Faculty or Health Sciences, Department of Medical Biology
Tumour Biology Research Group

Prognostic markers in oral squamous cell carcinoma

Oddveig G. Rikardsen
A dissertation for the degree of Philosophiae Doctor – April 2014
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1. **AKNOWLEDGEMENTS**

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Tromsø, April 2014    Oddveig G. Rikardsen
2. LIST OF PAPERS

I. Clincopathological characteristics of oral squamous cell carcinoma in Northern Norway. A retrospective study.
Oddveig G. Rikardsen, Inger-Heidi Bjerkli, Lars Uhlin-Hansen, Elin Hadler-Olsen, Sonja E. Steigen
(submitted)

II. Urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1) are potential predictive biomarkers in early stage oral squamous cell carcinomas (OSCC)
Synnøve Magnussen*, Oddveig Rikardsen*, Elin Hadler-Olsen, Jan-Olof Winberg, Lars Uhlin Hansen, Sonja E. Steigen, Gunbjørg Svineng
*The authors contributed equally to this work
(re-submitted)

III. Plectin as a prognostic marker in non-metastatic oral squamous cell carcinoma.
Oddveig G. Rikardsen, Synnøve Magnussen, Gunbjørg Svineng, Elin Hadler-Olsen, Lars Uhlin-Hansen, Sonja E. Steigen
(submitted)

IV. Characterisation and prognostic value of tertiary lymphoid organs in oral squamous cell carcinomas
Anna Wirsing, Oddveig G. Rikardsen, Sonja E. Steigen, Lars Uhlin-Hansen, Elin Hadler-Olsen
(submitted)

V. High-endothelial venules predict favourable outcome in patients with oral squamous cell carcinoma
Anna Wirsing, Oddveig G. Rikardsen, Sonja E. Steigen, Lars Uhlin-Hansen, Elin Hadler-Olsen
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>CSCs</td>
<td>Cancer stem like cells</td>
</tr>
<tr>
<td>DSD</td>
<td>Disease specific death</td>
</tr>
<tr>
<td>DSS</td>
<td>Disease specific survival</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGRF</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked-immunosorbent assay</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<tr>
<td>FDC</td>
<td>Follicular dendritic cell (FDC)</td>
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<tr>
<td>GC</td>
<td>Germinal centre</td>
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<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
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<td>HEV</td>
<td>High endothelial venule</td>
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<td>HIER</td>
<td>Heat-induced epitope retrieval</td>
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<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>ICD-10</td>
<td>International Classification of Diseases, 10th edition</td>
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<tr>
<td>ICD-O</td>
<td>International Classification of Diseases for Oncology</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
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<tr>
<td>PA</td>
<td>Plasminogen activator</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor type 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween</td>
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<tr>
<td>PNAd</td>
<td>Peripheral node addressin</td>
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<td>Rb</td>
<td>Retinoblastoma</td>
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<td>REK</td>
<td>Regional Etisk Komitet</td>
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<tr>
<td>REMARK</td>
<td>REporting recommendations for tumor MARKer prognostic studies</td>
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<tr>
<td>RT</td>
<td>Radiation therapy</td>
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<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
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<tr>
<td>TLO</td>
<td>Tertiary lymphoid organs</td>
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<tr>
<td>TMA</td>
<td>Tissue micro array</td>
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<tr>
<td>TNM</td>
<td>Tumor-node-metastasis</td>
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<tr>
<td>tPA</td>
<td>Tissue type plasminogen activator</td>
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<tr>
<td>UICC</td>
<td>Union for International Cancer Control (UICC),</td>
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<tr>
<td>UNN</td>
<td>University Hospital of North Norway</td>
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<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
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<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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4. PREFACE

Head and neck squamous cell carcinomas (HNSCC) in general are aggressive and unpredictable tumours with a tendency for early lymphatic spread. Despite all medical improvements over the last decades, the improvements in outcome have been very limited. The overall prognosis is poor, and the treatment is distressing with devastating side-effects for those affected.

HNSCCs are known to be heterogeneous tumours, both in growth pattern, aggressiveness and response to treatment, indicating biological differences, also within the same primary site and stage. Even estimated manageable early stage tumours often have the ability for aggressive local growth and metastasize to regional lymph nodes with a fatal outcome. This unpredictability raises a need for new tools that can provide better prognostic information. In addition, tumours arising at different primary sites in the head and neck region show essential etiological, molecular and biological differences, and cancer location has to be taken into account in both research and choice of treatment. The best known example in HNSCC reflecting the etiological and biological differences between primary sites is the impact of human papilloma virus (HPV)-infection. HPV has been shown to be of huge importance in the oropharyngeal location, and HPV- positive and negative tumours are in many aspects to be considered as different diseases. However, HPV is by far less frequent in tumours of the oral cavity, illustrating the need of accuracy in diagnostics and reporting.

The complex anatomy of the head and neck give rise to intricate patterns of local invasion and regional spread, which often makes it difficult to eradicate the tumour surgically once it has spread into adjacent tissues. This makes it even more important to
increase the knowledge of the biology of HNSCCs and their response to non-surgical approaches.

In this thesis we have focused on possible prognostic markers in human oral squamous cell carcinomas, and correlated immunohistochemical staining of putative marker proteins with clinical information to evaluate the prognostic impact. In clinical practice, the main goal is to give the patient the treatment needed to cure the disease, but at the same time not cause unnecessary harm due to overtreatment. Therefore it is important to provide the most accurate information on prognosis and expected course of the disease, to enable a more aggressive therapy to be selected for high-risk patients, and avoid overtreatment of those with more favourable prospects.
5. BACKGROUND

5.1 Oral squamous cell carcinoma

5.1.1 Global incidence

Squamous cell carcinoma account for more than 90% of the malignant neoplasm of the Head and Neck (1, 2). Globally, HNSCC represents about 2-5% of all cancers, though, due to aggressive behaviour they accounted for 4.5% of all cancer deaths, and was ranked as the eight leading cause of cancer death worldwide in 2000 (3, 4). The corresponding numbers for Europe was 2.3% of all cancer deaths and a ranking as number thirteen as cause of cancer death (4).

Globally, there is estimated 400 000 new case of HNSCC identified each year, the annual incidence for oral cancer only is around 275.000 (3), two-thirds of the cases reported in developing countries.

There is a pronounced geographical variation in incidence, the high risk countries being found in the South and Southeast Asia, parts of Latin America, Caribbean and the Pacific regions. In high-risk countries as Sri Lanka, India, Pakistan and Bangladesh, oral cancer is the most common cancer among the male population, and contributes to as much as a fourth of all new cancer cases (3).

In Europe, the incidence rates are higher in Western and Eastern Europe than in north or south; France and Hungary presenting the highest numbers, Greece and Cyprus the lowest (5-8). In northern France, oral and oropharyngeal cancers combined constitute the second most common cancer in men, after lung cancer (6).
Male/female ratio in Northern Europe, like Denmark and the UK, vary between 1.3 to 2.0:1 (7, 9, 10). There is however both a global and a national trend of decreasing gender differences (3, 6, 11). The difference in incidence between men and women is in general smaller for oral than oropharyngeal site (6).

Age-standardized incidence rates per 100 000 for oral cancer (including lip cancer) in Northern Europe is 5.1 for men and 2.5 for women (5, 10). From the Cancer Registry of Norway, which publish national data in cancer incidence, mortality, survival and prevalence, the equivalent figures are 4.3 and 2.5. Lip cancer account for approximately 40% of the cases (12), the remaining comprising the oral cavity including tongue, floor of the mouth, bucca, gingiva and soft palate (ICD-O, C01-C06)(11).

From the Cancer Registry of Norway we have received incidence data from 1995 including the oral cavity and mobile part of tongue only (ICD-O, C02-C06)(11), being 3.0 for men and 1.4 for women (rates per 100 000), giving a male/female ratio of a 2.1:1. For the three northernmost counties, representing our impact area, the numbers were 2.6 for men and 2.0 for women, giving a ratio of 1.3:1.

5.1.2 Risk factors

Globally, the large geographical variations in incidence are mostly explained by cultural differences and exposure to risk factors (2). An example of this is the chewing of betel quid and areca nuts in high risk areas such as South and Southeast Asia (13, 14).

The lifetime risk of developing oral or oropharyngeal cancer in Europe is estimated to be 1.85 % for men and 0.37 % for women (3). The two best known and most studied risk
factors in our part of the world are tobacco and alcohol. These factors act strongly synergistic, and are estimated to account for up to 75% of the disease burden in Western countries (8, 15-17).

According to Koch et al., smoking increases the risk for oral cancer 1.9 times for men and three times for women. Daily, moderate drinkers (2 drinks a day) are reported to have an increased risk of 1.7, rising up to 3 for heavy drinkers. Daily smoking and drinking are estimated to give an odds ratio of 35 (18, 19). If you stop smoking, the risk falls to non-smoker levels in 10 years (18). The pattern of risk after stopping drinking remains unclear, though it appears that an increased excess risk persists for several years (6, 8, 18).

The exposure to various risk factors also have different impact on different primary sites in OSCC, illustrated by betel nut chewing disposing for tumours arising in the buccal mucosa, while smokers and alcoholics are particularly disposed to tumours in the floor of mouth (1, 18, 20-22). Changes in alcohol consumption appear to have more influence on the incidence of cancer in the oral cavity than on those in the pharyngeal location, which on the other hand are closely related to HPV.

La Vecchia et al. described a rising trend in oral cancer up to the mid 1990 in Europe, reflecting the expanding epidemic of all tobacco-related neoplasms in these countries. But although the recent changes in smoking habits have showed a positive impact on the incidence of lung cancer, no such correlation has been seen in oral cancer, essentially attributable to changes in alcohol consumption (8). Additionally, several studies have shown that hard liquor have a larger impact on OSCC-development than wine and beer (19, 23, 24).
There have also been indications that nutrition factors can influence on the risk for OSCC. Lack of fruit, non-starchy vegetables and foods containing carotenoids are associated with oral cancer with some evidence, 10-15% of causes are attributable to low fruit and vegetable intake (25). There is also evidence of increasing odds ratio for oral cancer with lower Body Mass Index (26, 27).

5.1.3 Primary site, classification and staging

Both Freier et al. (28, 29) and Radhakrishnan et al (30) concluded that the oncogene amplification of HNSCCs varies among the different anatomic sites, concluding that different molecular pathways are involved in HNSCCs of different localization. Therefore it is of great importance to be precise, and distinguishing between the different primary sites in both clinical and biological research.

The knowledge about the impact of HPV on oropharyngeal tumours highlights the importance of distinguishing these from tumours arising in other sites. Traditionally, in publications and records, tumours of the oropharynx and the lip are often included in the oral cancer term. Over the last decade, researchers have become more aware of the need to split this group defined on more specific anatomical sites, due to the differences in behaviour and biology of the tumours (6). Also, due to this increased knowledge, there is a need to split the cancers of the tongue into tumours arising in the mobile part and tumours of the base of tongue, respectively. The mobile part of the tongue should be included in the group of oral cavity cancers, while tumours in the base of the tongue should be included with the oropharyngeal cancers. Thus, when focusing on cancer of the oral cavity, only cancers arising in the mobile tongue, floor of mouth, bucca, gingiva, palate, vestibulum and labial mucosa should be
included (ICD-O, C02-C06)(11). Cancers arising in the base of the tongue, tonsils and other sites in the oropharynx, or the vermillion zone of the lips should be excluded, the latter for having more in common with squamous cell carcinomas of the skin both in aetiology and behaviour (31, 32).

The most widely used classification-system for describing the anatomical extent of the disease is the TNM-system by the Union for International Cancer Control (UICC). The TNM-system grades the primary tumour size and invasion features (T), regional lymph node spread (N) and the presence of distant metastasis (M)(33). Survival of the OSCC patients is strongly associated with the TNM-stage, and the TNM-classification system is the most important system in clinical practice to guide treatment selection (34-37). The TNM-stage of the tumour at diagnosis is quite a good indicator of patient prognosis, where small tumours without metastasis (low TNM-stage) in general present a better outcome.
The TMN-system is also the base for stage grouping of patients according to the American Joint Committee on Cancer (AJCC) staging system (38).
By morphological assessment, tumours are also classified based on the cancer cells differentiation into well, moderately and poorly differentiated carcinoma (1). However, although the histological differentiation is usually specified in pathology reports, it provides limited information to guide treatment decisions because a strong association between grade of differentiation and clinical outcome or treatment response is lacking (1, 39, 40).

The TNM-classification, and to a lesser degree, the grade of differentiation, are often used as an indicator for outcome. Although tumours have the same cellular origin, the SCCs of this region are unexpectedly heterogeneous, both in growth pattern, clinical course and response to treatment (34), all these individual differences are seen within the same TNM-groups (39, 41, 42), and a large number of occult metastases are found in patients clinically classified as N0 (43-45). This heterogeneity defines a need for increased knowledge of the molecular characteristics of the tumours to support the staging system in aiding the clinicians to recognize patients with greater need of close follow up and potential additional adjuvant treatment (39).

5.1.4 Histopathology

Squamous cell carcinoma (SCC) accounts for over 90% of malignant tumour in the oral cavity. The remainder includes adenocarcinomas (of salivary gland origin), melanomas, lymphomas and various sarcomas (46).
A malignant tumour of any epithelial origin is termed a *carcinoma*, and as the name indicates a *squamous cell carcinoma* origin from any site of native stratified squamous cell epithelia, in this context the surface epithelium of the oral cavity. The term “differentiation” describes to what degree the tumour tissue resembles normal oral surface epithelium. In general, squamous cell carcinomas of the head and neck are known to be histological heterogeneous (47). The conventional histopathological grading of oral squamous cell carcinomas are defined as well (pG1), moderately (pG2) or poorly (pG3) differentiated (1), based on degree of keratinisation, cellular pleomorphism, mitotic activity and nuclear aberrations. The degree of differentiation can vary within a tumour, from highly differentiated areas to areas with low differentiation. As a general rule, the tumours are to be classified based on the area of the tumour showing the lowest grade of differentiation.

Well differentiated SCC resembles closely normal squamous epithelium and has cytological features similar to the prickle cell layer of the normal stratified epithelium. These tumours have large, slightly fusiform cells, arranged in broad sheets and large clumps. The nuclei exhibit a moderate degree of pleomorphism, and mitoses are not very abundant. The most characteristic feature of well-differentiated SCC is the formation of keratin, both in individual cells, but more prominent in lamellated masses known as keratin pearls. Moderately differentiated SCC contains distinct nuclear pleomorphism and higher mitotic activity, including abnormal mitoses; and usually less keratinization. Poorly differentiated SCCs lose their resemblance to normal prickle cells, immature cells predominate, with numerous typical and atypical mitoses, and a high nucleus-cytoplasmic ratio. Keratin pearl formation is not seen, though individual cell keratinisation might be present. In the most anaplastic SCC, the only evidence of its origin may be the intercellular bridges only visible at high magnification.
Although keratinization is more likely to be present in well- or moderately-differentiated SCC, it should not be considered an important histological criterion in grading SCC.

In a **well differentiated** OSCC (pG1) the cells are large and slightly fusiform, nuclei shows a moderate degree of pleomorphism, and few mitoses are seen. Lamelled keratin masses known as keratin pearls is a characteristic feature.

In the **moderately differentiated** SCC (pG2) nuclear pleomorphism are more distinct. There is also a higher mitotic activity, including abnormal mitoses. Keratinization is less prominent.

**Poorly differentiated** SCCs (pG3) are dominated by immature cells with numerous typical and atypical mitoses, and a high nucleus-cytoplasmic ratio. Keratin pearl formation is not seen, though individual cell keratinisation might be present.
As grading by differentiation has shown to have a lack of interobserver agreement (48), and the fact that most SCC is moderately differentiated, grading by differentiation is really of limited prognostic value, as compared to the pattern of invasion.

Tumour growth at the invasive front can either be expansive, characterized by large tumour islands with well-defined pushing margins, or infiltrative, characterized by scattered small irregular cords or single tumour cells and poorly defined infiltrating margins. Different regions of the same tumour often show different patterns of invasion, and the most aggressive pattern should be recorded. In general the more infiltrative pattern is associated with a more aggressive course and worse outcome (1, 49-54).

6.1.5 Diagnosis, treatment and prognosis

HNSCC in general is associated with severe disease- and treatment related morbidity and mortality. Globally, the 5 year survival rate is reported to be approximately 50% (5, 55, 56). Despite numerous advances in treatment utilizing the most recent protocols for surgery, radiation, and chemotherapy, these numbers for long-term survival has remained relatively unchanged for the past 50 years (3, 57, 58).

This dismal outlook is due to a number of factors. For example, oral cancer is often diagnosed when the disease has already spread to regional lymph nodes and reached an advanced stage. The 5 years survival rate of early stage oral cancer is approximately 80% while survival drops to 19% for late stage disease (59). In addition, the frequent development of multiple primary tumours markedly decreases survival. The rate of second primary tumours in these patients has been reported to be 4% per year, which is higher than for any other malignancy (60). Because of genetically altered epithelial cells
in this location (field cancerization) (34, 61), an individual patient who is fortunate to live 5 years after the initial primary tumour has about 20% chance of developing at least one new primary tumour within that period of time, and the chance for survival of the second primary is extremely limited (62-65).

The 5-year survival rates for patients with cancer in lip, oral cavity and oropharyngeal sites (ICD-10 C00-C14)(66) in Norway in 2009-2011 were estimated to be 60.8 for men and 70.1 for women. The equivalent numbers for 1977-78 were 64.7 and 59.8 respectively, indicating that there has been an improvement in prognosis for women, but actually a slight decrease for men during the last 30 years(12). This is somewhat lower than most global reports (55, 67), which probably is due to a well-functioning health-care system in Norway providing easy access and good treatment prospects.

In clinical practice, the specific diagnose of a suspected malignant tumour of the oral cavity is set by the pathologist after examination of a tissue biopsy, and the TNM-stage determined by the clinical and radiological findings. In some cases where the size, grade of invasion or operability is difficult to clarify, the patient undergo thorough examination of the primary site under general anaesthesia. In the diagnostic and assessment process, the collaboration between the clinician, the pathologist and the radiologist is of great importance.

When it comes to treatment of the tumours arising in the oropharyngeal region, there has been a shift in paradigm the last decade, due to the detection of the impact of HPV, and these tumours increased response to chemoradiotheraphy (68-70). For tumours arising in the oral cavity however, the primary choice of treatment is still surgery (71-73).
In Norway, there are no official national guidelines for treatment. Though, Denmark has developed well established guidelines though their Danish Head and Neck Cancer Group (DAHANCA)(73), and the great majority of Norwegian head and neck cancer surgeons and oncologist largely follow the DAHANCA guidelines in their daily clinical practice.

For the early stage tumours, surgery might be used as monotheraphy, and the choice to give adjuvant radiotherapy is mainly determined by the size of the primary tumour, the depth of invasion, surgical margins and the presence of lymph node metastasis and any extracapsular spread (40, 54, 74-76). The decision whether or not to treat the neck of a patient without lymph node metastasis (N0) with radiotherapy and/or surgery also depends on factor such as tumour size and depth, the presence of lymphovascular and perineural invasion (42, 71, 77). In the most advanced cases, where primary radical surgery and reconstruction seems unattainable, primary radiochemotherapy is the treatment of choice, often followed by neck dissection.

Depending on the location of the disease, both the surgical- and the radiochemotherapy treatment, have devastating side effects, affecting several vital functions. The surgical treatment of tumours arising in the oral cavity often gives functional complications related to the mobility of the tongue, trismus (reduced ability to open the mouth)(78), and reductions in the general mobility of the jaws and neck. Besides the general fibrosis in the tissue, the most troublesome side effect of radiotherapy of the oral cavity is xerostomia (reduced saliva production)(79). All this contributes to nutrition problem as dysphagia and aspiration (80), speaking problems and often dental health challenges
(81-83). In addition, these patients often encounter a reduced quality of life due to the social aspects related to these deficiencies (84).

### 5.2 Relevant biomarkers

A short introduction of the biomarkers discussed in this thesis, described in the order they appear in the papers:

#### 5.2.1 p16

The discovery of the connection between human papilloma virus (HPV)-infection and HNSCC is one of the most pioneering scientific finding of the head and neck oncology for decades. HPV has been shown to be an important risk factor for carcinomas arising in the oropharynx, affecting both the behaviour and response to treatment of the oropharyngeal SCC (69, 70, 85). HPV-positive tumours have been shown to differ to such a degree from HPV negative tumours that an increasing number of scientists claim that they should be considered as two different diseases (34, 69, 85, 86). In the oral cavity, though, the frequency of HPV-driven tumours is low, and the HPV-status has been shown to have by far less impact on outcome (87-92).

Tumor cells infected with a transcriptionally active oncogenic HPV-type are shown to overexpress the tumor suppressor protein p16\(^{INK4a}\). This is due to an inactivation of the retinoblastoma (Rb) gene by the E7 oncoprotein, which in turn leads to a compensatory upregulation of the p16\(^{INK4}\) protein (93). In clinical practice the HPV status of a tumor is often determined indirectly by immunohistochemical identification of the increased
expression of the p16\textsuperscript{INK4a} protein, as this is shown to be a reliable and cost-effective substitution for the more demanding PCR-based HPV-testing (94-97).

5.2.2 uPAR and PAI-1

Tumor invasion and metastasis are complex processes that involves cell migration, angiogenesis, extravasation, intravasation and survival at the new site (98, 99). Cancer cells are thought to exploit the plasminogen activation (PA) system and matrix metalloproteinases (MMPs) during many of these stages (100-102).

The key effector of the PA system is the serine protease plasmin. Plasmin is readily activated from its precursor plasminogen, by either \textbf{urokinase plasminogen activator (uPA)} or tissue type plasminogen activator (tPA), into the active serine protease plasmin, a broad spectrum serine protease that can degrade many different types of extracellular matrix (ECM) proteins in addition to release latent growth factors and cytokines from the ECM and activate several matrix metalloproteases (MMPs) (101, 103, 104). \textbf{Urokinase plasminogen activator receptor (uPAR)} is the cell surface receptor for uPA, and both uPA and uPAR are linked to increased proteolytic activity and migration of cancer cells. tPA is primarily thought to be involved in fibrinolysis, while uPA is mainly involved in wound healing and cancer invasion. By binding of uPA to uPAR, cancer cells can direct the proteolytic activity to the cell surface (105). Even though uPAR lacks a transmembrane domain, it has the ability to signal through interaction with several other transmembrane proteins such as the integrins (106),
receptor tyrosine kinases (104) and G-protein coupled receptors leading to cell
migration, proliferation and adhesion regulation (107, 108).

**Plasminogen activator inhibitor-1 (PAI-1)** and PAI-2 are involved in the regulation of uPA and tPA activity (103). In addition to regulation of proteolysis, both uPAR and PAI-1 have roles directly linked to cell adhesion and migration through their interactions with the ECM constituent vitronectin (109, 110).

Both uPAR, uPA and PAI-1 have previously been suggested prognostic biomarkers in OSCC (111-118). In addition, a significant correlation between the expression of these proteins and mode of invasion has been seen (111, 114, 119). Furthermore, increased expression of uPAR in tumor cells is associated with the process of epithelial to mesenchymal transition (EMT) (120-123), a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become more like mesenchymal cells (124).

### 5.2.3 Ki-67

**Ki-67** is a well known proliferation marker that is used to predict prognosis in several cancer types (125, 126). The Ki-67 protein was originally defined by the prototype monoclonal antibody Ki-67 which was generated by immunizing mice with nuclei of a Hodgkin lymphoma cell line, and the name is derived from the city of origin (Kiel, Germany) and the number of the original clone in the 96-well plate (127).

Ki-67 is expressed during all phases of active cell cycle except G0. Ki67 expression is known to increase with increasing malignancy grade in several cancer types (128-130), among them breast cancer (131-133) and malignant melanoma (134, 135). In the head and neck oncology, Ki67 has been found to be of predictive value related to outcome of
salivary gland malignancies (136), though the prognostic value of in head and neck SCC are still debated (41, 137-139).

5.2.4 Plectin

**Plectin** is a large 500-kDa dumbbell-shaped protein belonging to a growing family of structural and in part functionally related proteins, referred to as plakins or cytolinker proteins (140, 141) that has been found to be important in cytoskeleton network organization. Plectin is expressed in a wide variety of cells and tissue and is localized on the intracellular side of the plasma membrane, at attachment sites of intermediate filaments (IF), microtubules and microfilaments (141, 142). As expected, due to its main function as a cytolinker and stabilizer of the cytoskeleton, it is particularly abundant in tissue exposed to mechanical stress, such as epithelial and muscle cells in addition to endothelial cells of vessels (142-145).

In epithelia its main location is in relation to the hemidesmosomes in the basal layer and focal adhesions where it interacts with the cytoplasmic tail of the integrin β4 subunit (146, 147). Defects in the plectin gene have been found in the severe skin blistering hereditary disease epidermolysis bullosa simplex, emphasizing the importance of the protein in normal functioning cells (148). Plectin affects mechanical, as well as dynamic properties of the cytoskeleton, and in its absence, the IF network in keratinocytes has been found to be changed promoting increased migration rates (149). Plectin is a multidomain protein, and is able to interact with a wide range of different proteins via one or more of its domains. Besides reinforcing the cytoskeleton, plectin also function as a scaffold for proteins and molecules involved in cell signalling, in which their binding to plectin position them at specific sites within the cells.
Little is known about plectin and EMT, but the formation of podosomes has been proposed as a first step towards EMT in OSCC. Podosomes are actin-rich adhesion structures that are normally found in mesenchymal cells such as macrophages, endothelial cells and osteoclasts, and have a role in cell adhesion and extracellular matrix (ECM) degradation (150, 151). During SCC development original epithelial cells can develop structures very similar to podosomes (151) indicating a transformation into cells with mesenchymal properties.

As the SCC-cell acquires an even more aggressive and invasive phenotype through further EMT, these podosome-like structures can develop into the more invasive invadopodia. Plectin has been found to be localized in the podosomes in the peripheral ring corresponding to the hemidesmosomes surrounding the core of actin filaments (152) but not in the invadopodia (150). The formation of podosomes and invadopodia in epithelial cancer cells seems to be related to progression to a more invasive, mesenchymal phenotype (153), and high expression of plectin and formation of podosomes might indicate the start of transformation to a more aggressive and invasive phenotype.

In 2010 plectin was introduced as a biomarker for improving detection and staging of pancreatic cancer (154, 155). To our knowledge, there is only one study involving the prognostic value of plectin in HNSCC, showing a decreased survival rate and increased frequency of recurrences in patients with high plectin expressing tumours (156).
5.2.5 E-cadherin

Similar to plectin, the transmembrane glycoprotein E-cadherin has a key role in maintaining normal epithelial tissue. E-cadherin is a transmembrane glycoprotein involved in cell-cell adhesion. E-cadherin has an extracellular-, transmembrane- and an intracellular domain. The extracellular domain forms homophilic ligations with E-cadherin on neighbouring cells. The intracellular domain binds actin filaments via the linker proteins α- and β-catenin (157). The catenins are also heavily involved in intracellular signalling pathways, thus connecting cadherin expression and function to intracellular signalling (158).

During EMT, adhesion contact points are lost and the cytoskeleton is rearranged enabling cells to migrate through the ECM with more ease (159). The loss of E-cadherin expression is heavily involved in the EMT of epithelial cells (159, 160), and also in the process of invasion and metastasis in epithelial tumours in vivo (161).

Aberrant expression or down-regulation of E-cadherin has been shown to increase invasion and metastases in various carcinomas, including those of the head and neck region (156, 162-167) though there are some inconsistency (168) and poor reproducibility between studies. This might be due to the E-carherin having both extra- and intracellular domains and the choice of antibody used (169).

5.2.6 TLO and HEV

The lymphoid system can be divided into primary, or central, lymphoid organs and secondary, or peripheral, lymphoid organs. The primary lymphoid organs comprise the
thymus and the bone marrow. The spleen, lymph nodes and the clusters of lymphoid
tissue distributed in the digestive tract; Peyer's patches and mucosa-associated
lymphoid tissues (MALTs), including the tonsils and adenoids in the pharynx, are
included in the term secondary lymphoid structures. In addition, during chronic
inflammatory reactions, lymphoid cells can accumulate and organize themselves in
structures markedly similar to secondary lymphoid organs (SLOs), therefore called
tertiary lymphoid organ (TLO) (170, 171). Like in lymph nodes, T and B lymphocytes
are segregated into 2 distinct and adjacent regions. TLOs typically compose of B cell
follicles encompassing a ring of naïve B-cells around a germinal centre that mainly
contains B cells, but also T-cells, follicular dendritic cells (FDCs) and macrophages. The
distinct T cell compartments comprise clusters of T-cells and mature dendritic cells
(DCs). These lymphocyte clusters is surrounded by specialized blood vessels called high
endothelial venules (HEVs).

Unlike lymph nodes, TLOs are not encapsulated, resulting in constitutive, direct
antigenic stimulation from their surrounding inflammatory microenvironment (172). It
is well established that these structures can be found in several chronic inflammatory
diseases as rheumatoid arthritis and atherosclerosis. Despite detailed studies on ectopic
lymphoid tissue in autoimmunity, where TLO formation is suspected to have harmful
effects (172, 173), data on TLO development and correlation with clinical outcome in
cancer, are still limited (172, 173). Among other solid tumours, the de novo formation of
ectopic lymph node-like structures has been described in breast-, ovarian-, malignant
melanomas, non-small-cell lung- and colorectal cancer, where the presence of these
highly specialized structures is supposed to be associated with a favourable prognosis
(174-179). In oral cancer, chronically inflamed tissue precedes most of the tumours
(180, 181) providing favourable sites for TLO formation, but to the best of our knowledge, TLOs have not previously been reported in OSCC.

In humans, HEVs are specialized post-capillary venous swellings normally found in all secondary lymphoid organs, except the spleen (182). In contrast to the usual thin endothelial cells from other vessels, HEVs are characterized by high endothelial cells with a more cuboidal morphology. HEVs also have discontinuous ‘spot-welded’ junctions between the cells which differ from the tight-junctions that characterize capillary and arterial endothelium, but similar to the non-occluding junctions found in other post capillary venules. HEVs enable naïve lymphocytes to move in and out of the lymph nodes from the circulatory system and are able to support high levels of lymphocyte extravasation from the blood to the tissue (174, 182, 183). The endothelial cells of HEVs express various specialized ligands for lymphocytes at their surcafe, among them the lymphoid chemokine peripheral node addressin (PNAd). PNAd binds to L-Selectin on naive lymphocytes and anchor them to the HEV wall in preparation for crossing the endothelium, thus, provides lymphocyte recruitment to sites of chronically inflamed tissue (183). Previously, intratumoural HEVs were found to be independently associated with favourable clinical outcome in breast cancer (178).
6. AIMS OF THESIS

6.1 General aims

Despite enormous progress in fighting cancer, the prognosis of OSCC remains relatively unchanged. Surgery, often combined with radiotherapy, is still the treatment of choice, despite major side effects and functional loss. There is a great need for increased knowledge and understanding of the tumours biological characteristics that can supplement the TNM-classification to better predict outcome, and make it possible to tailor the grade of surgical treatment and consider specific additional medical treatment for each individual patient. The aim of this thesis was to contribute to this pursuit for knowledge.

6.2 Specific aims

Paper 1: The aim was to characterise a North Norwegian cohort and compare similarities and differences to other European groups of patients. The purpose was also to support that studies on biomarkers on this cohort can be applicable for patients with corresponding diseases in other populations.

Paper 2: The aim was to search for prognostic markers among factors of the plasminogen activator system; uPA, uPAR and PAI-1. We also wanted to compare these with the well-known proliferation marker Ki-67 which is a commonly used biomarker in several cancers.
**Paper 3:** The aim was to evaluate plectin and E-cadherin as possible prognostic markers for OSCC.

**Paper 4:** The aim was to characterize tertiary lymphoid organs (TLOs) in OSCC, and to evaluate if they could have any influence on outcome.

**Paper 5:** The aim was to characterize high endothelial venules (HEVs) in OSCC. Furthermore, the purpose of this study was to introduce an easy scoring model for HEVs, and investigate the relevance of these in a clinicopathological setting.
7. MATERIAL AND METHODS

7.1 Patient cohort

The patient material constituting the base of this thesis was retrieved from the archives of the department of pathology (Diagnostic Clinic – Clinical Pathology), University Hospital of North Norway (UNN). A register of all squamous cell carcinomas of the oral cavity in the period 1986-2002 was made on the basis of the pathology SymPathy system. Initially there were 179 patients included in the database. During the process of coordinating the collected clinical and pathological information, we found that some of these were incorrectly registered and turned out not to be a primary SCC of the oral cavity, and some were registered twice. All of the patients with recurrent disease, incorrect histological diagnosis or localization outside the oral cavity (such as oropharynx) were excluded. We also excluded patients with second primaries that had received prior radiotherapy to the head and neck area. After this review of the material, a total of 133 patients with primary OSCC were included in our database. Of these, we had representative tumour tissue from 120 patients. Five of these patients had tumours recorded as verrucous carcinoma. These five are included in paper 1, but excluded in the other papers, which therefore are based on 115 patients. In paper 4 and 5, only 80 out of the group of 115 patients are included, since availability of full size tumours was a prerequisite for these studies.
7.2 Clinicopathologic variables

The clinicopathological information and variables were extracted from the patients' paper files at The University hospital of North Norway (UNN), and entered into the database by OR. The registered information was gender, date of birth, date of diagnosis, primary site, T, N and M stage of the disease, tumour differentiation, treatment given and any recurrences of the disease. In addition the available information on smoking and drinking habits were collected, and the date and cause of death. For patients where the information about death was not available in the patient file, this information was retrieved from the Statistics of Norway, Cause of Death Registry.

7.3 Tissue Micro Array

7.3.1 TMA, general considerations

The tissue-micro-array (TMA) technique represents an efficient and cost effective method to analyse large numbers of tissue samples on a single slide. The TMA makes it possible to explore molecular targets on the DNA, RNA or protein level, from as many as 1000 samples at the time, prepared and stained under the same conditions. The technique can be both economical and time saving. Though, the construction of a TMA block is time consuming, once it is made, up to 300 consecutive sections from each TMA block can be obtained (184-186).

The technique of using a multi block for immunohistological staining was first introduced by Battifora in 1986 (187, 188), and further developed by Kononen, which presented the first TMA study in 1998 (185). The use of TMA rapidly became a frequently used method in molecular profiling studies (30, 184, 189-192).
Possible advantages and disadvantages with the use of TMA are listed in the table:

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time saving</td>
<td>Technically challenging</td>
</tr>
<tr>
<td>Cost saving</td>
<td>Setting up array time-consuming</td>
</tr>
<tr>
<td>Tissue saving</td>
<td>Small tissue volumes</td>
</tr>
<tr>
<td>Easier to score uniformly</td>
<td>Tissue may not be representative</td>
</tr>
<tr>
<td>Enables study of larger cohorts</td>
<td>Lower accuracy of heterogeneous tumours</td>
</tr>
<tr>
<td>Standardized staining conditions</td>
<td>Not suited for individual diagnosis</td>
</tr>
<tr>
<td>Research collaboration through sharing slides</td>
<td>Limited information of invasion and growth pattern</td>
</tr>
</tbody>
</table>

### 7.3.2 TMA-construction

The general principles of the construction of a TMA are illustrated in the figure below, and starts with a collection of paraffin embedded, formalin fixed blocks of the tissue of interest. Tissue cores of 0.6-2.0 mm in diameter are harvested by puncturing the tumour area of interest in the donor blocks, and transferring it to a prepared recipient paraffin block. A stylet is used to empty the needle and precisely insert the core into a predrilled well in the recipient block. Sections of the resultant tissue micro array block are cut and transferred to glass slides for processing and IHC or *in situ* hybridization techniques. Biomarker expression is assessed by manual or automated analysis (193).
Our TMAs were assembled using the manual Micro Tissue Arrayer (Beecher instruments). This consists of a thin-walled needle of stainless steel with an inner diameter of 0.6 mm. One representative tumour block from each tumour was selected, and a hematoxylin-eosin (HE)-slide from the block was used to identify morphological representative regions of the tumour. The selection of the tumour tissue block and the donor spot in the tumour tissue were done by OR. In case of doubt, the HE-slide was evaluated together with a trained pathologist. A total of 8 cores of 0.6 mm were taken from the selected regions of the donor block and inserted in pairs into a recipient blocks. Material from each primary tumour was divided into 3 separate blocks. Four parallels of each block (A, B, C and D) were made. Four μm thick sections of the fixed, paraffin embedded TMA tissue were cut with a microtome and placed on superfrost slides. HE slides and immunhistological cytokeratin-staining was performed to verify histology and the presence of tumour tissue.

A TMA containing cores from respective lymph node metastasis of the included patients was also made, however, due to the small number of patients included, this block was only used for the p16 IHC staining included in paper 1.
7.4 Immunohistochemistry and immunofluorescence

**Immunohistochemistry (IHC)** is a technique used to identify the presence of specific proteins as detected by an antibody (194). IHC is a two step process where the first phase involves binding of the specific primary antibody to the tissue antigen you want to identify, and the second phase involves binding of a labelled secondary antibody for the visualization in the microscope. Horseradish peroxidase (HPR) is the most common label giving a brown colour.

In principle, **immunofluorescence (IF)** is quite similar to IHC, but in IF the secondary antibody is tagged with fluorescent dyes that absorb ultra-violet rays and emits visible light, a process called fluorescence. The antigen-antibody complex is visualized using a ultra-violet (fluorescent) microscope. IF can be used on tissue sections or cultured cells, and may be used to analyze the presence and distribution of proteins.

7.4.1 IHC and IF procedures

Both manual and automated IHC staining procedures were used in this thesis, and performed on 4μm sections of the TMA or standard whole tissue sections from paraffin-embedded archival tissue.
The primary antibodies used are listed in the table:

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Manufacturer</th>
<th>Catalog #/clone</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>Mouse monoclonal</td>
<td>Ventana</td>
<td>825-4713/Clone E6H 4</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>uPAR</td>
<td>Mouse monoclonal</td>
<td>Sekisui Diagnostica</td>
<td>3936</td>
<td>1:10</td>
</tr>
<tr>
<td>uPA</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>Ab24121</td>
<td>1:75</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Rabbit polyclonal</td>
<td>Nordic BioSite</td>
<td>BT-BS3503</td>
<td>1:100</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Rabbit monoclonal</td>
<td>Ventana</td>
<td>790-4286/clone 30-9</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>Plectin</td>
<td>Rabbit monoclonal</td>
<td>Abcam</td>
<td>ab32528</td>
<td>1:200</td>
</tr>
<tr>
<td>Plectin</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>ab83497</td>
<td>1:10</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Rabbit monoclonal</td>
<td>Cell Signaling Tech.</td>
<td>Clone 3195</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>PNAd</td>
<td>Rat monoclonal</td>
<td>Biolegend</td>
<td>clone MECA-79</td>
<td>1:25</td>
</tr>
<tr>
<td>CD20</td>
<td>Mouse monoclonal</td>
<td>Ventana</td>
<td>clone L26</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>CD21</td>
<td>Mouse monoclonal</td>
<td>Ventana</td>
<td>clone 2G9</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>CD34</td>
<td>Mouse monoclonal</td>
<td>Ventana</td>
<td>clone QBEnd/10</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>CD3</td>
<td>Rabbit monoclonal</td>
<td>Ventana</td>
<td>clone 2GV6</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>Bcl-6</td>
<td>Mouse monoclonal</td>
<td>Ventana</td>
<td>clone GI191E/A8</td>
<td>Pre-diluted</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Mouse monoclonal</td>
<td>Ventana</td>
<td>760-2135/Clone AE1/AE3/ PKC26</td>
<td>Pre-diluted</td>
</tr>
</tbody>
</table>

The IHC staining for uPAR, uPA, PAI-1, plectin and PNAd were done manually. The rest of the IHC stainings were done in the automated slide stainer Ventana Benchmark, XT (Ventana) at UNN, which is accredited for the respective staining. The same protocols, positive and negative controls as in the clinical routines were used. All slides were deparaffinized and rehydrated in graded xylene/ethanol baths before different antigen retrieval. The staining protocols were optimized for each of the antibodies and described in detail in each paper.

**Validation and negative controls**

A negative control where the primary antibody was omitted was included for all antibodies used and showed no staining in all cases of both IHC and IF.
The uPAR antibody used, had previously been validated in several studies (195-197), in addition the specificity was validated using our IHC protocol and using Western blotting. Furthermore, the anti-uPAR antibody was used to stain pancreatic cancer known to be uPAR positive (198). The anti-uPA and PAI-1 antibodies were verified by staining human placenta tissue, as cytotrophoblasts are known to be positive for both (198-201).

The anti-plectin monoclonal antibody used has previously been thoroughly validated in pancreas cancer tissue (154). We validated the polyclonal antibody used in the IF by staining pancreas cancer tissue with both antibodies, and found them to stain tumour tissue equally. As a negative control, pancreatic carcinoma tissue where the primary antibody was omitted was treated according to the same staining protocol. No staining was seen in either muscle- or cancer cells, indicating that the secondary antibody gave no unspecific staining of the tissue. We also did Western blotting of whole muscle cell lysate, and found that the antibody gave a band of approximately 500 kDa corresponding to the size of plectin.

7.4.2 Immunohistochemical scoring

For paper 1-3 all stainings were examined by an experienced pathologist (SES) and a trained head and neck surgeon (OR) without knowledge of clinical outcome. The scoring was semi-quantitative (202, 203) as a product of proportion of stained tumour cells (no staining (0), <10% (1), 10–50% (2), 51–80% (3) or >80% (4)), multiplied with the staining intensity (none (0), weak (1), moderate (2) or strong (3)). The final score was determined by multiplication of these two variables, resulting in total score values
differing from a minimum of zero to a maximum value of 12. Ki-67 was scored in a modified version as percentage of nuclei stained; 1 (<10%), 2 (10-50%), 3 (>50%) (204).

Agreement between the two observers was tested on approximately 25 % of the cores stained with uPA and uPAR staining with a good Spearman’s correlation coefficient of 0.753 and 0.881 (p<0.001). A correlation of deviation between the cores was 33.8% (tested for uPA) which reflects the heterogeneity of the tumours. Cut-off points were determined to obtain binary variables for statistical analyses and were based on median values of staining product, or in the case of Ki-67, percentage of nuclei stained. Values below the median point were designated low-expression, while the values in the upper median part were designated as high-expression.

Evaluation of the IHC for paper 4 and 5 were done by EHO and AW. TLOs were identified by verifying B-cell aggregates by the B-cell marker CD20 containing CD21 positive FDCs cells. In addition, CD3 staining was performed to identify T-cells adjacent to B-cell clusters, and BCL-6 to verify the presence of GC B-cells in the TLOs. In paper 5, HEVs were recognized by positive staining for PNAd. Five hotspots in each slide were identified and micrographs of the hotspots were taken at high power magnification (400×). The micrographs were individually analysed by EHO and AMW. Positively stained cell clusters were considered as individual vessels, whereas positive single cells were not counted. The mean number of HEVs per hotspot was calculated for every section, sections with a mean of < 0.5 HEVs per hotspot as HEV-negative, and those with a mean number ≥ 0.5 as positive.
7.5 **Statistical analysis**

The statistical analyses were performed using IBM SPSS statistics for Windows (IBM Corporation Armonk, New York), version 21. In all papers, associations between the biological markers and the different categorical variables were assessed with Pearson’s chi-square test and Pearson correlation. Comparing of means was done using the ONE-Way ANOVA-test. Univariate analyses of time from diagnosis to disease specific death and survival curves were performed using the Kaplan Meier method. Differences between categories were estimated by the log-rank test, with the date of diagnosis as starting point. Variables that were statistically significant in the univariate analysis were entered into multivariate Cox regression analyses to assess the independent value of the prognostic factors on survival in the presence of other variables. The last day of follow-up was 01.01.2012. All results were considered significant if \( p \leq 0.05 \).
8. MAIN RESULTS

8.1 Paper I

Clinicopathological characteristics of oral squamous cell carcinoma in Northern Norway - A retrospective study

In this paper we describe clinicopathological characteristics of 133 North Norwegian patients diagnosed with squamous cell carcinoma of the oral cavity in the period 1986-2002. The cohort is the basis for all the papers included in this thesis.

Main results:

- The cohort consisted of 69 men and 64 women, giving male/female ratio of 1.1.
- Forty-seven of the 133 patients (35%) died of the disease within 5 years from diagnosis. There was no significant difference between the genders and disease specific death
- Men both smoked and drank more alcohol than women (p<0.001)
- The strongest predictors for disease specific death were tumour size and the presence of regional lymph node metastasis
- Heavy smokers and drinkers presented with more advanced disease, more often localized to the floor of mouth than nonsmoking and abstinent patients, whom more often presented tumours of the mobile tongue
- Our results correlate well with previously published clinicopathological data on comparable cohorts
8.2 Paper II

Urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1) are potential predictive biomarkers in early stage oral squamous cell carcinomas (OSCC)

In this TMA-based study of tumour tissue from 115 OSCCs we assessed the possibility to use components of the plasminogen activator system as prognostic markers for OSCC outcome and compared this to the commonly used biomarker Ki-67.

Main results:

- Low expression of uPAR (p=0.031) and PAI-1 (p=0.021) in the tumour cells was significantly associated with low disease specific death in patients with small tumours and no lymph node metastasis (T1N0)
- Ki-67 was not associated with disease specific death in any of the groups of patients analysed

8.3 Paper III

Plectin as a possible prognostic marker in non-metastatic oral squamous cell carcinoma

In paper III we evaluated the potential prognostic value of plectin and E-cadherin by a TMA-based immunohistochemical analysis of primary tumour tissue from 115 OSCC tissue samples.

Main results:

- Low expression of plectin in the tumour cells predicted a favourable outcome for patients with non-metastatic disease (p=0.008)
- Low expression of Plectin indicated a favourable prognosis in T1 tumours 
  \( p = 0.031 \). This result was also significant in a multivariate analysis of the N0-
  cases \( p = 0.014 \)
- The expression of plectin correlated with the expression of uPAR \( p < 0.001 \),
  which we have previously found to be a potential prognostic marker for T1N0
  tumours
- The expression level of E-cadherin did not show any correlation with outcome or
  with the expression of any of the other biomarkers

8.4 Paper IV
Characterisation and prognostic value of tertiary lymphoid organs in oral
squamous cell carcinoma

This paper was based on immunohistochemical analyses of full size tumour sections
from 80 of the patient in our cohort. The sections were stained for B-cells, follicular
dendritic cells, T-cells, germinal centre B-cells and high-endothelial venules to look for
TLO formation, some of the samples were sectioned at multiple levels.

Results:
- Tumour-associated tertiary lymphoid organs do exist in OSCC, and were detected
  in 21% of the tumour samples
- The presence of TLOs were associated with lower risk for 5-year disease-specific
  death \( P = 0.039 \)
- TLOs were unevenly distributed within a tumour.
8.5 Paper V

High-endothelial venules predict favourable outcome in patients with oral squamous cell carcinoma

This paper is based on the findings in paper IV, and includes the same patient-samples. Immunohistochemical staining for the lymphoid chemokine peripheral node addressin (PNAd) which is expressed on the surface of the endothelial cells of HEVs was performed to identify the presence of HEVs in the tumour tissue. Some of the samples were evaluated at multiple levels.

Main results:

- The presence of high-endothelial venules was significantly associated with lower disease-specific death in univariate analysis (P=0.001). In multivariate analyses, only the T status was significant

- High-endothelial venule count on a single tissue plane was representative for the whole tumour
9. DISCUSSION

9.1 Ethical considerations

The use of patient tissue samples and the collection of clinicopathological data from the patients’ files were approved by the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007). All clinical data were kept de-identified, and OR was the only involved who had access to the link to the identifying data.

We got an exemption from the requirement of written consent from the patients before implementation of the study, because about half of the patients were dead by the start of the data collection. The patients still alive were of high age and the burden of a consent form was found not to be necessary.

9.2 Methodological considerations

9.2.1 Patient cohort and study design

The material and information, on which this thesis is based, were collected retrospectively, a design that involves the risk of certain selection and misclassification biases and the lack of validation of the recorded information. All collection and registration of the clinicopathological information and variables were done by searching through the patients’ paper files at UNN. Among the problems with the study design was the unstandardised reporting of tobacco and alcohol consumption in the patients’ files, making it impossible to specify the use in pack-years or units. This also led to a relatively
large proportion of 'unknown's in the material, which of course limit the reliability of the results. There were also some inaccuracies in the TNM-classification of the tumours resulting in some ‘unknown's also in this variable.

In some of the patients’ files there was limited information about relapses and further clinical course as a proportion of the patients were followed by their local hospital after the initial treatment.

In the period 1986-2002, the regional Head and neck cancer-care was not as well organised as it is today and about half of the patients were transferred to hospitals outside the region, especially from Nordland, the southernmost county of the region. Calculations of incidence based on our cohort were therefore not possible.

**Site specificity**

This thesis has been based on squamous cells carcinomas from the oral cavity proper. We have described the anatomical localisation of the tumours in accordance to the International Classification of Diseases for Oncology (ICD-0). Tumours arising in the tonsils, base of the tongue and the lip has been excluded from the study. In many studies the information about the tumours’ localisation is insufficient, as the material is often reported to be from the oral cavity without further specification. When comparing prognostic factors between different studies the reproducibility can be hampered by the fact that tumours are collected from different locations (205).
9.2.2 Tissue Micro Array

OSCCs are known to be histologically heterogeneous (47). The degree of differentiation can vary within a tumour, from highly differentiated areas to areas with low differentiation. Due to this heterogeneity, the use of the TMA technique can be questioned in OSCC. However, even though the TMA reduces the amount of tissue to a limited fraction of each tumour, several studies have shown that the technique is applicable also in OSCC (206, 207).

Monteiro et al (208) did immunohistological staining with EGFR and Ki67 on both TMA and whole sections from the same oral cancers, and found a high degree of correlation between the TMA and whole section stained. When using a single core only, the correlation decreased. Radhakrishnan et al (30) concluded that from a theoretical point of view, a single spot per case may be adequate for a homogenous marker. However, four cores from each tumour maximize the usefulness of TMA for any biomarker. Concerning the size of the core, most authors claims that the diameter has little impact on the reliability of the TMA-technique (209).

Mucci et al. (210) argued that the use of TMA in heterogeneously expressed markers actually gives a more correct result than whole section analysis. Protein expression can be difficult to quantify histopathologically, and in this respect, TMAs might be easier to evaluate uniformly, and can even be used to assess heterogeneity. They conclude that the TMA-technique are more reliable when screening for new antibodies, while in a clinical setting where prognosis is based on a single event, standard whole sections should be evaluated.
We experienced a loss of 16.5% of the cores due to technical issues during preparation, and of the preserved cores in the TMA, 8.4% contained too little tumour tissue to be evaluated. This is about the same numbers as reported in other studies. Mucci et al (210) reported that 73% of the cores could be evaluated as 18% of the cores were lost during processing and 9% did not contain tumour tissue. They do report though, that the proportion of evaluable cores increased to about 90% as they got more experienced with the technique. Chen et al (206) concluded that when using the TMA-technique, 25% loss of tissue cores should be expected. They reported 13% loss during processing; another 13% contained no tumour cells or too few tumour cells for scoring. Therefore they recommend that at least three cores from each tumour should be included.

9.2.3 Tissue quality and immunohistochemistry

The tissue investigated was collected between 1996 and 2002. The antigenicity of the tissue could be altered because of long storage time, thus giving false negative or false positive results. In a study by Bertheau et al they reported somewhat altered immunohistochemical reactivity in stored, precut slides over time for some antibodies, but not for others. Freshly cut slides from old blocks did not seem to have altered staining pattern (211). The tissue from our cohort was fixed and stored in paraffin, and also the cores in the TMA are embedded in paraffin. The staining was performed on freshly cut slides and the occurrence of antigen degradation should therefore be at a very low level if any.

The influence of “prestaining” variables might complicate the comparison of staining pattern and intensity in archival material (205, 212), and this potential source of bias requires additional consideration. Quality control of the material used has been debated
in the Norwegian pathology society (personal communications). There is often little information about the time a tissue sample has been fixated, and this might inflict on the quality of the immunohistochemical staining. The fixed tissue is embedded in paraffin, but information about storage conditions (temperature and humidity among others) is rarely, if ever recorded. In our study information about fixation time and storage conditions were not specified, but in the future such information might be available.

Some of the biomarkers used on our material are well established and staining has been performed according to standardised protocols used in the diagnostic routine at the Department of Clinical Pathology, UNN. Others have been used only in scientific work, and have not been verified for diagnostic use. These biomarkers were validated by several methods during the work of this thesis, and especially uPAR and PAI-1 have been tested extensively. The result from these analyses might also be of use also for other researchers.

9.2.4 Statistical analysis

Size of material

Although the total amount of patients in our studies is 133, when splitting into subgroups, some of them become too small to conduct meaningful subgroup- analyses. Group sizes are also a limiting factor in the multivariate analyses. Another problem is the proportion of “unknown”s in some of the categories. Nevertheless, as presented in paper 1, the results of the univariate and multivariate analyses of the clinicopathological variables in the present cohort are in accordance with
the published literature, indicating a representative patient population and a good basis for biomarker analyses.

**Cut-off values**

There are different approaches for selection of cut-off values. Either you can use already established values based on previous research, which makes sense in conformational studies. For novel studies, the mean, median or percentiles are often chosen as cut-off values, which increase the reproducibility and makes the results easier to evaluate in following studies. Another possibility in a novel study is to use the cut-off for each biomarker that gives the most significant differences in DSS between the subgroups. When using the cut-off that yield the groups with the largest possible difference in the end-point under investigation, one aims to identify the biologically significant cut-offs, but this increases the risk of false positive results (type 1 errors)(213) and may lead to problems in maintaining large enough subgroups.

We decided to use the mean value as the cut-off point in all the investigated markers in paper 1, 2 and 3. This makes the method easier to reproduce, but increases the chance of false negative results (type 2 errors)(213).

In this setting a false negative result means a false acceptation of the null-hypothesis, which in our studies was that there are no differences in outcome between patients with high and low expression of the investigated marker. Making a type 2 error, we falsely accept the null hypothesis concluding that the investigated marker has no prognostic value, even though it actually has, but as a result of the chosen cut-off this doesn’t show in the statistical analyses.
9.3 Discussion of main results

This thesis is based on a patient cohort from the northernmost region of Norway. In paper 1 we describe the clinicopathological characteristics of the cohort, and found our results to correlate well with previously published data on comparable European cohorts (18, 88, 90, 214). To our knowledge, no previous epidemiological study describing OSCC patients from this region has been published. It was important to characterize the cohort as we wanted our results on biomarkers to be useful for other cohorts. Likewise, it is important for clinicians to have a basic knowledge of the characteristics of the patients in their own impact area when considering the applicability of studies from other parts of the world, made on other cohorts.

Paper 2 and 3 are based on TMAs made of tissue samples from 115 of the patients in the cohort described in paper 1, all with tumours from the oral cavity. Our research group have for several years had an special interest for proteolytic enzymes, and done research on SCC-cell lines both in culture and in vivo experiments in mice (103, 162, 215, 216). The plasminogen-activator (PA)-system is one of these areas of interest, thus we wanted to assess expression and prognostic value of members of the PA system on human cancer samples. Interestingly, we found that low uPAR expression was significantly correlated with low PAI-1 expression, and that low expression of uPAR and PAI-1 in the tumour cells were significantly associated with low disease specific death in patients with small tumours and no lymph node metastasis (T1N0). Based on this, we suggested that uPAR and PAI-1 could be potential predictive biomarkers in early stage tumours.

Previously, a significant correlation between the expression of these proteins and mode of invasion has been seen (114, 119), but the use of TMA in our study, did not allow the
evaluation of invasion pattern, hence further studies on whole tumour sections are needed in order to analyse this association.

In addition to the interest for the PA-system, a member of our group (SES) has been involved in research on plectin in pancreatic cancer tissue. The prognostic value of plectin in OSCC has, to our knowledge, previously only been studied by Katada et al. (156), which investigated a cohort consisting of 62 HNSCC, among them only 23 from the oral cavity. They found significantly lower survival of patients with high expression of plectin and low expression of E-cadherin in the tumour. We wanted to analyse the prognostic value of plectin and E-cadherin in our group of patients which is larger and more homogenous, as described in paper 3. We could not reproduce the results from Katada on E-cadherin-expression, which might be due to the use of different antibodies (169). The E-cadherin antibody used in our study was specific for a cytoplasmic region of the protein, while Katada et al. used an antibody that recognizes an epitope located on the extracellular domain (156).

However, we found a highly significant correlation between low expression of plectin and longer DSS both for patients with N0-disease and those with T1- primary tumours. For the T1N0 patients, multivariate analyses showed that plectin was an independent prognostic marker.

As high uPAR and plectin expression both were associated with shorter DSS in the same subgroup of patients, we evaluated the possibility of a correlation between these two biomarkers. There was indeed a strong correlation between the uPAR and plectin expression, with many of the same tumours expressing a high level of both proteins. In
the light microscope, we got the impression that these two markers were located in the same areas of the tumour, though having different location within the cells. Double immunofluorescence staining of the two markers on full sections of some of the tumours, confirmed this result.

In the literature, it is difficult to find publications that offer of a functional link between the two proteins. However, both uPAR and plectin are connected to the epithelial mesenchymal transition (EMT) of epithelial cancer cells (120-123, 150-153), and in paper 3 we suggest EMT as a possible link.

In paper 2 we also wanted to evaluate the proliferation marker Ki-67 which is used to predict prognosis in several cancer types. The results from studies of OSCC have been inconsistent (41, 126, 137-139). We did not find any significant correlation with Ki-67 and survival, and we conclude that Ki-67 has no prognostic value in OSCC.

Ectopic lymphoid formation is a common feature in chronically inflamed tissues and has been found at various anatomical sites in a number of autoimmune disorders, where tertiary lymphoid organs (TLOs) might contribute to disease progression (172, 173). In oral cancer, chronically inflamed tissue is associated with cancer development (180, 181) providing favourable sites for TLO formation. To the best of our knowledge, paper 4 is the first report on the existent TLOs in OSCC. We found TLO formation to be a positive prognostic factor for these patients. However, in our attempts to identify TLO formation in OSCC patients by the use of IHC analysis, we faced several problems. To confirm the presence of TLOs staining for several different markers had to be performed and the tumours had to be evaluated at several levels for
reliable results. Therefore, our method for TLO detection does not seem to be applicable in clinical practice as a prognostic factor in individual clinical management.

All TLOs are surrounded by specialized blood vessels called high endothelial venules (HEVs). In search of an alternative method for indirect detection of TLOs, we assessed the prognostic value of tumour associated HEVs. HEVs were present also in some of the tumours where no TLO formation was detected.

We found the presence of HEVs to be significantly associated with lowered 5-year DSD. The PNAd staining was highly specific for the high endothelial cells in the HEVs, and our study indicated that staining of a single slide from each tumour was enough, as the HEVs were evenly distributed within the tumour.

In general terms, reflecting its biological significance, the ideal marker would be easy to score and display large differences between groups (205). Our experience from the study described in paper 5, was that the identifying of HEVs by IHC staining for PNAd meet this criteria, and seems to be a reliable marker easy to score, and might also be a potential positive prognostic marker for patients with oral cancer.

We found TLOs to be most frequently found in well-differentiated tumours, thus one could speculate that dedifferentiation makes the tumour cells less antigenic and thereby elicits a milder inflammatory reaction with lower induction of TLOs. Further, HEVs were preferentially located in T1 and T2 tumours, suggesting that development of HEVs take place in the early phases of tumour growth. HEVs have been shown to be plastic and able to differentiate and dedifferentiate upon various stimuli (217). Thus, it is possible that HEVs in larger tumours could dedifferentiate to resemble normal blood vessels and gradually lose their specific marker PNAd on their surface.
As described in paper 4 we found no correlation between HPV-status and TLO formation. Though, in paper 5, a significantly higher proportion of the HPV-negative tumours were HEV-positive. However, due to the low number of HPV-positive tumours in our study, no reliable conclusions could be drawn. Although HPV probably plays only a minor role in cancers arising in the oral cavity (218), the relation between HPV-infection and the immunological reaction in the tumour and its environment is an interesting issue that remains to be fully understood. In our cohort, the number of HPV positive tumours was similar to previous reports, and HPV-status did not correlate with outcome. In general, the role of HPV infection in oral cavity cancer initiation and progression remains disputable, and we were not able to address this issue with only 10 p16 positive samples in our material.

Although the prognosis in OSCC is mainly determined by the stage of the tumour at presentation (34, 180), there is a need for reliable prognostic biomarkers that can be used for stratification of treatment options within subgroups of patients. The most interesting and promising results in this thesis is in patients with early stage tumours. In this group of patients’ additive information as prognostic biomarkers can be of great value in the difficult challenge to cure without over treating.
10. CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

Even though the incidence of OSCC is low, at least in our part of the world, it is a major health problem. Survival rates have shown little improvement over the last decades, and the treatment has major side effects. For those who survive, the disease often makes devastating interventions in quality of life and severe reductions in vital functions. The main goal is to cure, but also to avoid harmful overtreatment of patients with a favourable prognosis. In general, OSCC are heterogenous tumours, and the course of the disease can be unpredictable. When it comes to decision making about the extent of treatment, the discussion is often most divergent when it comes to the small tumours.

In conclusion of this thesis, we suggest uPAR, PAI-1 and plectin as potential prognostic markers in early stage OSCC. Of these, plectin seems to be the most promising one to discriminate between tumours of high and low malignancy potential. Though, all of these markers need to be tested in larger, conformational studies to evaluate their practical applicability.

The inflammatory process in the OSCCs and the formation of TLOs and HEVs are also interesting results that also deserves to be followed up in larger studies. First, as it increases the knowledge of the interaction between the host' immune system and the tumour cells, and also as the detection of HEVs in the tumour might be of prognostic value.
In my opinion, the most likely future perspective is that not one single, but a battery of biological markers are to be implemented to clinical practice.

As it would be of great interest to validate all of our results in a larger study, collaboration with several other research groups in other head and neck cancer centres in Scandinavia is currently being established to be able to perform a multicentre study based on the findings presented in this thesis. For further investigations we want to expand the TMAs to increase the statistical strength of the results, and also include investigations of full sections of the early stage tumours. This will give us more information on the variety in the expression of the biomarkers, which is important for implementation in clinical practice. Full sections will also make it possible to evaluate the pattern of invasion and any variations in biomarker expression related to the invasive front.
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Clinicopathological characteristics of oral squamous cell carcinoma in Northern Norway. A retrospective study.
Oddveig G. Rikardsen, Inger-Heidi Bjerkli, Lars Uhlin-Hansen, Elin Hadler-Olsen, Sonja E. Steigen
Urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1) are potential predictive biomarkers in early stage oral squamous cell carcinomas (OSCC)

Synnøve Magnussen*, Oddveig Rikardsen*, Elin Hadler-Olsen, Jan-Olof Winberg, Lars Uhlin Hansen, Sonja E. Steigen, Gunbjørg Svineng

*The authors contributed equally to this work
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