on VM formation was compared with the clinically approved antiangiogenic drug (sorafenib). Treatment with sorafenib showed reduction in tube formation by HSC-3 cells at concentrations of 1-5 μM. Treatment with IL-17F inhibited the formation of the tube-like structures in HSC-3 cells in a manner similar to sorafenib, with the greatest effect observed at 100 ng/ml. Magnification 4x.

8.7.2. The prognostic value of MVD markers in TSCC

The number of studies that reported the prognostic value of MVD markers in TSCC patients was as follows: CD34 (n = 5 studies); CD31 (n = 1 study); CD105 (n = 1 study); and FVIII (n = 2 studies). While two studies (Huang et al. 2012; Toyoda et al., 2014) did not find any significant correlation between CD34 and patient survival, Sasahira et al. revealed that CD34 overexpression was associated with shorter DFS in TSCC patients (P = 0.02) (Sasahira et al., 2010). A similar observation was reported by Shao et al., who showed a shorter OS in TSCC patients with higher CD34 expression (Shao et al., 2008). Forootan et al. concluded that patients with a low CD34-positive vessel count (VC) had a better prognosis (P = 0.023) (Forootan et al., 1999).

In one study that used anti-CD31 staining in TSCC specimens, the authors found that statistical analysis failed to demonstrate any correlation between CD31-expressing MVD with either the 5-year survival or the recurrence rate (Fernández et al., 2007). Chuang et al. found that an induced level of CD105 was significantly correlated with advanced tumour stage (P<0.001), positive lymph node status (P<0.001), and the presence of perineural invasion (P=0.003). Furthermore, the authors found that the 5-year DFS rate was correlated with low expression of CD105 (P<0.001), and that the immunoexpression of

CD105 could be suggested as a useful prognosticator in early-stage TSCC patients (Chuang et al., 2006).

The prognostic value of FVIII in TSCC was assessed by two reports. No association between FVIII expression and the survival outcome in TSCC patients was found in one study (Kantola et al., 2000). In another study, Vora et al. reported that early TSCC stages (i.e. I and II) with induced FVIII-expressing microvessel count were associated with a significant reduction of OS and RFS. However, the multivariate analysis failed to support the FVIII as a significant independent prognosticator in patients with TSCC (Vora et al., 2003).

8.7.3. The prognostic value of LVD markers in TSCC

Two LVD markers were assessed in TSCC patients as follows: D2-40 (n = 3 studies); and LYVE-1 (n = 2 studies). Using the anti-human D2-40 antibody for LVD, Al-Shareef et al. showed that TSCC patients with higher LVD status had a worse prognosis, as OS and DFS were associated with intra- and peritumoral LVD. The authors suggested that LVD expression may serve as an independent prognostic factor to predict the survival of TSCC patients (Al-Shareef et al., 2016). In agreement with these findings, a higher LVD level was significantly associated with lymph node metastasis and shorter OS in a cohort of OTSCC patients (Yan et al., 2014). In another study by Seppälä et al., only the relative density of lymphatic vessels (RDLV) was shown to be associated with worse OS in TSCC patients, whereas the LVD level did not affect patient survival. The RDLV was calculated by dividing the average of D2-40-expressing LVD by the average of VWF-expressing LVD per microscopic field (Seppälä et al., 2016). Two studies assessed the prognostic value of the specific LVD marker, LYVE-1, in TSCC patients. However, while LYVE-1 overexpression was found to predict poor DFS (Sasahira et al., 2010), another study failed to find a significant correlation between LYVE-1 and OS in TSCC patients (Ding et al., 2014).

9. DISCUSSION

The incidence and mortality rates of OTSCC continue to increase worldwide. In spite of the substantial advancement in cancer management, patients with OTSCC still show a relatively poor prognosis. Therefore, current research has been devoted to identifying novel therapeutic targets not only in tumour cells but also in the other components of TME including inflammatory cells and cytokines. In fact, cytokines showed a promising success in other chronic disorders such as rheumatoid arthritis and psoriasis, and they have been an attractive target in cancer therapeutics research and immunotherapy (Lee and Margolin 2011; Conlon et al., 2019). In this doctoral thesis, we aimed for the first time to investigate the potential involvement of IL-17F in OTSCC.

The inflammatory component of the tumour microenvironment includes potent inflammatory cells, which can affect the prognosis of cancer patients. This influence has been previously studied in OTSCC, where patients with higher levels of $CD163^+$, $Foxp3^+$, and $CD80^+$ cells had more cancer recurrence than patients with a smaller population of these cells (Dayan et al., 2012). Additionally, in the first study, we showed for the first time that MC-derived IL-17F at the invasion front predicted improved DSS. We also found that IL-17F staining was most intense in MCs compared with lymphocytes, which is in line with previous reports showing that IL-17F is derived mainly from human dermal MCs (Lin et al., 2011). This low immunoreactivity of IL-17F in OTSCC samples (>75% of samples) was further confirmed by our gene expression analysis, which showed that OTSCC cell lines (HSC-3 and SCC-25) exhibit low mRNA levels of IL-17F. In spite of their controversial role in carcinogenesis, tumour infiltrating MCs have been shown to promote an antitumorigenic effect and improve the survival outcome in different cancers including colorectal cancer and HNSCC (Nielsen et al., 1999; Hedström et al. 2007; Attramadal et al., 2016). In fact, MCs contain and release a variety of strong inflammatory molecules. We have shown previously that supernatants from activated human MCs are able to downregulate oral oncogenes (Salem et al., 2017). It is now well documented that the invasion front is critical for many protumorigenic events including invasion, increased cell proliferation and initiation of angiogenesis (Sharma et al., 2013). Therefore, MC-released IL-17F at the tumour borders could be directed to slowdown the invasion of tumour cells deep into the stromal tissue. Thus, it was important to investigate the potential antitumorigenic role of IL-17F in depth using in vitro models of OTSCC.

In the second part of this doctoral project, we reported an inhibitory effect of IL-17F on the proliferation and random migration of one highly aggressive OTSCC cell line (HSC-3). We also reported that the CAF-mediated pro-invasive effects on HSC-3 were eliminated by human IL-17F in the spheroid model. Moreover, IL-17F inhibited EC-mediated angiogenesis in vitro. Consistent with our report, previous studies have shown that IL-17 family cytokines can influence the proliferation of cancer cell lines, reduced metastasis, and enhance antitumour immunity in mouse models of colon cancer and

melanoma (Kryczek et al., 2009; Martin-Orozco et al., 2009). Furthermore, Th17 cells were shown to have more therapeutic efficacy than Th1 cells in a murine model of lung melanoma, which resulted in a strong CD8-response (Martin-Orozco et al., 2009). However, although IL-17A shares high homology with IL-17F, it was shown to promote procancerous effects by inducing cancer cell proliferation such as in nasopharyngeal carcinoma and skin SCC (Cai et al., 2017; Wang et al., 2010). Supporting these studies, we report that IL-17A showed a slight induction of OTSCC cell proliferation compared with the inhibitory effect of IL-17F. Furthermore, IL-17F promoted the apoptotic caspase 3/7 signal in the same OTSCC cell line. Cell invasion describes the ability of aggressive tumour cells to migrate and change their position to penetrate basement membranes and tissue barriers. Importantly, tumour cell migration and invasion allow them to intravasate into lymphatic and blood vasculature and consequently undergo metastasis (Friedl and Wolf 2003). The TME plays an important role in this process. For instance, CAFs derived from the TME of colon cancer promoted cancer cell invasion through the laminin basement membrane (Glentis et al., 2017). In this regard, it is interesting that IL-17F treatment inhibited CAF-mediated invasion of OTSCC cells in vitro, which suggests that IL-17F may orchestrate cell-cell interactions in the TME.

Tumour-induced angiogenesis is a hallmark and therapeutic target of cancer. Tumours of HNSCC express high levels of angiogenic factors, such as VEGF, which is also associated with worse OS in patients with HNSCC (Vassilakopoulou et al., 2015; Kyzas et al., 2005). Our findings indicate that IL-17F can influence HUVEC-derived tube formation in a dose-dependent manner, which showed a similar trend even when we used a very low concentration (1 ng/mL) of IL-17F. In agreement with this, it has been shown that IL-17F over-expressing colon tumours had significantly lower VEGF levels and CD31-expressing cells. In contrast, the VEGF levels were increased in the tumour tissues obtained from IL-17F(-/-) mice (Tong et al., 2012). Furthermore, a supernatant from an IL-17F-transfected HCC cell line directly inhibited vascular EC growth, and showed a significant decrease in tumour size and MVD in vivo. On the other hand, IL-17A did not produce any consistent effect on HUVEC tube formation, which contrasts with a previous report where it was shown to promote angiogenesis (Pan et al., 2015).

In addition to tumour-induced angiogenesis, VM was described as a tumour cell-formed vascularization model, which is believed to provide nutrients as well as an alternative metastatic route for aggressively growing tumours (Maniotis et al., 1999; Hendrix et al., 2003). Recently, we showed that tumoral VM-positive staining is associated with shorter OS in HNSCC patients (Hujanen et al., 2020). Furthermore, increasing evidence suggests the involvement of VM in tumour invasion, metastasis, and poor prognosis (Luo et al., 2020). Therefore, identifying new antivascular therapies targeting VM would be important to improve the survival of cancer patients. Our findings confirm the ability of invasive OTSCC-derived cell lines to form VM networks similar to HUVEC tubes on Matrigel. However, the inability of the low-invasive SCC-25 cells to form VM structures in

vitro supports previous studies that VM is a feature of the highly aggressive tumours (Seftor et al., 2012). Furthermore, we showed that OTSCC cells express EC-specific markers in vitro. In agreement with our findings, a strong staining of EC-specific markers has been shown in human melanoma and gastric adenocarcinoma, which suggests a role of these proteins in VM development (Kim et al., 2019; Pisacane et al., 2007). Furthermore, Tong et al. reported that cultured HNSCC cells exhibit EC markers and functional features including the uptake of low-density lipoprotein, generating tube-like structures in vitro and responsiveness to VEGF signalling (Tong et al., 2013). Therefore, our findings suggest that in addition to the traditional $PAS^{+}/CD31^{-}$ staining method, the mosaic pattern may also be important when identifying VM structures. In addition, these findings highlight the phenotypic plasticity in HNSCC, which is also supported by the ability of HNSCC cells to undergo epithelial- and endothelial-mesenchymal transitions (Tong et al., 2013).

The lack of effective anti-angiogenic therapies for HNSCC remains a challenge. Importantly, current angiogenesis inhibitors such as anginex, TNP-470, and endostatin are not effective against the formation of a VM tubular network by human melanoma cells in vitro. Moreover, human endothelial cells expressed higher levels of endostatin receptors than melanoma cells, which highlights the need for new anti-angiogenic drugs that target both angiogenesis and VM (van der Schaft et al., 2004). Therefore, we investigated the effect of IL-17F on VM formation in vitro and compared it with sorafenib, which is a clinically approved kinase inhibitor targeting the VEGF receptor family (Yadav et al., 2011). Interestingly, similar to sorafenib, IL-17F showed a clear inhibition of the VM tubular network on Matrigel and at comparable concentrations that inhibited tumour cell invasion, migration and angiogenesis in vitro. IL-17F has previously been shown to negatively affect the expression of important pro-angiogenic factors including IL-6, IL-8, and VEGF, which eventually inhibit tumour angiogenesis (Tong et al., 2012; Xie et al., 2010). However, further studies are still needed to evaluate whether IL-17F can inhibit VM development using an in vivo model.

The identification of biomarkers that associate with tumour vasculature could improve the prognostic and risk-stratification approaches in patients with HNSCC. In the final part of this thesis, and based on the anti-angiogenic role of IL-17F in cancer, we conducted a systematic review to evaluate the prognostic value of MVD and LVD markers in TSCC. Analyses of the selected 13 clinical studies (with a total of 973 patients) revealed controversial results. Only 7 studies (53.84%) indicated a prognostic significance of one or more of the MVD or LVD markers, with D2-40 having the most promising results. Therefore, we concluded that more prognostic studies with a larger number of TSCC patients are needed before recommending these markers for prognostic purposes.

The following markers have been used to identify MVD in patients with TSCC: CD31 (platelet endothelial cell adhesion molecule 1), CD34, CD105 (endoglin),

FVIII, and VWF. Apart from TSCC, many investigators have studied the prognostic value of angiogenic-related molecules in different cancers. For instance, there is a general agreement that CD34 can be used as a sensitive biomarker in cancers including hepatocellular carcinoma and lung cancer. Therefore, it could become an integral part of a more reliable staging system (Salizzoni et al., 2003; Saad et al., 2004; Mineo et al., 2004). However, the results were not always consistent in TSCC. While two studies showed that stronger CD34 expression was associated with worse survival (Sasahira et al. 2010; Shao et al., 2008), two later studies were not able to reproduce the significance of CD34 as a prognostic marker in TSCC patients (Huang et al. 2012; Toyoda et al., 2014). A CD105-positive MVD count was correlated with more tumour cell dissemination in HNSCC patients (Martone et al., 2005). Furthermore, another study found that CD105 positive expression in OSCC tissue was significantly higher than in normal mucosa and it was correlated with higher T stage (Schimming and Marmé, 2002). In the included studies on TSCC, CD31 and CD105 were studied only once. In this study, the statistical analysis failed to demonstrate any correlation between CD-31 expressing MVD with either the 5-year survival or the recurrence rate (Fernández et al., 2007). However, this might be due to a relatively small sample size (n = 43) used in the study. Additionally, two studies on the prognostic value of FVIII in TSCC patients showed contradictory findings (Vora et al., 2003; Kantola et al., 2000).

Recently, several markers of lymphatic vessels have been identified including D2-40 and LYVE-1. D2-40 (or podoplanin) is a transmembrane glycoprotein that is expressed on lymphatic EC, which make up lymphatic vessels and are essential for lymph node metastasis (Seppälä et al., 2016). The prognostic role of these two markers has been investigated and studies showed that they could be useful in predicting survival in TSCC. Seppälä et al. did not find such significance using LVD, however, RDLV predicted poor OS in TSCC patients (Seppälä et al., 2016). While Ding et al. found no correlation between the expression of either intratumoral or peritumoral LYVE-1 and TSCC patient survival, Sasahira et al. reported that peritumoral LYVE-1-expressing LVD was associated with shorter DFS when combined with positive staining of VEGF (Ding et al., 2014; Sasahira et al. 2010). Interestingly, a recent study by our group showed that OTSCC patients showing intratumoral LYVE-1+ mimicry vessels had shorter OS compared to the LM-negative group. However, this difference was not statistically significant due to the small size of the patient sample (Karinen et al., 2021). Taking the results of this systematic review together, there is currently insufficient evidence to suggest the incorporation of any of the MVD or LVD markers into the staging system of TSCC patients. Although there was some evidence that overexpression of these markers could negatively affect the survival outcomes, serious limitations exist. These include small cohorts, variable assessment criteria, heterogeneity of the study samples and the lack of key prognostic information in the majority of the studies.

Therefore, we recommend more well-designed prognostic studies of the role of MVD and LVD in patients with TSCC.

10. CONCLUSIONS

The findings of this doctoral dissertation, overall, reveal an anticancerous role of IL-17F in OTSCC. Extracellular mast cell-derived IL-17F at the tumour invasive front represents a promising prognosticator for risk stratification approaches. The in vitro assays revealed inhibitory effects on tumour cell proliferation, migration, invasion, angiogenesis and VM formation. Due to the potential significance of tumour-related vasculature in tumour metastasis, further preclinical studies on IL-17F using in vivo models are warranted. In addition, the currently available MVD and LVD markers may exhibit some prognostic potential, however, more well-designed clinical studies are needed before they can be recommended for reliable clinical use. Among the strengths of this study were the use of various experimental approaches including in vitro and in vivo models, clinical samples from OTSCC patients, and a comprehensive systematic review using multiple databases. However, several limitations should be acknowledged including the limited number of patient's samples, the lack of functional assessment of VM, and there was no evaluation of the therapeutic effect of IL-17F in vivo. Additionally, although lymph node metastasis and higher tumour stages are usually associated with poor prognosis, this phenomenon was not observed in study I. The reason is not clear, however, it could partly be due to the fact that small node metastases may not always be detected in previous decades (the clinical data were obtained during 1990-2010) when the imaging modality was less sensitive compared with the MRI. Finally, IL-17 cytokines have already been used to develop effective, and clinically approved, medications for treating chronic immune diseases such as psoriasis. Therefore, development of an IL-17F-based drug, or targeting one of its regulatory pathways, may have prognostic implications as well as serve as a promising therapeutic approach in patients with OTSCC.

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Helsinki, December 2021

Rabeia A Imahmoudi

12. REFERENCES

Aghbari SMH, Abushouk AI, Attia A, et al. Malignant transformation of oral lichen planus and oral lichenoid lesions: A meta-analysis of 20095 patient data. *Oral Oncol*. 2017; 68:92-102.

Ahmed SP, Jayan L, Dineshkumar T, et al. Oral squamous cell carcinoma under microscopic vision: A review of histological variants and its prognostic indicators. *SRM J Res Dent Sci.* 2019;10:90-7

Al Obeed OA, Vaali-Mohamed MA, Alkhayal KA, et al. IL-17 and colorectal cancer risk in the Middle East: gene polymorphisms and expression. *Cancer Manag Res*. 2018 Sep 5;10:2653-2661.

Al-Samadi A, Moossavi S, Salem A, et al. Distinctive expression pattern of interleukin-17 cytokine family members in colorectal cancer. *Tumour Biol*. 2016;37(2):1609-15.

Al-Shareef H, Hiraoka SI, Tanaka N, et al. Use of NRP1, a novel biomarker, along with VEGF-C, VEGFR-3, CCR7 and SEMA3E, to predict lymph node metastasis in squamous cell carcinoma of the tongue. *Oncol Rep*. 2016;36(5):2444-2454.

Araghi M, Galanti MR, Lundberg M, et al. No association between moist oral snuff (snus) use and oral cancer: pooled analysis of nine prospective observational studies. *Scand J Public Health*. 2020 May 28:1403494820919572.

Åström P, Juurikka K, Hadler-Olsen ES, et al. The interplay of matrix metalloproteinase-8, transforming growth factor-β1 and vascular endothelial growth factor-C cooperatively contributes to the aggressiveness of oral tongue squamous cell carcinoma. *Br J Cancer*. 2017 Sep 26;117(7):1007-1016.

Attramadal CG, Kumar S, Gao J, et al. Low mast cell density predicts poor prognosis in oral squamous cell carcinoma and reduces survival in head and neck squamous cell carcinoma. *Anticancer Res*. 2016;36:5499-5506.

Bagan J, Sarrion G, Jimenez Y. Oral cancer: clinical features. *Oral Oncol*. 2010 Jun;46(6):414-7.

Balermpas P, Michel Y, Wagenblast J, et al. Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer. *Br J Cancer*. 2014 Jan 21;110(2):501-9.

Barnes L, Eveson JW, Reichart P, et al. Pathology and Genetics of Head and Neck Tumours. World Health Organization Classification of Tumours. IARC Press, Lyon. 2005.

Bedoui SA, Barbirou M, Stayoussef M, et al. Association of interleukin-17A polymorphisms with the risk of colorectal cancer: A case-control study. *Cytokine*. 2018;110:18-23.

Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018 Nov;68(6):394-424.

Brierley J, Gospodarowicz M, O'Sullivan B. The principles of cancer staging. *Ecancermedicalscience*. 2016 Nov 24; 10:ed61.

Brierley J, Gospodarowicz M, Wittekind C editors. TNM Classification of Malignant Tumours. 8th ed.: Wiley-Blackwell; 2017.

Brinkman BM, Wong DT. Disease mechanism and biomarkers of oral squamous cell carcinoma. *Curr Opin Oncol*. 2006 May; 18(3):228-33.

Cai K, Wang B, Dou H, et al. IL-17A promotes the proliferation of human nasopharyngeal carcinoma cells through p300-mediated Akt1 acetylation. *Oncol Lett*. 2017 Jun;13(6):4238-4244.

Carreras-Torras C, Gay-Escoda C. Techniques for early diagnosis of oral squamous cell carcinoma: Systematic review. *Med Oral Patol Oral Cir Bucal*. 2015 May 1;20(3):e305-15.

Chang SH, Mirabolfathinejad SG, Katta H, et al. T helper 17 cells play a critical pathogenic role in lung cancer. *Proc Natl Acad Sci U S A*. 2014 Apr 15;111(15):5664-9.

Chang SH. T helper 17 (Th17) cells and interleukin-17 (IL-17) in cancer. *Arch Pharm Res*. 2019 Jul;42(7):549-559.

Chen Y, Yang Z, Wu D, et al. Upregulation of interleukin-17F in colorectal cancer promotes tumor invasion by inducing epithelial-mesenchymal transition. Oncol *Rep. 2019 Sep;42(3):1141-1148.*

Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma--an update. *CA Cancer J Clin*. 2015 Sep-Oct;65(5):401-21.

Chuang HC, Su CY, Huang HY, et al. High expression of CD105 as a prognostic predictor of early tongue cancer. *Laryngoscope*. 2006 Jul;116(7):1175-9.

Chung L, Thiele Orberg E, et al. Bacteroides fragilis Toxin Coordinates a Pro-carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells. *Cell Host Microbe*. 2018 Feb 14;23(2):203-214.e5.

Cianfriglia F, Di Gregorio DA, Manieri A. Multiple primary tumours in patients with oral squamous cell carcinoma. *Oral Oncol*. 1999 Mar;35(2):157-63.

Coffelt SB, Kersten K, Doornebal CW, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. 2015 Jun 18;522(7556):345-348.

Coletta RD, Yeudall W, Salo T. Grand Challenges in Oral Cancers. *Front Oral Health*. 2020. 1. 10.3389/froh.2020.00003.

Conlon KC, Miljkovic MD, Waldmann TA. Cytokines in the Treatment of Cancer. *J Interferon Cytokine Res*. 2019 Jan;39(1):6-21.

Dayan D, Salo T, Salo S, et al. Molecular crosstalk between cancer cells and tumor microenvironment components suggests potential targets for new therapeutic approaches in mobile tongue cancer. *Cancer Med*. 2012 Oct;1(2):128-40.

D'Cruz AK, Vaish R, Kapre N, et al. Elective versus Therapeutic Neck Dissection in Node-Negative Oral Cancer. *N Engl J Med*. 2015 Aug 6;373(6):521-9.

De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer*. 2017 Aug;17(8):457-474.

Ding L, Hu EL, Xu YJ, et al. Serum IL-17F combined with VEGF as potential diagnostic biomarkers for oral squamous cell carcinoma. *Tumour Biol*. 2015;36(4):2523-9.

Ding L, Zhang Z, Shang D, et al. α-Smooth muscle actin-positive myofibroblasts, in association with epithelial-mesenchymal transition and lymphogenesis, is a critical prognostic parameter in patients with oral tongue squamous cell carcinoma. *J Oral Pathol Med*. 2014 May;43(5):335-43.

Dourado MR, Korvala J, Åström P, et al. Extracellular vesicles derived from cancer-associated fibroblasts induce the migration and invasion of oral squamous cell carcinoma. *J Extracell Vesicles*. 2019 Feb 13;8(1):1578525.

Drost J, Clevers H. Organoids in cancer research. Nat Rev Cancer. 2018 Jul;18(7):407-418.

Du M, Nair R, Jamieson L, et al. Incidence Trends of Lip, Oral Cavity, and Pharyngeal Cancers: Global Burden of Disease 1990-2017. *J Dent Res*. 2020;99(2):143-151.

Durr ML, Li D, Wang SJ. Oral cavity squamous cell carcinoma in never smokers: analysis of clinicopathologic characteristics and survival. *Am J Otolaryngol*. 2013;34(5):388-93.

Elpek GO, Gelen T, Aksoy NH, et al. The prognostic relevance of angiogenesis and mast cells in squamous cell carcinoma of the oesophagus. *J Clin Pathol*. 2001 Dec; 54(12):940-4.

Elshazli RM, Salman DO, Kamel MM, et al. Genetic polymorphisms of IL-17A rs2275913, rs3748067 and IL-17F rs763780 in gastric cancer risk: evidence from 8124 cases and 9873 controls. *Mol Biol Rep*. 2018 Oct;45(5):1421-1444.

Fernández MM, García-Rozado A, Parente PL. Is microvascular density an independent prognostic factor in squamous cell carcinoma of the tongue? *Acta Otorrinolaringol Esp*. 2007;58:341-346.

Forootan SS, Jones AS, Helliwell TR. Neoangiogenesis and squamous cell carcinoma of the tongue. *Cancer Detect Prev*. 1999;23(2):137-46.

Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer*. 2003 May;3(5):362-74.

Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol*. 2013 Oct;14(10):1014-22.

Galli F, Aguilera JV, Palermo B, et al. Relevance of immune cell and tumor microenvironment imaging in the new era of immunotherapy. *J Exp Clin Cancer Res*. 2020 May 18;39(1):89.

Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: a meta-analysis. *Int J Cancer*. 2008 Jan 1;122(1):155-64.

Garcia C, Crowson AN. Acantholytic squamous cell carcinoma: is it really a more-aggressive tumor? *Dermatol Surg*. 2011 Mar;37(3):353-6.

Glentis A, Oertle P, Mariani P, et al. Cancer-associated fibroblasts induce metalloprotease-independent cancer cell invasion of the basement membrane. *Nat Commun*. 2017 Oct 13;8(1):924.

Gu C, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. *Cytokine*. 2013 Nov;64(2):477-85.

Guha N, Boffetta P, Wünsch Filho V, et al. Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. *Am J Epidemiol*. 2007 Nov 15;166(10):1159-73.

Gupta B, Johnson NW. Systematic review and meta-analysis of association of smokeless tobacco and of betel quid without tobacco with incidence of oral cancer in South Asia and the Pacific. *PLoS One*. 2014 Nov 20;9(11):e113385.

Gupta PC, Warnakulasuriya S. Global epidemiology of areca nut usage. *Addict Biol*. 2002 Jan;7(1):77-83.

Hakulinen T, Tryggvadóttir L, Gislum M, et al. Trends in the survival of patients diagnosed with cancers of the lip, oral cavity, and pharynx in the Nordic countries 1964-2003 followed up to the end of 2006. *Acta Oncol*. 2010 Jun;49(5):561-77.

Hamari AK, Toljamo TI, Kinnula VL, et al. Dual use of cigarettes and Swedish snuff (snus) among young adults in Northern Finland. *Eur J Public Health*. 2013;23(5):768-71.

Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000 Jan 7;100(1):57-70.

Hashibe M, Brennan P, Benhamou S, et al. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled

analysis in the International Head and Neck Cancer Epidemiology Consortium. *J Natl Cancer Inst*. 2007 May 16;99(10):777-89.

Haughey BH, Gates GA, Arfken CL, et al. Meta-analysis of second malignant tumors in head and neck cancer: the case for an endoscopic screening protocol. *Ann Otol Rhinol Laryngol*. 1992 Feb;101(2 Pt 1):105-12.

Haya-Fernández MC, Bagán JV, Murillo-Cortés J, et al. The prevalence of oral leukoplakia in 138 patients with oral squamous cell carcinoma. *Oral Dis*. 2004;10(6):346-8.

Hedström G, Berglund M, Molin D, et al. Mast cell infiltration is a favourable prognostic factor in diffuse large B-cell lymphoma. *Br J Haematol*. 2007;138:68-71.

Hendrix MJ, Seftor EA, Hess AR, et al. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer*. 2003 Jun;3(6):411-21.

Hirata E, Sahai E. Tumor Microenvironment and Differential Responses to Therapy. *Cold Spring Harb Perspect Med*. 2017 Jul 5;7(7):a026781.

Huang C, Sun Z, Sun Y, et al. Association of increased ligand cyclophilin A and receptor CD147 with hypoxia, angiogenesis, metastasis and prognosis of tongue squamous cell carcinoma. *Histopathology*. 2012 Apr;60(5):793-803.

Hujanen R, Almahmoudi R, Karinen S, et al. Vasculogenic Mimicry: A Promising Prognosticator in Head and Neck Squamous Cell Carcinoma and Esophageal Cancer? A Systematic Review and Meta-Analysis. *Cells*. 2020 Feb 24;9(2):507.

International Agency for Research on Cancer (IARC). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. List of Classifications by Cancer Site. monographs.iarc.fr/ENG/Classification/index.php. Accessed June 3, 2015.

Iocca O, Sollecito TP, Alawi F, et al. Potentially malignant disorders of the oral cavity and oral dysplasia: A systematic review and meta-analysis of malignant transformation rate by subtype. *Head Neck*. 2020 Mar;42(3):539-555.

Isayeva T, Li Y, Maswahu D, et al. Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. *Head Neck Pathol*. 2012 Jul;6 Suppl 1(Suppl 1):S104-20.

Ishigame H, Kakuta S, Nagai T, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity*. 2009 Jan 16;30(1):108-19.

Jain PV, Sharan R, Manikantan K, et al. Redefining adequate margins in oral squamous cell carcinoma: outcomes from close and positive margins. *Eur Arch Otorhinolaryngol*. 2020 Apr;277(4):1155-1165.

Jang WI, Wu HG, Park CI, et al. Treatment of patients with clinically lymph node-negative squamous cell carcinoma of the oral cavity. *Jpn J Clin Oncol*. 2008;38(6):395-401.

Jiang X, Ye J, Dong Z, et al. Novel genetic alterations and their impact on target therapy response in head and neck squamous cell carcinoma. *Cancer Manag Res*. 2019;11:1321-1336. Published 2019 Feb 8.

Jin MZ, Jin WL. The updated landscape of tumor microenvironment and drug repurposing. *Signal Transduct Target Ther*. 2020 Aug 25;5(1):166.

Johnson DE, Burtness B, Leemans CR, et al. Head and neck squamous cell carcinoma. *Nat Rev Dis Primers*. 2020 Nov 26;6(1):92.

Kantola S, Parikka M, Jokinen K, et al. Prognostic factors in tongue cancer - relative importance of demographic, clinical and histopathological factors. *Br J Cancer*. 2000 Sep;83(5):614-9.

Karinen S, Juurikka K, Hujanen R, et al. Tumour cells express functional lymphatic endothelium-specific hyaluronan receptor in vitro and in vivo: Lymphatic mimicry promotes oral oncogenesis? *Oncogenesis*. 2021 Mar 5;10(3):23.

Kim HS, Won YJ, Shim JH, et al. Morphological characteristics of vasculogenic mimicry and its correlation with EphA2 expression in gastric adenocarcinoma. *Sci Rep*. 2019 Mar 4;9(1):3414.

Knochelmann HM, Dwyer CJ, Bailey SR, et al. When worlds collide: Th17 and Treg cells in cancer and autoimmunity. *Cell Mol Immunol*. 2018 May;15(5):458-469.

Korn T, Bettelli E, Oukka M, et al. IL-17 and Th17 Cells. *Annu Rev Immunol*. 2009;27:485-517.

Kreimer AR, Villa A, Nyitray AG, et al. The epidemiology of oral HPV infection among a multinational sample of healthy men. *Cancer Epidemiol Biomarkers Prev*. 2011 Jan;20(1):172-82.

Kryczek I, Wei S, Szeliga W, et al. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood*. 2009 Jul 9;114(2):357-9.

Kuen DS, Kim BS, Chung Y. IL-17-Producing Cells in Tumor Immunity: Friends or Foes? *Immune Netw*. 2020 Feb 7;20(1):e6.

Kyzas PA, Cunha IW, Ioannidis JP. Prognostic significance of vascular endothelial growth factor immunohistochemical expression in head and neck squamous cell carcinoma: a meta-analysis. *Clin Cancer Res*. 2005 Feb 15;11(4):1434-40.

Lambert R, Sauvaget C, de Camargo Cancela M, et al. Epidemiology of cancer from the oral cavity and oropharynx. *Eur J Gastroenterol Hepatol*. 2011 Aug;23(8):633-41.

Lee EJ, Park HJ, Lee IJ, et al. Inhibition of IL-17A suppresses enhanced-tumor growth in low dose pre-irradiated tumor beds. *PLoS One*. 2014 Sep 2;9(9):e106423.

Lee S, Margolin K. Cytokines in cancer immunotherapy. *Cancers* (Basel). 2011; 13;3(4):3856-93.

Lereclus E, Tout M, Girault A, et al. A possible association of baseline serum IL-17A concentrations with progression-free survival of metastatic colorectal cancer patients treated with a bevacizumab-based regimen. *BMC Cancer*. 2017; 27;17(1):220.

Lin AM, Rubin CJ, Khandpur R, et al. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. *J Immunol*. 2011;187:490-500.

Liu M, Wang SJ, Yang X, et al. Diagnostic Efficacy of Sentinel Lymph Node Biopsy in Early Oral Squamous Cell Carcinoma: A Meta-Analysis of 66 Studies. *PLoS One*. 2017 Jan 20;12(1):e0170322.

Liu SY, Chang LC, Pan LF, et al. Clinicopathologic significance of tumor cell-lined vessel and microenvironment in oral squamous cell carcinoma. *Oral Oncol*. 2008;44(3):277-85.

Liu Y, Sun X, Zhao X, et al. Expression and location of IL-17A, E, F and their receptors in colorectal adenocarcinoma: Comparison with benign intestinal disease. *Pathol. Res. Pract*. 2018 Apr;214(4):482–91.

Lotti F, Jarrar AM, Pai RK, et al. Chemotherapy activates cancer-associated fibroblasts to maintain colorectal cancer-initiating cells by IL-17A. *J Exp Med*. 2013 Dec 16;210(13):2851-72.

Luo Q, Wang J, Zhao W, et al. Vasculogenic mimicry in carcinogenesis and clinical applications. *J Hematol Oncol*. 2020 Mar 14;13(1):19.

Macey R, Walsh T, Brocklehurst P, et al. Diagnostic tests for oral cancer and potentially malignant disorders in patients presenting with clinically evident lesions. *Cochrane Database Syst Rev*. 2015 May 29;2015(5):CD010276.

Maggioni D, Pignataro L, Garavello W. T-helper and T-regulatory cells modulation in head and neck squamous cell carcinoma. *Oncoimmunology*. 2017 May 4;6(7):e1325066.

Maniotis AJ, Folberg R, Hess A, et al. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol*. 1999;155(3):739-52.

Markopoulos AK. Current aspects on oral squamous cell carcinoma. *Open Dent J*. 2012;6:126-30.

Marques LA, Eluf-Neto J, Figueiredo RA, et al. Oral health, hygiene practices and oral cancer. *Rev Saude Publica*. 2008 Jun;42(3):471-9.

Martin-Orozco N, Muranski P, Chung Y, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity*. 2009 Nov 20;31(5):787-98.

Martone T, Rosso P, Albera R, et al. Prognostic relevance of CD105+ microvessel density in HNSCC patient outcome. *Oral Oncol*. 2005 Feb;41(2):147-55.

Marur S, D'Souza G, Westra WH, et al. HPV-associated head and neck cancer: a virus-related cancer epidemic. *Lancet Oncol*. 2010 Aug;11(8):781-9.

Marur S, Forastiere AA. Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment. *Mayo Clin Proc*. 2016; 91:386-396.

Massey C, Dharmarajan A, Bannuru RR, et al. Management of N0 neck in early oral squamous cell carcinoma: A systematic review and meta-analysis. *Laryngoscope*. 2019 Aug;129(8):E284-E298.

McCullough MJ, Farah CS. The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes. *Aust Dent J*. 2008 Dec;53(4):302-5.

McShane LM, Altman DG, Sauerbrei W, et al. REporting recommendations for tumour MARKer prognostic studies (REMARK). *Br J Cancer*. 2005 Aug 22;93(4):387-91.

Merchant A, Husain SS, Hosain M, et al. Paan without tobacco: an independent risk factor for oral cancer. *Int J Cancer*. 2000 Apr 1;86(1):128-31.

Mineo TC, Ambrogi V, Baldi A, et al. Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB-IIA non-small cell lung cancer. *J Clin Pathol*. 2004 Jun;57(6):591-7.

Miossec P. Local and systemic effects of IL-17 in joint inflammation: a historical perspective from discovery to targeting. *Cell Mol Immunol*. 2021 Apr;18(4):860-865.

Miranda-Filho A, Bray F. Global patterns and trends in cancers of the lip, tongue and mouth. *Oral Oncol*. 2020 Mar;102:104551.

Muto M, Nakane M, Katada C, et al. Squamous cell carcinoma in situ at oropharyngeal and hypopharyngeal mucosal sites. *Cancer*. 2004 Sep 15;101(6):1375-81.

Ng JH, Iyer NG, Tan MH, et al. Changing epidemiology of oral squamous cell carcinoma of the tongue: A global study. *Head Neck*. 2017 Feb;39(2):297-304.

Nguyen N, Bellile E, Thomas D, et al. Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. *Head Neck*. 2016 Jul;38(7):1074-84.

Nielsen HJ, Hansen U, Christensen IJ, et al. Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. *J Pathol*. 1999;189:487-495.

O'Grady JF, Reade PC. Candida albicans as a promoter of oral mucosal neoplasia. *Carcinogenesis*. 1992 May;13(5):783-6.

Olmos M, Glajzer J, Büntemeyer TO, et al. Neoadjuvant Immunotherapy of Oral Squamous Cell Carcinoma: Case Report and Assessment of Histological Response. *Front Oncol*. 2021 Jul 21;11:720951.

Omura K. Current status of oral cancer treatment strategies: surgical treatments for oral squamous cell carcinoma. *Int J Clin Oncol*. 2014;19(3):423-30.

Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005 May 6;121(3):335-48.

O'Sullivan B, Brierley J, Byrd D, et al. The TNM classification of malignant tumours-towards common understanding and reasonable expectations. *Lancet Oncol*. 2017 Jul;18(7):849-851.

Pałasz P, Adamski Ł, Górska-Chrząstek M, et al. Contemporary Diagnostic Imaging of Oral Squamous Cell Carcinoma -A Review of Literature. *Pol J Radiol*. 2017;82:193-202.

Pan B, Shen J, Cao J, et al. Interleukin-17 promotes angiogenesis by stimulating VEGF production of cancer cells via the STAT3/GIV signaling pathway in non-small-cell lung cancer. *Sci Rep*. 2015 Nov 3;5:16053.

Pavia M, Pileggi C, Nobile CG, et al. Association between fruit and vegetable consumption and oral cancer: a meta-analysis of observational studies. *Am J Clin Nutr*. 2006 May;83(5):1126-34.

Piemonte ED, Lazos JP, Brunotto M. Relationship between chronic trauma of the oral mucosa, oral potentially malignant disorders and oral cancer. *J Oral Pathol Med*. 2010 Aug 1;39(7):513-7.

Pisacane AM, Picciotto F, Risio M. CD31 and CD34 expression as immunohistochemical markers of endothelial transdifferentiation in human cutaneous melanoma. *Cell Oncol*. 2007;29:59-66.

Pitkäniemi J, Malila N, Tanskanen T, et al. Cancer in Finland 2019. Cancer Society of Finland Publication No. 98, Helsinki 2021.

Puel A, Cypowyj S, Bustamante J, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science*. 2011 Apr 1;332(6025):65-8.

Punt S, Langenhoff JM, Putter H, et al. The correlations between IL-17 vs. Th17 cells and cancer patient survival: a systematic review. *Oncoimmunology*. 2015;6;4(2):e984547.

Radoï L, Luce D. A review of risk factors for oral cavity cancer: the importance of a standardized case definition. *Community Dent Oral Epidemiol*. 2013;41(2):97-109.

Ramirez-Garcia A, Rementeria A, Aguirre-Urizar JM, et al. Candida albicans and cancer: Can this yeast induce cancer development or progression? *Crit Rev Microbiol*. 2016;42(2):181-93.

Raphael I, Nalawade S, Eagar TN, et al. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*. 2015 Jul;74(1):5-17.

Regezi J, Sciubba J, Jordan R. Oral Pathology: Clinical Pathologic Correlations. 2017; 7th Ed. (3) 80-113; St. Louis MO, Elsevier.

Ribatti D, Annese T, Ruggieri S, et al. Limitations of Anti-Angiogenic Treatment of Tumors. *Transl Oncol*. 2019 Jul;12(7):981-986.

Ribatti D, Tamma R, Crivellato E. The dual role of mast cells in tumor fate. *Cancer Lett*. 2018 Oct 1; 433:252-258.

Roma-Rodrigues C, Mendes R, Baptista PV, et al. Targeting Tumor Microenvironment for Cancer Therapy. *Int J Mol Sci*. 2019 Feb 15;20(4):840.

Rosen RD, Sapra A. TNM Classification. 2021 Feb 23. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021.

Saad RS, Luckasevic TM, Noga CM, et al. Diagnostic value of HepPar1, pCEA, CD10, and CD34 expression in separating hepatocellular carcinoma from metastatic carcinoma in fine-needle aspiration cytology. *Diagn Cytopathol*. 2004;30(1):1-6.

Sahai E, Astsaturov I, Cukierman E, et al. A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer*. 2020 Mar;20(3):174-186.

Sahovaler A, Kim MH, Mendez A, et al. Survival Outcomes in Human Papillomavirus-Associated Nonoropharyngeal Squamous Cell Carcinomas: A Systematic Review and Meta-analysis. *JAMA Otolaryngol Head Neck Surg*. 2020;146(12):1158-1166.

Sakaguchi S, Yamaguchi T, Nomura T, et al. Regulatory T cells and immune tolerance. *Cell*. 2008 May 30;133(5):775-87.

Salem A, Almahmoudi R, Hagström J, et al. Human β-Defensin 2 Expression in Oral Epithelium: Potential Therapeutic Targets in Oral Lichen Planus. *Int J Mol Sci*. 2019 Apr 10;20(7):1780.

Salem A, Almahmoudi R, Listyarifah D, et al. Histamine H4 receptor signalling in tongue cancer and its potential role in oral carcinogenesis - a short report. *Cell Oncol (Dordr)*. 2017 Dec;40(6):621-630.

Salem A, Salo T. Nothing to sneeze at: Histamine and histamine receptors in oral carcinogenesis. *Oral Dis*. 2021b. Jul;27(5):1090-1096.

Salem A, Salo T. Vasculogenic Mimicry in Head and Neck Squamous Cell Carcinoma-Time to Take. *Frontiers in Oral Health*.2021a.

Salizzoni M, Romagnoli R, Lupo F, et al. Microscopic vascular invasion detected by anti-CD34 immunohistochemistry as a predictor of recurrence of hepatocellular carcinoma after liver transplantation. *Transplantation*. 2003;15;76(5):844-8.

Salo T, Sutinen M, Hoque Apu E, et al. A novel human leiomyoma tissue derived matrix for cell culture studies. *BMC Cancer*. 2015 Dec 16;15:981.

Samiei G, Yip WK, Leong PP, et al. Association between polymorphisms of interleukin-17A G197A and interleukin-17F A7488G and risk of colorectal cancer. *J Cancer Res Ther*. 2018 Jun;14(Supplement):S299-S305.

Sankari SL, Gayathri K, Balachander N, et al. Candida in potentially malignant oral disorders. *J Pharm Bioallied Sci*. 2015 Apr;7(Suppl 1):S162-4.

Sano D, Myers JN. Metastasis of squamous cell carcinoma of the oral tongue. *Cancer Metastasis Rev*. 2007 Dec; 26(3-4):645-62.

Sasahira T, Kirita T, Kurihara M, et al. MIA-dependent angiogenesis and lymphangiogenesis are closely associated with progression, nodal metastasis and poor prognosis in tongue squamous cell carcinoma. *Eur J Cancer*. 2010 Aug;46(12):2285-94.

Schilling C, Shaw R, Schache A, et al. Sentinel lymph node biopsy for oral squamous cell carcinoma. Where are we now? *Br J Oral Maxillofac Surg*. 2017 Oct;55(8):757-762.

Schimming R, Marmé D. Endoglin (CD105) expression in squamous cell carcinoma of the oral cavity. *Head Neck.* 2002 Feb;24(2):151-6.

Scoccianti C, Cecchini M, Anderson AS, et al. European Code against Cancer 4th Edition: Alcohol drinking and cancer. *Cancer Epidemiol*. 2016 Dec;45:181-188.

Seftor RE, Hess AR, Seftor EA, et al. Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. *Am J Pathol*. 2012 Oct;181(4):1115-25.
Selvamani M, Yamunadevi A, Basandi PS, et al. Prevalence of oral squamous cell carcinoma of tongue in and around Davangere, Karnataka, India: A retrospective study over 13 years. *J Pharm Bioallied Sci*. 2015 Aug;7(Suppl 2):S491-4.
Seppälä M, Pohjola K, Laranne J, et al. High relative density of lymphatic vessels predicts poor survival in tongue squamous cell carcinoma. *Eur Arch Otorhinolaryngol*. 2016 Dec;273(12):4515-4524.
Shao Z, Zhang WF, Chen XM, Shang ZJ. Expression of EphA2 and VEGF in squamous cell carcinoma of the tongue: correlation with the angiogenesis and clinical outcome. *Oral Oncol*. 2008 Dec;44(12):1110-7.
Sharma M, Sah P, Sharma SS, Radhakrishnan R. Molecular changes in invasive front of oral cancer. *J Oral Maxillofac Pathol*. 2013 May;17(2):240-7.
Sharp SP, Avram D, Stain SC, Lee EC. Local and systemic Th17 immune response associated with advanced stage colon cancer. *J Surg Res*. 2017 Feb;208:180-186.
Shieh YS, Lee HS, Shiah SG, et al. Role of angiogenic and non-angiogenic mechanisms in oral squamous cell carcinoma: correlation with histologic differentiation and tumor progression. *J Oral Pathol Med*. 2004 Nov;33(10):601-6.
Sim YC, Hwang JH, Ahn KM. Overall and disease-specific survival outcomes following primary surgery for oral squamous cell carcinoma: analysis of consecutive 67 patients. *J Korean Assoc Oral Maxillofac Surg*. 2019 Apr;45(2):83-90.
Søland TM, Bjerkli IH, Georgsen JB, et al. High-risk human papilloma virus was not detected in a Norwegian cohort of oral squamous cell carcinoma of the mobile tongue. *Clin Exp Dent Res*. 2021 Feb;7(1):70-77.
Spector ME, Bellile E, Amlani L, et al. Prognostic Value of Tumor-Infiltrating Lymphocytes in Head and Neck Squamous Cell Carcinoma. *JAMA Otolaryngol Head Neck Surg*. 2019 Nov 1;145(11):1012-1019.
Speight PM, Farthing PM. The pathology of oral cancer. Br Dent J. 2018; 9;225(9):841-847.
Speight PM. Update on oral epithelial dysplasia and progression to cancer. *Head Neck Pathol*. 2007 Sep;1(1):61-6.
Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. *Nat Rev Immunol*. 2017 Sep;17(9):535-544.
Stoeckli SJ. Sentinel node biopsy for oral and oropharyngeal squamous cell carcinoma of the head and neck. *Laryngoscope*. 2007 Sep;117(9):1539-51
Stuelten CH, DaCosta Byfield S, Arany PR, et al. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta. *J Cell Sci*. 2005 May 15;118(Pt 10):2143-53.
Sun C, Kono H, Furuya S, et al. Interleukin-17A Plays a Pivotal Role in Chemically Induced Hepatocellular Carcinoma in Mice. *Dig Dis Sci*. 2016 Feb;61(2):474-88.
Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*. 2021 May;71(3):209-249.
Sutton DN, Brown JS, Rogers SN, et al. The prognostic implications of the surgical margin in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg*. 2003 Feb;32(1):30-4.

Syrjänen S, Lodi G, von Bültzingslöwen I, et al. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review. *Oral Dis*. 2011;17 Suppl 1:58-72.

Tasche KK, Buchakjian MR, Pagedar NA, Sperry SM. Definition of "Close Margin" in Oral Cancer Surgery and Association of Margin Distance With Local Recurrence Rate. *JAMA Otolaryngol Head Neck Surg*. 2017 Dec 1;143(12):1166-1172.

Thiagarajan S, Nair S, Nair D, et al. Predictors of prognosis for squamous cell carcinoma of oral tongue. *J Surg Oncol*. 2014;109(7):639-44.

Thompson L. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. *Ear Nose Throat J*. 2006 Feb; 85(2):74.

Tong M, Han BB, Holpuch AS, et al. Inherent phenotypic plasticity facilitates progression of head and neck cancer: endotheliod characteristics enable angiogenesis and invasion. *Exp Cell Res*. 2013 Apr 15;319(7):1028-42.

Tong Z, Yang XO, Yan H, et al. A protective role by interleukin-17F in colon tumorigenesis. *PLoS One*. 2012;7(4):e34959.

Toyoda M, Kaira K, Ohshima Y, et al. Prognostic significance of amino-acid transporter expression (LAT1, ASCT2, and xCT) in surgically resected tongue cancer. *Br J Cancer*. 2014 May 13;110(10):2506-13.

Tuomainen K, Al-Samadi A, Potdar S, et al. Human Tumor-Derived Matrix Improves the Predictability of Head and Neck Cancer Drug Testing. *Cancers (Basel)*. 2019;30;12(1):92.

Turati F, Garavello W, Tramacere I, et al. A meta-analysis of alcohol drinking and oral and pharyngeal cancers. Part 2: results by subsites. *Oral Oncol*. 2010 Oct;46(10):720-6.

Ustrell-Borràs M, Traboulsi-Garet B, Gay-Escoda C. Alcohol-based mouthwash as a risk factor of oral cancer: A systematic review. *Med Oral Patol Oral Cir Bucal*. 2020 Jan 1;25(1):e1-e12.

Valdivia A, Mingo G, Aldana V, et al. Fact or Fiction, It Is Time for a Verdict on Vasculogenic Mimicry? *Front Oncol*. 2019 Aug 2;9:680.

van der Schaft DW, Seftor RE, Seftor EA, et al. Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells. *J Natl Cancer Inst*. 2004 Oct 6;96(19):1473-7.

van der Waal I, de Bree R, Brakenhoff R, Coebergh JW. Early diagnosis in primary oral cancer: is it possible? Med Oral Patol Oral Cir Bucal. 2011 May 1; 16(3):e300-5.

Varricchi G, Galdiero MR, Loffredo S, et al. Are Mast Cells MASTers in Cancer? *Front Immunol*. 2017 Apr 12; 8:424.

Vassilakopoulou M, Psyrri A, Argiris A. Targeting angiogenesis in head and neck cancer. *Oral Oncol*. 2015 May;51(5):409-15.

Vitiello GA, Miller G. Targeting the interleukin-17 immune axis for cancer immunotherapy. *J Exp Med*. 2020 Jan 6;217(1):e20190456.

Vora HH, Shah NG, Patel DD, et al. Prognostic significance of biomarkers in squamous cell carcinoma of the tongue: multivariate analysis. *J Surg Oncol*. 2003 Jan;82(1):34-50.

Wang K, Kim MK, Di Caro G, et al. Interleukin-17 receptor a signaling in transformed enterocytes promotes early colorectal tumorigenesis. *Immunity*. 2014 Dec 18;41(6):1052-63.

Wang L, Yi T, Zhang W, et al. IL-17 enhances tumor development in carcinogen-induced skin cancer. *Cancer Res*. 2010 Dec 15;70(24):10112-20.

Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. *J Oral Pathol Med.* 2007; 36:575-580.

Watnick RS. The role of the tumor microenvironment in regulating angiogenesis. *Cold Spring Harb Perspect Med*. 2012 Dec 1;2(12):a006676.

Wei T, Cong X, Wang XT, et al. Interleukin-17A promotes tongue squamous cell carcinoma metastasis through activating miR-23b/versican pathway. *Oncotarget*. 2017 Jan 24;8(4):6663-6680.

Weijers M, Snow GB, Bezemer PD, et al. The clinical relevance of epithelial dysplasia in the surgical margins of tongue and floor of mouth squamous cell carcinoma: an analysis of 37 patients. *J Oral Pathol Med*. 2002 Jan;31(1):11-5.

Whiteside TL. The Local Tumor Microenvironment. In: Kaufmann H, Wolchok JD, editors. General Principles of Tumor Immunotherapy: Basic and Clinical Applications of Tumor Immunology. Springer; 2007. pp. 145–167.

Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene*. 2008 Oct 6;27(45):5904-12.

Wolff KD, Follmann M, Nast A. The diagnosis and treatment of oral cavity cancer. *Dtsch Arztebl Int*. 2012 Nov;109(48):829-35.

Woolgar JA, Triantafyllou A. Pitfalls and procedures in the histopathological diagnosis of oral and oropharyngeal squamous cell carcinoma and a review of the role of pathology in prognosis. *Oral Oncol*. 2009 Apr-May;45(4-5):361-85.

Xie Y, Sheng W, Xiang J, et al. Interleukin-17F suppresses hepatocarcinoma cell growth via inhibition of tumor angiogenesis. *Cancer Invest*. 2010 Jul;28(6):598-607.

Yadav A, Kumar B, Teknos TN, Kumar P. Sorafenib enhances the antitumor effects of chemoradiation treatment by downregulating ERCC-1 and XRCC-1 DNA repair proteins. *Mol Cancer Ther*. 2011 Jul;10(7):1241-51.

Yamaguchi H, Wyckoff J, Condeelis J. Cell migration in tumors. *Curr Opin Cell Biol*. 2005 Oct;17(5):559-64.

Yan J, Jiang Y, Ye M, et al. The clinical value of lymphatic vessel density, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 expression in patients with oral tongue squamous cell carcinoma. *J Cancer Res Ther*. 2014;10; 125-130.

Yang L, Liu F, Wu Y, et al. Predictive Value of Occult Metastasis and Survival Significance of Metabolic Tumor Volume Determined by PET-CT in cT1-2N0 Squamous Cell Carcinoma of the Tongue. *Front Oncol*. 2020 Dec 4;10:542530.

Yang XO, Chang SH, Park H, et al. Regulation of inflammatory responses by IL-17F. *J Exp Med*. 2008 May 12;205(5):1063-75.

Zanoni DK, Migliacci JC, Xu B, et al. A Proposal to Redefine Close Surgical Margins in Squamous Cell Carcinoma of the Oral Tongue. *JAMA Otolaryngol Head Neck Surg*. 2017 Jun 1;143(6):555-560.

Zhang Y, Zoltan M, Riquelme E, et al. Immune Cell Production of Interleukin 17 Induces Stem Cell Features of Pancreatic Intraepithelial Neoplasia Cells. *Gastroenterology*. 2018 Jul;155(1):210-223.e3.

Zhao J, Chen X, Herjan T, Li X. The role of interleukin-17 in tumor development and progression. *J Exp Med*. 2020 Jan 6;217(1):e20190297.

Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol*. 2010;28:445-89.