Oral Squamous Cell Carcinoma

Role of the Plasminogen Activation System in Tumour Progression

Synnøve Norvoll Magnussen

A dissertation for the degree of Philosophiae Doctor – Month 20xx
Oral Squamous Cell Carcinoma (OSCC)
Role of the Plasminogen Activation System in Tumour Progression

Doctoral Thesis
Synnøve Norvoll Magnussen

Tumour Biology Research Group
Department of Medical Biology
Faculty of Health Sciences
UiT - The Arctic University of Norway
Acknowledgements

The current work was performed at the Tumour Biology Research Group at the Department of Medical Biology, Faculty of Health Sciences, University of Tromsø (UiT) – The Arctic University of Norway. The work was funded by UiT, and grants from the Norwegian Cancer Society, the Erna and Olav Aakre Foundation and The North Norwegian Regional Health Authorities (HelseNord).

Firstly, I would like to express my sincere gratitude to my principal supervisor Associate Professor Gunbjørg Svineng. Thank you for giving me the opportunity to do my PhD at the Tumour Biology Research Group, and thank you for letting me finish it! Thank you for always being available, always being positive and patient, and for sharing all your knowledge with me. You have been my supervisor, “sister”, friend and college all these years. I would also like to thank my array of talented co-supervisors: Associate Professor Elin Hadler-Olsen, Professor Jan-Olof Winberg and Professor Lars Uhlin-Hansen. Thank you for sharing all your knowledge, for your patience, for proofreading manuscripts and for always finding time. I have learnt so much from you all. Even more so, I would like to thank all my supervisors for believing in me and supporting me, especially when all hope seemed lost!

I would also like to thank Sonja Eriksson Steigen who has guided me into the world of TMAs and SPSS which was completely new to me. Thank you for always trying to make everything as clear as possible. Thanks to Oddveig Rikardsen for bringing the patient into it the work, and for bringing life to the workplace. A big thank you goes to all my co-authors for valuable contributions and insightful comments. A big thank you to all my wonderful colleagues. Thank you all for creating such a pleasant and fruitful working environment. A special thanks for all the lab(orious) support given by Eli Berg, Cristiane Jacobsen, Bente Mortensen and Nadezhda Latysheva. Thank you for always helping with a smile, especially at the most desperate periods of my PhD! Thanks also to my officemate Gry, for always having a smile.

I would like to give a warm thank you to my friends Tina, Jennifer, Mari and Stine who have been present at different times during the project, and who have always been
supportive. Thank you for listening to all my complaints and worries, and sharing my joys! It has really meant a lot to me.

To all my friends and family I thank you all for the encouragements. It has sometimes been a necessity. To my mother and father for always encouraging me and believing in me. To my brother, my hero, you’re the best! I’ve always looked up to you! Thank you. To my extended family for taking me in so open heartedly. Thank you for supporting and encouraging me. Last but certainly not least, a big thank you to my Bjørn. You have supported me ALL THE WAY through! You are the best person I know, and you make me a better person every day!

Tromsø, October 2014.

Synnøve Norvoll Magnussen
Abstract

Human kind has battled with diseases for centuries, and conquered both small pox and polio. Even though the global battle against cancer has been ongoing for decades, it is still one of the leading causes of death worldwide. Oral squamous cell carcinoma (OSCC) is an aggressive and unpredictable cancer with a high tendency to recur and metastasise. Despite increasing efforts to improved treatment, the 5-year survival rate is still low. Early intervention gives, as with many types of cancer, the best prognosis. However even small early stage tumours can behave aggressively.

We discovered that low expression levels of the urokinase plasminogen activator receptor (uPAR) and the plasminogen activator inhibitor-1 (PAI-1) two proteins normally involved in the process of wound healing, were associated with low disease specific death in patients with early stage OSCC. PAI-1 and uPAR were therefore suggested as potential biomarkers to aid clinicians in treatment stratification. High expression of uPAR and PAI-1 was therefore implicated in the early stages of OSCC progression. In vivo studies further showed that the tumour microenvironment was involved in the induction of uPAR expression, which simultaneously resulted in enhanced activity of gelatinolytic enzymes. In vivo tumour growth in a syngeneic mouse model of OSCC did not show metastasis, however the tumour growth pattern reflected that of early stage OSCC. uPAR locates the proteolytic enzyme urokinase plasminogen activator (uPA) to the cell surface of cancer cells, and such pericellular proteolysis is thought to be required for cancer cell invasion and metastasise to distant organs. Cleavage of uPAR terminates its ability to bind uPA, however gives cells a different set of functions. These include directional migration which is also needed for cancer cells to spread. We could show that the stromal derived factor transforming growth factor-β1 (TGF-β1) up-regulated the expression of PAI-1, and furthermore down-regulated cleavage of uPAR. Taken together, the current study shows that uPAR and PAI-1 are involved in early stage of OSCC progression, and that factors in the tumour microenvironment are important regulators of both the expression and posttranslational modifications such as glycosylation and proteolytic cleavage.
List of papers

Paper I


Paper II


Paper III

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>ATF</td>
<td>Amino terminal fragment</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CAF</td>
<td>Carcinoma/cancer-associated fibroblast</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>GFD</td>
<td>Growth factor domain</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Hepatocyte growth factor/Scatter factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor 1α</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia-responsive element</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LRP-1</td>
<td>Low density lipoprotein receptor-related protein-1</td>
</tr>
<tr>
<td>M6PR/IGF2R</td>
<td>Mannose 6-phosphate receptor/Insulin-like growth factor II receptor</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSP</td>
<td>Macrophage-stimulating protein</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PA System</td>
<td>Plasminogen Activation System</td>
</tr>
<tr>
<td>PAI-1 and -2</td>
<td>Plasminogen activator inhibitor -1 and -2</td>
</tr>
<tr>
<td>PAP</td>
<td>Preactivation peptide</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>pEGFR</td>
<td>Phosphorylated epidermal growth factor receptor</td>
</tr>
<tr>
<td>Plg</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>Plm</td>
<td>Plasmin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PN1</td>
<td>Protease Nexin-1</td>
</tr>
<tr>
<td>REMARK</td>
<td>Recommendations for tumour marker prognostic studies</td>
</tr>
<tr>
<td>suPAR</td>
<td>soluble urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour-associated macrophages</td>
</tr>
<tr>
<td>TGFB1</td>
<td>Transforming growth factor-beta 1</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNFα and β</td>
<td>Tumour necrosis factor α and β</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VN</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>I</td>
</tr>
<tr>
<td>Abstract</td>
<td>III</td>
</tr>
<tr>
<td>List of papers</td>
<td>IV</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>V</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Oral squamous cell carcinoma (OSCC)</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Epidemiology and Etiological factors</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Clinical features and histology</td>
<td>2</td>
</tr>
<tr>
<td>1.1.3 Treatment of OSCC</td>
<td>6</td>
</tr>
<tr>
<td>1.1.4 Prognostic and predictive factors</td>
<td>6</td>
</tr>
<tr>
<td>1.2 The tumour microenvironment</td>
<td>10</td>
</tr>
<tr>
<td>1.2.1 The extracellular matrix (ECM)</td>
<td>11</td>
</tr>
<tr>
<td>1.2.2 Secreted soluble factors</td>
<td>12</td>
</tr>
<tr>
<td>1.2.3 Tumour-associated macrophages (TAMs)</td>
<td>13</td>
</tr>
<tr>
<td>1.2.4 Carcinoma-associated fibroblasts (CAFs)</td>
<td>13</td>
</tr>
<tr>
<td>1.2.5 Endothelial cells</td>
<td>14</td>
</tr>
<tr>
<td>1.3 Plasminogen activation system</td>
<td>14</td>
</tr>
<tr>
<td>1.3.1 Plasminogen and plasmin</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2 Tissue-type plasminogen activator (tPA)</td>
<td>16</td>
</tr>
<tr>
<td>1.3.3 Urokinase plasminogen activator (uPA)</td>
<td>16</td>
</tr>
<tr>
<td>1.3.4 Plasminogen activator inhibitors (PAI)</td>
<td>18</td>
</tr>
<tr>
<td>1.3.5 Urokinase plasminogen activator receptor (uPAR)</td>
<td>19</td>
</tr>
<tr>
<td>1.4 uPAR in migration, invasion and metastasis</td>
<td>26</td>
</tr>
<tr>
<td>1.4.1 In vitro and in vivo</td>
<td>27</td>
</tr>
<tr>
<td>1.4.2 Expression of the PA-system in OSCC</td>
<td>28</td>
</tr>
<tr>
<td>2. Background and aims for the study</td>
<td>29</td>
</tr>
<tr>
<td>3. Summary of results</td>
<td>30</td>
</tr>
<tr>
<td>Paper I</td>
<td>30</td>
</tr>
<tr>
<td>Paper II</td>
<td>30</td>
</tr>
<tr>
<td>Paper III</td>
<td>31</td>
</tr>
<tr>
<td>4. General discussion</td>
<td>32</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.1 Methodological considerations</td>
<td>32</td>
</tr>
<tr>
<td>4.1.1 Prognostic biomarkers</td>
<td>32</td>
</tr>
<tr>
<td>4.1.2 Model systems in cancer research</td>
<td>39</td>
</tr>
<tr>
<td>4.2 Discussion of results</td>
<td>43</td>
</tr>
<tr>
<td>4.2.1 Prognostic biomarkers</td>
<td>43</td>
</tr>
<tr>
<td>4.2.3 The tumour microenvironment</td>
<td>45</td>
</tr>
<tr>
<td>5. Conclusions and future perspectives</td>
<td>50</td>
</tr>
<tr>
<td>6. References</td>
<td>52</td>
</tr>
</tbody>
</table>
1. Introduction

The aim of the introduction is to give relevant background information to the study. The introduction is divided into four separate parts, where information about the disease of interest, oral squamous cell carcinoma (OSCC), is given in the first section. In the second section, an introduction is given to some aspects of the tumour microenvironment that are relevant for the study. In the third section, the plasminogen activation (PA) system is introduced, with special emphasis on the urokinase plasminogen activator receptor (uPAR). Finally, in the last section, the role of uPAR in migration, invasion and metastasis is summarized based on the current literature.

1.1 Oral squamous cell carcinoma (OSCC)

Oral squamous cell carcinomas (OSCC) are cancers originating from the squamous epithelium in the oral cavity. Locations include the lip, mobile tongue, buccal mucosa, labial mucosa, floor of the mouth, gingiva, hard palate and soft palate. OSCC belongs to a larger subgroup of tumours termed head and neck squamous cell carcinomas (HNSCC), comprising carcinomas arising in the oral cavity, oropharynx, larynx, hypopharynx, nasal cavity, nasopharynx, paranasal sinuses, salivary glands and the ear [1], where OSCCs are the most common oral malignancy with a poor 5-year survival rate [1-4].

1.1.1 Epidemiology and Etiological factors.

In 2008, more than 260,000 new cases of oral cavity cancers were predicted worldwide and over 130,000 of these patients were estimated to die from the disease (approximately 50%). More than 60% of these cases occur in the developing countries, where the male population by far displays the highest prevalence [3]. In Norway, the number of cancers arising in the oral cavity have gradually increased during the last decades, with an approximate 300 new cases each year. From these 300, it is estimated that 30-40% will die from the disease [5].

Gender, race and age have all been associated with differences in OSCC incidence, mortality, site, grade, histological type and tumour stage at diagnosis [6]. As with many other types of cancer, OSCC most commonly occurs in the middle aged and elderly population [7,8].
male population have traditionally had a higher incidence in OSCC, typically 1:2 compared to women. This is however evening out, probably due to increased alcohol consumption and tobacco use among the female population [8]. In 2001, the highest mortality rates for OSCC were reported to be in France, the Indian subcontinent, Brazil and central/eastern Europe [7]. The lowest survival rates have been ascribed patients of African-American origin living in the United States [6]. Also among South-African Indians, living in Natal, the mortality rates from OSCC were high [7,9]. Most often, such differences in mortality rates are explained by cultural traditions, ethnic differences and socioeconomic circumstances [7].

Certain risk factors such as tobacco use, alcohol consumption and human papillomavirus (HPV) infections, increases the HNSCC incidence [3,7]. Furthermore, heavy consumption of alcohol combined with smoking functions synergistically, multiplying the risk of developing OSCC [3,7,10]. Snuff and chewing tobacco has also been associated with higher risk of developing OSCC. The Swedish snus on the other hand is a non-fermented moist snuff that contains less nitrosamine, and is therefore less carcinogenic than snuff, chewing tobaccos and smoking [11]. Though still debated, no large scale epidemiological studies have been able to prove an association between snus use and elevated risk of oral cancer [8,11]. A high percentage of oropharyngeal cancers are HPV positive (90% in Sweden, 60% in the USA), and HPV is thought to be a major cause of cancers in the oropharynx [12], though far less important for the development of cancers in the oral cavity. In the United States and Europe, an increase in HPV-related HNSCC has been reported. This trend was hypothesized to be related to an increase in oral sex [6], even though many patients with HPV-positive tumours, reported few or no oral sexual partners [12]. Other risk factors believed to have an impact on the development of OSCC are poor oral hygiene, gastro-oesophageal reflux disease, dietary factors, use of marijuana and environmental contaminants such as paint fumes, plastic by-products and gasoline fumes [10].

1.1.2 Clinical features and histology

OSCCs gradually progress from normal epithelium, via precursor stages, to invasive and metastatic cancers [1,10,13]. Oral cancers often develop from precancerous lesions such as leukoplakias and erythroplakias, which are often subtle, painless and asymptomatic. These often present as identifiable red (erythroplakia) or white (leukoplakia) patches, where development of leukoplakias in the tongue and floor of the mouth are associated with
Introduction

higher risk of malignant transformation [8]. Leukoplakias in these sites are more frequently diagnosed with malignant changes such as epithelial dysplasia, carcinoma in situ and invasive SCC [6,8]. The most common site for the development of erythroplakias is the floor of the mouth, lateral tongue, retromolar pad and the soft palate. Erythroplakias are more frequently diagnosed as malignant than leukoplakias [8].

Gradually the tumour presents as an exophytic mass or can display an endophytic growth pattern associated with the development of SCCs (figure 1). As the tumour develops, non-healing ulcers appear. Ulcers can partially heal and later reappear, and eventually the cancer evolves to a crusted, non-tender, indurated ulcer or mass. Verrucous carcinoma represents about 3% of all OSCCs. It is a low-grade variant of OSCC, which displays slow and exophytic growth, is well differentiated and has a much better prognosis than conventional OSCC [8].

Figure 1: Oral squamous cell carcinoma (OSCC). The image to the left shows an OSCC located underneath the tongue. The tumour has a white appearance and shows an exophytic growth. The image to the right shows and OSCC located in the buccal mucosa, and presents as an exophytic mass.

Late stage symptoms include bleeding, loosening of teeth, difficulty wearing dentures, difficulties in swallowing (dysphagia), painful swallowing (odynophagia), speech impairment (dysarthria), and development of a neck mass as a sign of lymph node metastasis. Metastasis from OSCC usually develops in the ipsilateral (on the same side) cervical lymph nodes, and distant metastasis to the lung, though any part of the body may be affected [8].
OSCCs are histopathologically characterized according to the degree of differentiation [14]. In well differentiated tumours (grade 1; pG1), the tumour cells resemble normal epithelial cells, arranged in an orderly stratification. Heavy keratinization can be found in pearl formations (figure 2A). In moderately differentiated tumours (grade 2; pG3), the cells are less stratified, less keratinized and the tumour contains prickle cells (figure 2B). In grade 3 (pG3) tumours the cells are poorly differentiated but still identifiable as squamous cell carcinomas (figure 2C) [10,14,15]. It is estimated that >50% of OSCC are moderately differentiated [4].

Figure 2: Differentiation of SCC. A: Well differentiated SCC (pG1). B: Moderately differentiated SCC (pG2). C: Poorly differentiated SCC (pG3). Reprinted and modified image from [4] with permission.
OSCC may also be characterized according to the growth pattern, which refers to growth at the invasive tumour front [15]. The tumour growth is categorized according to four different types of invasion patterns (type I-IV). When several different growth patterns are observed within the same tumour, the most aggressive pattern is reported. Type I: Tumours showing a pushing- or expansive boarder, where the intracellular connections (cohesions) are still intact, resulting in a well delineated infiltrating border. Type II: The malignant keratinocytes are arranged as solid rounded cords or bands. The tumour front is asymmetrically aligned, penetrating the surrounding tissue at different levels. Type III: Very similar to the type II growth pattern, except for small groups or cords of infiltrating cells. Type IV: The tumour shows an ill-defined and irregular border with satellite cells infiltrating the tumour stroma [15,16].

Approximately 10-35% of patients suffering from oral cancer are at risk of developing second tumours [17]. Patients can present with multiple premalignant and malignant lesions in the oral cavity, a concept explained by field cancerization. The field cancerization theory was presented as early as in 1953 to explain the common local re-occurrence of OSCC after treatment [18]. Tabor and colleagues found, by analysing genetic markers, that the oral mucosa surrounding the resected tumour often displayed similar genetic mutations [19]. They hypothesized that “fields” of genetically altered cells could explain the high propensity for local recurrences and second field tumours (previously termed second primary tumours), later underscored by other studies [20-23]. While local recurrences develop from residual cancer cells not removed through treatment, second field tumours develop from the predisposed “field” surrounding the resected tumour. Tumours arising from new independent “fields” has been proposed as the source of “true” second primary tumours (figure 3) [1,24], which may be explained by the fact that the aerodigestive tract is chronically exposed to potential carcinogens, whereupon tumours may readily develop independently of each other [17].
Relapse occurs in 10-35% of patients where the primary tumour has been removed. These relapses often occur within “fields” of genetically altered cells (light blue), in close proximity to the excised primary tumour (dark blue), and could explain the high propensity for relapse. These tumours are termed second field tumours. Residual cancer cells (dark blue single cells) after primary tumour resection, is a source for local recurrence, while a second field of cancerization may give rise to second primary tumours.

Image reprinted from [1] with permission.

1.1.3 Treatment of OSCC

The treatment strategy is determined by several factors such as primary tumour size and location, lymph node status, presence or absence of distant metastasis, the patient’s ability to tolerate the treatment, and the patient’s desire [8]. Treatment of OSCC include surgery, radiation therapy and chemotherapy [10], but the most common treatment is the combination of radiation therapy and surgery, as is most often used for advanced stages of the disease [8].

1.1.4 Prognostic and predictive factors

Even though the prevalence of OSCC is higher in men than women [8], the prognosis is similar between the sexes. The importance of age at the time of diagnosis however is controversial. Some reports say that age influences the outcome, where older patients display worse prognosis [25], while others find an even distribution of prognosis across the age groups [6]. When it comes to alcohol and smoking there are conflicting reports on
Introduction

prognosis. Some claim it has no effect and others claims it does [25]. Poor prognosis is often associated with lower socioeconomic status and lower education, and early detection of (pre)malignant lesions is of importance for improving prognosis [25,26].

**TNM-staging.** TNM-staging classifies tumours according to tumour size (T; T0-T4), lymph node metastasis (N; N0-N3) and distant metastasis (M; M0-M1) (see table 1) [4,10]. The tumours TNM-stage is strongly correlated to the prognosis of the patient, and is the main contributor to establishing the proper treatment strategy [8]. The individual T, N and M values classifies the tumour to either of four stages (stage I-IV) (see table 1), where a higher stage is strongly associated with worse prognosis [8,25]. Patients baring cervical lymph node metastasis (N+) are classified as stage III. Metastases to lymph nodes is widely accepted as a major prognostic factor, where (N+) patients display worse prognosis and are far more susceptible to recurrence [25]. If distant metastasis (M+) is found, the disease is classified as stage IV, reducing the 5-year survival rate from approximately 45% to 20% [8]. A common problem is that patients with tumours of the same stage often respond differently to the same treatment [10]. This might in part be explained by the molecular heterogeneity of these tumours [1,27], hence better prognostic markers are needed, especially biomarkers predicting invasive and metastatic tumour behaviour.
Table 1: TNM staging of OSCC.

<table>
<thead>
<tr>
<th>TNM staging of OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary tumour size (T-status)</strong></td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td><strong>Lymph node metastasis (N-status)</strong></td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>N2a</td>
</tr>
<tr>
<td>N2b</td>
</tr>
<tr>
<td>N2c</td>
</tr>
<tr>
<td>N3</td>
</tr>
<tr>
<td><strong>Distant metastasis (M-status)</strong></td>
</tr>
<tr>
<td>M0</td>
</tr>
<tr>
<td>M1</td>
</tr>
<tr>
<td><strong>Stage grouping</strong></td>
</tr>
<tr>
<td>Stage I</td>
</tr>
<tr>
<td>Stage II</td>
</tr>
<tr>
<td>Stage III</td>
</tr>
<tr>
<td>Stage IV</td>
</tr>
</tbody>
</table>

**Histopathological grading.** Histopathological grading (pG1-pG3) of OSCC was in the 70’s suggested as an indicator of prognosis [28,29]. However, tumour grade alone is now recognised as a poor tool for predicting outcome and treatment strategy in OSCC [10,30,31]. To improve the prognostic value of tumour grading, it has now been recommended to combine it with tumour growth pattern (type I-IV), as described in the “clinical features and histology” section [14-16,32,33]. A tumour-induced “reactive” stroma (also termed desmoplasia) is necessary for tumourigenesis and metastasis. Desmoplasia is characterized by the presence of fibroblasts and myofibroblasts surrounding the invasive tumour island. However little is known about how the tumour stroma affects prognosis and henceforth treatment stratification of OSCC [15].

**Molecular heterogeneity and prognostic biomarkers.** OSCC has proven to be a molecular heterogeneous type of tumour. All head and neck tumours may be subdivided into two main classes: those infected with high-risk HPV, especially HPV 16 and 18, comprising approx. 20% of all the tumours, and those that are not, approx. 80% (figure 4) [1,34,35]. Interestingly, patients infected with high-risk HPV show improved disease specific survival, with these tumours preferentially locating to the oropharynx [36]. HPV-infected tumours will most
Introduction

often harbour a wild-type TP53 gene, which encodes the apoptosis regulating protein p53. During an HPV infection, the viral oncogenes E6 and E7 will encode proteins that result in the loss of apoptotic control through degradation of p53 and the binding of retinoblastoma (Rb) proteins, respectively [10]. This leads to a loss of regulation during the cell cycle, leading to an accumulation of DNA damage and cancer progression. In tumour cells not infected with HPV, it is estimated that approx. 60-80% of the tumours will have mutations in the p53 or Rb-pathways [1,37,38]. Mutations in the tumour suppressor gene TP53 are frequently observed in tumours from patients that are smokers and drinkers [10]. The p53 protein has therefore been suggested as a prognostic marker, predicting recurrence [25]. However, a review of published literature on popular OSCC biomarkers, p53 was found correlated with poor survival in only six of the 11 studies [39].

For HNSCC in general, other genes have been shown to be important in development and progression: CDKN2A encoding p16INK4A, CCND1 encoding cyclin D1, RB1/RBL1 encoding p170 and RBL1 encoding p130 [1].

Figure 4: Molecular heterogeneity of HNSCC. HNSCC may be subdivided in those tumour cells infected with high-risk HPV (approx. 20%) and those that are not infected with HPV (approx. 80%). The majority of HPV-negative tumours will harbour a p53 mutation, driving the tumourigenesis. Image from [1], with permission.

Several studies also report that the epidermal growth factor receptor (EGFR) is overexpressed in HNSCC [40-43]. Overexpression of EGFR in OSCC has been correlated with increased tumour size, advanced pathological stage, increased incidence and severity of recurrence, decreased disease-free survival, and hence functions as a promising prognostic
marker. Furthermore, EGFR has been a hot target in the development of new treatment strategies [44]. In a study using expression profiling of 60 tumours, 56 were found positive for EGFR expression. Out of the 56 EGFR-positive tumours, 34 also showed positive immunostaining for phosphorylated EGFR (pEGFR) [45]. In a study including 82 NHSCC patients, where only 14 displayed phosphorylation of EGFR, a significant correlation was found between pEGFR and prognosis [41]. Both tyrosine kinase inhibitors and EGFR-targeted antibodies have been tested in clinical trials, resulting in merely 5-15% response rates when used as a sole treatment in recurrent and metastatic disease [46]. A phase III clinical trial, where the use of radiotherapy and an anti-EGFR antibody (cetuximab) was combined, resulted in prolonged progression-free survival in patients with HNSCC [47]. However, contradictive results are published on the prognostic value of EGFR. As described by Søland and Brusevold only two of the seven studies reviewed showed a correlation between EGFR expression and survival [39].

The Ki67 marker is located in the nucleus of cells undergoing proliferation, and it is thought to indicate how fast the tumour is growing [48]. Some studies find that Ki67 correlates with poor prognosis, although contradictive findings do exist [27,39,49,50].

Expression of certain matrix metalloproteinases (MMPs) in the primary tumour have been correlated with tumour stage [48] and poor prognosis in a subgroup of patients lacking lymph node metastasis [51]. Also proteins of the plasminogen activation (PA) system have been suggested as prognostic markers and therapeutic targets in OSCC [52-56], which will be discussed in more detail later.

1.2 The tumour microenvironment

The tumour microenvironment has gained increasing interest in the cancer research field over the last decades, and it is now generally accepted that the microenvironment plays a part in the development and progression of cancer [57]. The tumour microenvironment is a complex network of secreted soluble factors, non-cellular material and stromal cells that can modulate tumour progression. The stromal cells include many different cell types (summarized in figure 5) including neutrophils, mast cells, fibroblasts, macrophages and
endothelial cells [58,59]. As the tumour microenvironment is complex and consists of a multitude of factors that can influence on tumour progression, only a selection is presented below, which will be relevant for the study at hand.

Figure 5: The tumour microenvironment. The tumour microenvironment contains numerous stromal cells that can either promote or suppress tumour progression. BMDC = Bone marrow-derived cell; MDSC = myeloid-derived suppressor cell; MSC = mesenchymal stem cell; TEM = TIE2-expressing monocytes. The images is modified and reprinted with permission [59].

1.2.1 The extracellular matrix (ECM)

The ECM includes the interstitial matrix and the basement membrane, and consists of a diversity of proteins such as collagens, elastin, fibronectin, fibrillin and proteoglycans, giving the ECM structure and organ specific functions [58,60]. The basement membrane is a specialized type of ECM containing a complex network of collagen IV, laminin, entactin/nidogen and heparin-sulphate proteoglycans [60]. During cancer progression, the
normal ECM is transformed into a reactive stroma by either stromal cells, such as fibroblasts, or the tumour cells. Desmoplasia, a reactive stroma often observed in OSCC, is characterized by a dense deposition of ECM interspersed with activated fibroblasts (myofibroblasts) [61,62]. During tissue homeostasis, fibrin is not present, however in wounds; fibrin is deposited and is strongly proangiogenic. Fibrin is also present in the tumour microenvironment. Dvorak postulated in 1986 that tumour were like “wounds that do not heal” [63]. This was based on the observations that the tumour stroma was populated with numerous proliferating fibroblasts, showed large deposits of complex ECM proteins and displayed angiogenesis.

1.2.2 Secreted soluble factors

There are many soluble factors in the TME that may influence of tumour progression: VEGFs [64], TGF-α and EGF (in EGFR signalling) [65,66], PDGF [67] and the TGF-β [68]. Secreted soluble factors may also include MMPs and proteins of the PA system [58]. However, only TGF-β will be the focus of the following section. The TGF-β superfamily consist of over 40 proteins, including the three highly conserved human isoforms of TGF-β (TGF-β1-3), activins (A, AB, B, C and E), inhibins (A and B), bone morphogenetic proteins (BMPs) and growth/differentiation factors (GDFs) [69]. The TGF-β cytokines have roles in cell growth, migration, proliferation, differentiation and activation of gene transcription of a wide range of genes [68,70]. The TGF-β1 isoform is pleiotropic, and produced by almost all cells, but mainly by the platelets, regulatory T cells (Tregs), monocytes/macrophages, lymphocytes, fibroblasts, epithelial cells and dendritic cells [69]. It is now well known that TGF-β can function both as a tumour suppressor in early tumour development, and a tumour promoter during later stages of progression [68,71], and a role of TGF-β has also been associated with changes occurring in the tumour microenvironment [72-74].

During its production, TGF-β is associated with the latency-associated peptide (LAP) in the ER. Together they form the small latent complex (SLC). LAP shields the sites in TGF-β involved in receptor binding. Before secretion, SLC binds to the single latent TGF-β binding protein (LTBP) in the ER, forming the large latent complex (LLC). Once secreted, the LTBP promotes extracellular sequestration of TGF-β [75]. Before TGF-β can bind to it receptor, it must be released from its latent complex [76]. This activation of TGF-β can be performed by for instance plasmin (see figure 11), but also many more [76-78]. Integrins are also involved
in TGF-β activation, either through a protease-independent mechanism (involving αVβ6), or a protease-dependent mechanism (involving αVβ8). Upon activation, TGF-β dimers form a complex with two TGFβ type II (TGFβRII) and two TGFβ type I (TGFβRI) receptors located at the cell surface of the target cell [76]. This receptor complex formation leads to the phosphorylation of TGFβRI by the constitutively active TGFβRII. TGFβRI then phosphorylates SMAD2 and SMAD3 proteins and the signal is transmitted to the cell nucleus through what is known as the canonical signalling pathway [76]. TGF-β signalling may also involve an alternative non-canonical signalling pathway, involving PI3K-Akt, RhoA and MAPK pathways [68].

1.2.3 Tumour-associated macrophages (TAMs)

Under normal conditions, macrophages play an important role in the non-adaptive immune system, functioning as scavenger cells, ridding the body of pathogens and tumour cells, but also stimulating the innate immune system [79]. There are two main macrophage phenotypes, M1 and M2. M1 macrophages are activated through factors such as IFN-ϒ, while M2 is activated through different interleukins and TGF-β [79]. The macrophage phenotype M1 will encourage inflammation, while the M2 phenotype suppresses the immune system, encouraging tissue repair through processes such as angiogenesis and matrix remodelling [80]. However, during carcinogenesis, the macrophage phenotype M2 may have a prometastatic effect, enhancing tumour cell migration, invasion and intravasation [81,82]. The M2 macrophage has therefore been termed the tumour-associated macrophage (TAM) [83,84]. High TAM content has been correlated with poor prognosis in ovarian cancer [85] and OSCC [86]. TAMs located in hypoxic regions of the tumour microenvironment induce angiogenesis through up-regulated production of VEGF [87,88].

1.2.4 Carcinoma-associated fibroblasts (CAFs)

The primary purpose of fibroblasts is maintaining the tissue integrity and homeostasis by synthesizing structural ECM proteins and proteases [58]. However, in wounds or in the tumour microenvironment, fibroblasts become activated and are termed myofibroblasts or cancer/carcinoma-associated fibroblasts (CAFs) [62,89]. TGF-β can activate fibroblasts, and once active they can be recognised through their expression of α-smooth-muscle actin.
Furthermore, CAFs can promote tumour progression, as shown when non-invasive cancer cells co-injected with CAFs in mice resulted in increased invasion [89]. Through the secretion of MMPs and serine proteases such as uPA, hepatocyte growth factor/scatter factor (HGF/SF) and VEGF, myofibroblasts can promote migration, invasion and metastasis of the tumour cells [90]. Through the secretion of VEGF, myofibroblasts can attract endothelial cells. CAFs also produce other factors that contribute to angiogenesis, such as IL-8, secreted protein acidic and rich in cysteine (SPARC) and TGF-β [62]. In OSCC, when staining for CAFs, an abundant staining pattern was associated with poor prognosis [86]. Furthermore, CAFs have been shown to promote tumour formation and invasion [91].

### 1.2.5 Endothelial cells

As a tumour grows in size, the need for nutrients and the need for waste removal increases. Without a sufficient blood supply, tumours only develop to a size of 1-2 mm³ [92,93]. For tumours to develop beyond this point, they must progress through the “angiogenic switch”. The angiogenic switch is controlled through a balance between pro-angiogenic factors such as VEGF and PDGF, and anti-angiogenic factors such as thombospondin, endostatin, vasculostatin and angiostatin [93]. With a poor oxygen supply the tumour tissue becomes hypoxic and the transcription factor hypoxia-inducible factor 1 (HIF1) is stabilized and induces the expression of VEGF which is involved in recruitment of vascular endothelial cells [93]. Numerous *in vitro* studies have shown that the presence of fibroblasts enhances endothelial cell sprouting and promotes tubulogenesis [94-96]. Taken together, this shows that there is a complex interplay between the tumour cells and the tumour microenvironment, where the tumour cells recruit stromal cells that can eventually promote tumour progression.

### 1.3 Plasminogen activation system

The plasminogen activation (PA) system has been implicated in wound healing, tissue regeneration, clot lysis and cancer progression. Several proteins comprise the PA system, where the main effector is the broad spectrum serine proteinase plasmin (summarized in figure 6). Plasmin is activated from its precursor plasminogen (plg), by either the urokinase plasminogen activator (uPA) or the tissue-type plasminogen activator (tPA). The
Introduction

Plasminogen activator inhibitors (PAI-1 and PAI-2) regulate the activity of uPA and tPA. tPA and uPA are secreted as inactive zymogens; pro-tPA and pro-uPA. While tPA is activated in solution, uPA is efficiently activated by plasmin when bound to its cell surface receptor uPAR [97]. In addition, plasmin can activate plasminogen [98], tPA [99], and cleave uPAR [100,101]. The functions of uPA and tPA are overlapping, though tPA is mainly involved in fibrinolysis, while uPA is involved in cell invasion as seen during wound healing and cancer invasion [102,103].

**Figure 6: The plasminogen activation (PA) system.** Plasminogen can be activated by both tPA and uPA in the pericellular environment as well as intravascular. Plasminogen is secreted as the proenzyme Glu-plasminogen, and in a feed-back loop, plasmin cleaves Glu-plasminogen into Lys-plasminogen by removing the PAP-domain. tPA and uPA can then activate Lys-plasminogen into fully active plasmin, a process that is accelerated when both uPA and plg are bound to their respective cell surface receptors [104], not shown in image. Reprinted from [103] with permission from DeGruyter.

**PERICELLULAR**
- Activators:
  - uPAR
  - tPA
  - uPA
- Inhibitors:
  - uPAR
  - tPA
  - uPA

**INTRAVASCULAR**
- Activators:
  - tPA
  - uPA
  - plg
- Inhibitors:
  - PAI-1
  - PAI-2
  - SERPINs

Substrates:
- ECM molecules
- Adhesion molecules
- Protease receptors
- Growth factors
- Neurotransmitters
- Neurotransmitter receptors

Inhibitors:
- uPAR
- tPA
- PAI-1
- SERPINs
1.3.1 Plasminogen and plasmin

Plasminogen (plg) is mainly produced by the hepatocytes in the liver and circulates in the blood (approx. 2 μM) in its native proenzyme form, Glu-plasminogen (93 kDa). In a feed-back loop, plasmin can activate Glu-plasminogen to Lys-plasminogen (84 kDa) by cleaving off the preactivation peptide (PAP) (figure 6). Lys-plasminogen is more readily activated by uPA and tPA than Glu-plasminogen, and binds fibrin with greater affinity. Cleavage of the Arg561-Val562 peptide bond of Lys-plasminogen creates the fully active two-chain plasmin; held together by a disulphide bridge [103,105,106]. Active plasmin is a broad spectrum proteinase that degrades a variety of ECM proteins such as vitronectin (VN) [107], fibronectin (FN), fibrinogen [108,109], fibrin [108], aggrecan [110] and laminins [111-113].

1.3.2 Tissue-type plasminogen activator (tPA)

The human serine protease tPA (PLAT) is secreted as a 72 kDa inactive single-chain zymogen (pro-tPA), by mainly vascular endothelial cells, keratinocytes, melanocytes and neurons [108,114]. When the Arg275-Ile276 peptide bond is proteolytically cleaved, tPA is transformed into the active two-chain protease (chain A and B), held together by a single disulphide bridge (Cys264-Cys396). Chain A of tPA harbours a fibronectin type II domain/N-terminal finger domain, a growth factor domain resembling the epidermal growth factor (EGF), and two kringle domains. Chain B contains the serine protease domain, similar to that of uPA, and contains the active site triad His322, Asp371 and Ser478 [106,115]. tPA is mainly involved in the fibrinolysis of blood clots, where binding to fibrin increases the tPA-activation of plg [108]. In the clinic, intravenous injection of tPA is used in the treatment of acute ischemic stroke, myocardial infarction, and pulmonary embolism [99,116].

1.3.3 Urokinase plasminogen activator (uPA)

The serine protease uPA (PLAU) is secreted as a 55 kDa one-chain zymogen (pro-uPA) [106], mainly by endothelial cells, epithelial cells, leukocytes, monocytes, fibroblasts and cancer cells [117,118]. Pro-uPA consists of three domains: the growth factor domain (GFD), a kringle domain and the proteolytic serine protease domain. The growth factor domain and the kringle domain together constitute the amino terminal fragment (ATF), sometimes termed as chain A (figure 7) [115,119]. The remaining part of the enzyme, also termed chain B, contains the serine proteinase domain [120] with the active site triad His204, Asp255 and
Ser356 [121]. The concentration of uPA in plasma is approx. 20 pM, where most of it is in complex with PAI-1, and a small fraction is present in the pro-uPA form. Pro-uPA is by cleavage of the Lys158-Ile159 peptide bond, giving a two chain high molecular weight (HMW) form of uPA (figure 7) [122], linked by a single disulphide bond between Cys148 and Cys279 [123]. Activation of pro-uPA can be performed by several proteinases such as plasmin [97], trypsin [124], cathepsin B and -L [125,126], MMP-7 [127], as well as kallikreins 2, 4 and 12 [128]. Even though activation of pro-uPA can be performed in liquid phase, the activation is far more efficient when pro-uPA is bound to its cell surface receptor uPAR [106,122]. Active uPA can then activate plasminogen to plasmin, where the activation is much more efficient when both plg and uPA are bound to the cell surface. Active plasmin can then in a feed-back loop activate more uPA [129,130]. Only trace amounts of plasmin are needed to initiate the activation reaction, and uPA can remain active at the cell surface for several hours [131,132]. HMW-uPA can further be cleaved into the low-molecular weight (LMW) uPA which contains the protease domain and thus remains active, but cannot bind uPAR (figure 7) [106]. The remaining inactive part of uPA, the amino terminal fragment (ATF), can still bind uPAR [133,134]. Active uPA not only activates plasmin, but also HGF/SF, the macrophage-stimulating proteins (MSP) [106], and can additionally cleave uPAR, rendering it unable to bind uPA [135,136].
Introduction

Figure 7 Plasmin-induced activation of uPAR-bound uPA. uPA bind uPAR via it amino terminal fragment (ATF) comprising the growth factor domain (GFD) and the kringle domains. Receptor associated plasmin cleaves receptor-bound pro-uPA at Lys158-Ile159, producing the active two-chain form of uPAR, termed high-molecular weight (HMW) uPA. Plasmin can further cleave active HMW-uPA, producing an enzymatically active low-molecular weight (LMW)-uPA, and an inactive ATF-fragment. The ATF-fragment can bind uPAR, however LMW-uPA cannot.

1.3.4 Plasminogen activator inhibitors (PAI)

The main inhibitors of uPA and tPA are PAI-1 (SERPINE1) and PAI-2 (SERPINB2), in addition to neuroserpin (SERPINI1) and protease nexin-1 (PN1) (SERPINE2) [137], protein C inhibitor (PAI-3), thrombin and leukocyte elastase [103]. PAI-1 and -2 both belong to the serpin superfamily of serine protease inhibitors [138], and both PAIs perform similar physiological functions [139]. PAI-1 is a 379 amino acid protein of approx. 52 kDa [140], while PAI-2 exists either as a secreted 60 kDa glycosylated protein, or as a 47 kDa non-glycosylated
intracellular form not involved in regulation of proteolysis [141]. Both inhibitors bind uPA or tPA in a 1:1 complex, where PAI-1 acts faster and is more widely expressed than PAI-2 [99,102,142].

PAI-1 is either active, latent or cleaved, where only the active form can bind and inhibit uPA or tPA [143]. Most of PAI-1 is bound to the ECM protein VN [144], and has been found to induce multimerization of VN [145]. While the VN-bound PAI-1 stays active for longer periods of time, free active PAI-1 is rapidly converted to the latent form [146]. When PAI-1 binds uPAR bound uPA an low-density lipoprotein receptor-related protein-1 (LRP-1) assisted internalization of the complex is triggered. After uPA has been removed and routed for degradation, uPAR and LRP-1 are recycled back to the cell surface [102,147-149]. It was initially thought that PAI-1 could function as a good anticancer drug, by inhibiting proteolytic activity [150-153], and it was therefore surprising to find that PAI-1 expression conveyed poor prognosis in several types of cancer; breast cancer [154-156], pulmonary adenocarcinoma [157] and ovarian cancer [158]. Later, it was shown that the PAI-1 was involved in regulating tumour angiogenesis in a concentration dependent manner [102,159,160].

1.3.5 Urokinase plasminogen activator receptor (uPAR)

**Introduction.** uPAR is a multifunctional protein involved in pericellular proteolysis, cell adhesion, cell migration, and cell signaling through a spectrum of membrane partners (summarized in figure 8). Human uPAR consists of a single polypeptide chain that contains five N-linked glycosylation sites (Asn52, Asn162, Asn172, Asn200 and Asn233). The heavy and heterogenous glycosylation gives uPAR a broad band on SDS-PAGE (approx. 50-60 kDa). When treated with N-glycanase the size is reduced to approx. 35 kDa [161,162]. The cysteine rich glycoprotein is bound to the extracellular part of the membrane via a glycosylphosphatidylinositol (GPI) anchor (figure 8) [131,132], hence uPAR has no membrane spanning nor intracellular domain, and therefore lacks inherent signalling properties [163].

Three homologous domains constitute uPAR, domain 1-3 (D1-D3, also termed domain I, II and III), each connected through small inter-domain linker regions, where all three domains must be present in order for uPA to bind (figure 7) [164-166]. The interaction between uPAR and the ATF of uPA involves all three domains of uPAR, but the major binding site is located within domain I and involves the residues Trp30, Ile28, Phe25, Asn22 and Val20 in human
Both uPA and pro-uPA are able to bind uPAR, and dissociation from the receptor is slow, giving potential for focused and persistent proteolytic activity at the cell surface [102,115,120,167]. In addition to binding and facilitating in the activation of uPA, uPAR can bind the ECM protein VN (figure 8), causing changes in cell morphology and increased cell motility [168,169]. The uPAR-VN binding is stimulated when uPAR simultaneously binds pro-uPA, uPA, ATF and the uPA-PAI-1 complex, PAI-1 alone inhibits the interaction [106].

**Expression of uPAR.** The expression of uPAR in tissues is mainly restricted to tissues undergoing remodelling [171], such as during embryogenesis [172,173], wound healing [171,174], ischaemia [175] and during inflammation [176]. *In vivo*, cells reported to express uPAR are hematopoietic stem cells, monocytes/macrophages, peripheral blood leukocytes, B-lymphocytes, activated T-lymphocytes, neutrophils, granulocytes, activated keratinocytes, trophoblasts, myofibroblasts/fibroblasts and some endothelial cells. Furthermore, uPAR is expressed in many different cultured tumour cells [100,171,177,178], and increased uPAR expression is found in grafted tumours during cancer cell invasion [179,180]. In tumours, uPAR may be expressed by the cancer cells as well as by stromal cells such as
fibroblasts/myofibroblasts, neoangiogenic endothelial cells, neutrophils and macrophages [181-187]. Several extracellular factors, such as growth factors (GF), cytokines and chemokines, are involved in regulating the expression and/or availability of uPAR at the cell surface as summarized in table 2.

Table 2: Extracellular factors shown to regulate uPAR expression in cells.

<table>
<thead>
<tr>
<th>Growth factors, cytokines, chemokines and ECM proteins</th>
<th>Cells used</th>
<th>Regulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal Growth Factor (EGF)</td>
<td>Colon cancer cells (CBSsf), lung carcinoma cells (A549).</td>
<td>Up</td>
<td>[188,189]</td>
</tr>
<tr>
<td>Basic Fibroblast Growth Factor (bFGF)</td>
<td>Bovine vascular endothelial cells (BME).</td>
<td>Up</td>
<td>[190]</td>
</tr>
<tr>
<td>Vascular Endothelial Growth Factor (VEGF)</td>
<td>Bovine vascular endothelial cells (BME)</td>
<td>Up</td>
<td>[192]</td>
</tr>
<tr>
<td>Transforming Growth Factor-Beta 1 (TGFβ-1)</td>
<td>A549</td>
<td>Up</td>
<td>[188]</td>
</tr>
<tr>
<td>Hepatocyte Growth Factor (HGF)/Scatter Factor (SF)</td>
<td>Canine kidney epithelial cells (MDCK)</td>
<td>Up</td>
<td>[193]</td>
</tr>
<tr>
<td>Interferon α (IFN-α)</td>
<td>Colon cancer cells (HCT116).</td>
<td>Up</td>
<td>[194]</td>
</tr>
<tr>
<td>Interferon γ (IFN-γ)</td>
<td>U937 mononuclear phagocytes, colon cancer cells (HCT116).</td>
<td>Up</td>
<td>[194,195]</td>
</tr>
<tr>
<td>Tumour Necrosis Factor α (TNFα)</td>
<td>Colon cancer cells (HTC116, KM12SM and LM1215), U937 (suPAR).</td>
<td>Up</td>
<td>[195,196]</td>
</tr>
<tr>
<td>TNFβ</td>
<td>Macrophage-like cell (U937)</td>
<td>Up</td>
<td>[197]</td>
</tr>
<tr>
<td>Interleukin (IL)-1α</td>
<td>Macrophage-like cell (U937)</td>
<td>Slight increase</td>
<td>[197]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Human chondrocytes</td>
<td>Up</td>
<td>[198,199]</td>
</tr>
<tr>
<td>IL-2</td>
<td>Natural killer cells, Macrophage-like cell (U937)</td>
<td>Up/slight increase</td>
<td>[197,200]</td>
</tr>
<tr>
<td>IL-3</td>
<td>Macrophage-like cell (U937)</td>
<td>Unchanged</td>
<td>[197]</td>
</tr>
<tr>
<td>IL-4</td>
<td>Macrophage-like cell (U937)</td>
<td>Slight increase</td>
<td>[197]</td>
</tr>
<tr>
<td>IL-6</td>
<td>Macrophage-like cell (U937)</td>
<td>Up</td>
<td>[197]</td>
</tr>
<tr>
<td>Complement Cα5 (chemoattractant)</td>
<td>Macrophage-like cell (U937)</td>
<td>Up</td>
<td>[197]</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Macrophage-like cell (U937)</td>
<td>Slight increase</td>
<td>[197]</td>
</tr>
<tr>
<td>Collagen IV, laminin</td>
<td>Macrophage-like cell (U937)</td>
<td>Unchanged</td>
<td>[197]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other factors</th>
<th>Cells used</th>
<th>Regulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone (immunosuppressant)</td>
<td>Macrophage-like cell (U937)</td>
<td>down</td>
<td>[197]</td>
</tr>
<tr>
<td>Amiloride</td>
<td>Colon cancer cells (HTC116, KM12SM and LM1215)</td>
<td>Down/ inhibited</td>
<td>[196]</td>
</tr>
<tr>
<td>Sodium butyrate</td>
<td>Colon cancer cells (HCT116, LM1215)</td>
<td>Down/ inhibited</td>
<td>[203]</td>
</tr>
<tr>
<td>Forskolin</td>
<td>HUVEC</td>
<td>Up</td>
<td>[201]</td>
</tr>
<tr>
<td>Ethanol</td>
<td>HUVEC</td>
<td>Up</td>
<td>[204]</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Colon cancer cells (HCT116, GEO)</td>
<td>Up</td>
<td>[205]</td>
</tr>
<tr>
<td>Asbestos</td>
<td>Mesothelial cells (MeTSa)</td>
<td>Up</td>
<td>[206]</td>
</tr>
<tr>
<td>Okadaic acid (Serine/threonine phosphatase inhibitor)</td>
<td>U937, WI-38, HeLa999, 8387, A549, HeLa, HeP-2, MIAL.</td>
<td>Up</td>
<td>[207]</td>
</tr>
<tr>
<td>Lipopolysaccharides</td>
<td>Human gingival fibroblasts</td>
<td>Up</td>
<td>[208]</td>
</tr>
<tr>
<td>Hyaluronan (HA)</td>
<td>Basal-like breast cancer cells (MDA-MB-231)</td>
<td>Up</td>
<td>[209]</td>
</tr>
</tbody>
</table>
Many different signalling pathways are involved in regulating the expression of uPAR (summarized in table 3). The human gene for uPAR (PLAUR) is located on chromosome 19q13 and spans across 7 exons, where both a full version, and a truncated version lacking the GPI-anchor, are known to exist [210]. The promoter region of PLAUR contains neither TATA- nor CAT-boxes [211,212]. Instead, the promoter contains a GC-rich proximal sequence, where several specificity protein 1 (Sp1) consensus elements are present [190], in addition to activator protein (AP) -1, AP-2, nuclear factor (NF)-κB, GATA-2, NF-1 and PEA3 motifs [178,210]. Transcription factors such as Jun-D, c-Jun, c-Fos and Fra-1 have been shown to bind to the AP-1 consensus motif when cells were stimulated with PMA [213]. The promoter also contains a hypoxia-responsive element (HRE) where the hypoxia-inducible factor 1α (HIF-1α) can bind and induce transcription [214,215].

<table>
<thead>
<tr>
<th>Intracellular signalling pathways</th>
<th>Cell type</th>
<th>Regulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Kinase C (PKC)</td>
<td>Human umbilical vein endothelial cells (HUVEC).</td>
<td>Up</td>
<td>[201]</td>
</tr>
<tr>
<td>Protein Kinase A (PKA) and cAMP</td>
<td>HUVEC, U937.</td>
<td>Up</td>
<td>[201,216]</td>
</tr>
<tr>
<td>Mitogen Activated Protein Kinases (MAPKs): Extracellular Signal-Regulated Kinase 1 (ERK1) and ERK2</td>
<td>Colon cancer cells (RKO, GEO).</td>
<td>Up</td>
<td>[217]</td>
</tr>
<tr>
<td>JNK</td>
<td>Ovarian carcinoma (OVCAR-3).</td>
<td>Up</td>
<td>[202]</td>
</tr>
<tr>
<td>c-Src</td>
<td>Colon cancer cells (SW480).</td>
<td>Up</td>
<td>[218]</td>
</tr>
</tbody>
</table>

**uPAR cleavage.** A truncated form of uPAR, uPAR (II-III) (also known as uPAR D2+D3) can be produced through cleavage between domain I and II (figure 9). This cleavage can be performed by uPA, plasmin, trypsin, chymotrypsin, cathepsin G, elastase and several MMPs [101,135,219-221] and renders uPAR unable to bind uPA [222,223] and vitronectin (VN) [224]. Cleavage of uPAR is most efficiently performed when uPAR is GPI-anchored to the cell surface, and the process can be inhibited by saturating uPAR with inactive uPA. Hence, uPAR cleavage is performed mostly by uPA bound to neighbouring uPAR molecules [136,220]. This suggests that uPAR cleavage functions as a self-regulatory mechanism to avoid overactive proteolysis. uPAR may also be released from the cell surface through cleavage of the GPI-anchor, producing soluble uPAR (suPAR) (figure 9) [225,226]. Plasmin, trypsin, phospholipase C and -D are all able to produce suPAR. While the phospholipases cleave the GPI-anchor,
plasmin and trypsin cleavage within the C-terminal end of the amino acid chain of uPAR [101,227-229]. SuPAR may also be cleaved by uPA between domains I and II, although to a much lesser extent than membrane bound uPAR [135,136,220].

![Figure 9: Different forms of uPAR.](image)

**Figure 9: Different forms of uPAR.** uPAR exists in many forms at the cell surface, where the full-length version of uPAR, termed uPAR (I-III) is the only form that can bind uPA. uPAR (I-III) may be cleaved between domains I and II releasing domain I, termed uPAR (I). The remaining uPAR (II-III) is GPI-anchored to the cell surface, where the GPI-anchor may be cleaved, producing either the cleaved soluble uPAR, suPAR (II-III), or the soluble full-length version, suPAR (I-III).

**uPAR induced cellular signalling.** uPAR has no inherent signalling properties as it lacks both a membrane spanning domain, and an intracellular domain [163]. However, it has for a long time been known that uPAR triggers different signalling pathways in the cell through interactions with adjacent signalling molecules. The most studied signalling partners of uPAR
are the integrins [170,225]. uPAR has been found to associate with α3β1, α5β1, αVβ3, αIIbβ3 and αMβ2 integrins, leading to cell signalling through focal adhesion kinase (FAK) and Src, Ras-mitogen-activated protein kinase (MAPK) pathway and extracellular signal-regulated kinase 1 (ERK1) and ERK2 and the Rho family small GTPase Rac [170]. Research over the last two decades has brought another type of signalling receptor into focus, the G-protein coupled receptors (GPCR); N-formyl peptide receptor (FPR), FPR-like 1 (FPRL1) and FPRL2 [230-232]. The linker region connecting domains I and II of uPAR human contains a chemotactic epitope, the Ser-Arg-Ser-Arg-Tyr (SRSRY)-peptide (uPAR88-92). This peptide can be exposed through cleavage of uPAR between domain I and II of uPAR [233], but also through a conformational change in uPAR when it is bound to uPA or ATF [233,234]. Through interaction with the GPCRs, the SRSRY-peptide induces chemotaxis (figure 10), as seen in monocytes and basophils [230-232]. suPAR (I-III) however does not expose the SRSRY-peptide, as it is not recognized by an antibody specific for this sequence [220].

Figure 10: uPAR signalling through GPCRs. uPAR has the ability to associate with G-protein coupled receptors (GPCR) (e.g. FPR, FPRL1 and FPRL2) and induce cell signalling and chemotaxis. Either through uPAR cleavage, or through binding of ATF or uPA, may the chemotactic sequence SRSRY be revealed and associate with specific GPCRs.
uPAR has also been found to initiate cell signalling through several different types of receptor tyrosine kinases (RTKs), such as the platelet derived growth factor receptor-β (PDGFR-β) [235], insulin-like growth factor receptor (IGF1-R), c-Met [236,237] and the epidermal growth factor receptor (EGFR) [238-240]. In a study using an uncleavable mutant uPAR or a wild-type (wt) uPAR, it was found that uPAR (II-III) associated with GPCRs, while the uncleavable (full-length) uPAR preferably signalled through EGFR [241]. It is not clear whether uPAR can directly signal through EGFR alone, as several studies indicate that integrins functions as a link between the two [238,242].

Endocytosis and recycling of uPAR. The two major cell surface receptors involved in endocytosis and recycling of uPAR are the urokinase receptor associated protein (uPARAP, also known as Endo-180 and MRC-2) [243,244] and LRP-1 [245]. uPARAP/Endo180 belongs to the macrophage mannose receptor (MMR) protein family, is an endocytic receptor for collagen and involved in matrix turnover [244,246,247]. uPARAP/Endo180 has been reported to play a role in uPAR-dependent cell migration [244] and increased expression has been reported in stromal fibroblasts of HNSCC [248]. LRP-1 mediates internalization of uPAR and integrins through binding to the uPA:PAI-1 inhibitor complex, where PAI-1 functions as a bridge between uPAR and LRP-1 [245]. When active PAI-1 binds to uPA, the LRP-1-specific binding site within PAI-1 is exposed through a conformational change that enables endocytosis via LRP-1 [249]. While uPA is routed for degradation, uPAR, LRP-1 and integrins are returned to the cell surface [250,251]. Endocytosis of the uPAR/uPA/PAI-1/integrin complexes reduces migration [252], while inhibiting LRP-1-induced endocytosis results in increased migration [253,254]. Also the mannose 6-phosphate receptor/insulin-like growth factor II receptor (M6PR/IGF2R, also termed CD222) has been reported involved in the endocytosis, as well as cleavage of uPAR [255]. Furthermore, it has been shown that uPAR may be endocytosed and recycled via a clathrin and LRP-1-independent mechanism, mimicking micropinocytosis [256].

Cell surface distribution of uPAR. GPI-anchored proteins preferably partition into cholesterol rich and detergent resistant membrane microdomains termed lipid rafts [257]. The majority of uPAR however seems to be located in the detergent soluble fraction of the membrane, with only a small portion located within lipid rafts [258,259]. Interestingly, uPAR
dimers are preferentially lipid raft associated, displaying increased affinity for vitronectin, and increased susceptibility for uPA-induced cleavage [258]. Also, raft associated uPAR engages different signalling partners than non-raft associated uPAR, and binding to uPA or ATF induces raft partitioning [259-262]. Additionally, in stromal-derived factor 1α (SDF1-α) stimulated lymphocytes, certain types of lipid rafts containing uPAR and chemokine receptors have been shown to locate to the leading edge (lamellipodia) of migrating cells [263], which shows that external factors can control both the distribution and functions of uPAR.

**1.4 uPAR in migration, invasion and metastasis**

The components of the PA system are related to the invasive process of cells and thought to be important for cancer invasion in OSCC [264]. The fact that plasmin can activate several MMPs interlinks these two proteolytic systems, giving cancer cells increased ability to invade the tumour stroma (summarized in figure 11) [265,266]. Furthermore, uPAR, uPA and PAI-1 are involved in regulating cell signalling, migration and invasion both in vitro and in vivo [133,151,160,170,225,252]. Constituents of the PA system have therefore been suggested as promising prognostic biomarkers and as potential therapeutic targets [49,123,182,264].

**Figure 11: The role of uPAR in cancer cell invasion.** uPAR-bound uPA locates proteolytic activity to the cell membrane, enabling activation of plasmin. Plasmin can activate several matrix metalloproteases (MMPs) and latent growth factors such as TGF-β1. From [225] with permission.
1.4.1 In vitro and in vivo

Expression of uPAR not only provides cancer cells with the ability to regulate the activity of proteolytic enzymes, but also regulates cell signalling in a spatiotemporal manner and henceforth cell behaviour. Both uPA+/− and uPAR+/− mice exist and are viable and fertile [267,268]. The uPA+/− mice did however display occasional fibrin deposition [267]. This implies that there is functional redundancy of both the uPA and uPAR protein, and that they are not crucial for survival. This was shown in a study of wound healing, where the proteolytic functions of the PA system and MMPs overlap [266]. However, tumour development in mice lacking uPA (or PAI-1) is retarded [153], and the role of the uPAR-uPA binding in vivo has also been linked to inflammation [269].

The non-proteolytic functions of uPAR have, through several studies, been shown to be important for invasion and metastasis. By blocking the interaction between uPAR and uPA, using an ATF-like molecule, breast cancer- and gastric cancer in vivo cell growth, angiogenesis and metastasis to the liver was inhibited [134]. Similar results were obtained using an anti-uPAR antibody in the study of prostate cancer cells. In contradiction to the previously mentioned study, the antibody did not specifically block the uPAR-uPA binding, but epitopes located on uPAR important for other biological functions of the receptor.

Invasion and migration in vitro, tumour growth in vivo, and experimental metastasis in vivo was reduced [270]. Using human kidney epithelial cells (HEK-293) either lacking uPAR and uPA, or expressing the human uPAR, but not uPA, Jo et al. could show that metastasis was unrelated to the proteolytic functions of uPAR and uPA. Cells lacking uPAR showed little metastasis, while cells expressing uPAR but not uPA metastasized frequently. Because human uPAR does not bind the murine stromal produced uPA, cells must therefore metastasise independently of the uPAR-uPA interaction [271]. Other features of uPAR might therefore be more important for the invasive and metastatic process, where uPAR-integrin interactions are believed to be important [170].

In vitro studies have shown that uPAR interacts with both integrins α3β1 [272,273] and α5β1 [274,275]. Clustering of uPAR and the α3β1 integrin in cultured oral keratinocytes and OSCC cells induced expression of uPA, and activation of uPA enhanced invasion [56,276]. The importance of the uPAR-integrin interaction in vivo was shown using SCC-25 cells expressing either high or low levels of uPAR. Cells were injected orthotopically into the tongues of nude
mice. In areas of the tumours where uPAR and the α3β1 integrin co-localized, ERK was phosphorylated, showing that uPAR and α3β1-integrin interact in vivo [277]. The finding that inhibition of the uPAR-α5β1 integrin interaction led to tumour dormancy, through loss of FAK phosphorylation [278], supports the notion of uPAR-integrin interactions are important for tumour growth in vivo.

### 1.4.2 Expression of the PA-system in OSCC

Experiments using ELISA showed that uPA, uPAR, PAI-1, and PAI-2, but not tPA, were elevated in OSCC tumour tissue compared to healthy oral mucosa [53]. Furthermore high expression of uPA and PAI-1 have been shown to be significantly correlated to poor overall survival [55]. SuPAR was elevated in blood samples from patients suffering from HNSCC, compared to healthy individuals [279]. Immunohistochemical (IHC) studies have shown that high uPAR expression correlated with high tumour grading in OSCC, and that patients with uPAR positive tumours displayed a worse overall survival than patients with uPAR negative tumours [183]. It was also shown that low grade (G1) tumours with high uPAR expression predicted worse outcome than G1 tumours with low uPAR expression [183]. In another study, concomitant expression of uPA and uPAR in the OSCC tumours correlated with increased invasive behaviour, and expression of uPA correlated with secondary lymph node metastasis [52]. High uPA and uPAR expression has been correlated with worse overall survival [280], while high PAI-1 expression correlated with increasing tumour stage and tumour size [54]. Positive PAI-1 staining of tumour cells has also been suggested as a good indicative marker of invasive OSCC behaviour, even more so when co-localized with uPAR and the γ2-chain of laminin 5 [281].

Due to the fact that both stromal cells and tumour cells can display increased expression of uPAR [186,187,282,283], and cleavage products of uPAR may be indicative of a proteolytically active tumour, the measuring of cleavage products in patient blood, urine or ascites could function as a prognostic tool [284]. Different forms of uPAR, including suPAR (I-III), suPAR (II-III) and suPAR (I), have been detected in patients’ blood and urine, and elevated levels have been correlated with survival in a spectrum of different cancers [283,285-294].
2. Background and aims for the study

The role of uPAR in cancer has been thoroughly documented and extensively studied over the last three decades since its discovery. uPARs involvement in OSCC progression is less documented, but more studies have emerged in the recent years. OSCC is a heterogeneous disease and still patients suffer from relapse and a poor 5-year survival rate. This underscores the importance of developing better prognostic tools, better treatment strategies and finding new therapeutic targets. It is therefore vital with a deeper understanding of the molecular mechanisms involved. uPAR seems to play an important part in the progression of OSCC observed through in vivo and in vitro studies. In light of this, the goal of this work was to gain a better understanding of the role of uPAR in OSCC, characterize it as a prognostic marker, and gain a deeper understanding of how the tumour microenvironment is involved in the regulation of uPAR expression and function. More specifically, we wanted to:

- Study the distribution of uPAR, uPA, PAI-1 and Ki-67 in tumour tissue from patients with OSCC, and examine whether these factors were correlated with disease specific death.
- Establish a syngene mouse model for tongue SCC and study the effect of high- and low expression of uPAR in tumours in vivo.
- Study different tumour microenvironments in relation to expression and regulation of uPAR.
- Understand how stroma-derived factors are involved in the regulation of uPAR expression and cleavage.
3. Summary of results

Paper I

Urokinase Plasminogen Activator Receptor (uPAR) and Plasminogen Activator Inhibitor-1 (PAI-1) Are Potential Predictive Biomarkers in Early Stage Oral Squamous Cell Carcinoma (OSCC).


In this paper we used a tissue microarray (TMA) based immunohistochemical analysis of OSCC to test if components of the PA system could be used as predictive biomarkers. The expression of uPAR, PAI-1 and uPA was compared with clinicopathological variables and disease specific death. The principal findings were:

- Low expression of uPAR and PAI-1 were correlated with low disease specific death in early stage OSCC.
- uPA and the proliferation marker Ki-67 were not correlated with disease specific death.

Paper II

Tumour Microenvironments Induce Expression of Urokinase Plasminogen Activator Receptor (uPAR) and Concomitant Activation of Gelatinolytic Enzymes.


In this paper we studied the effect high- and low expression uPAR had on invasion and metastasis of a murine OSCC cell line, AT84, in vivo in a syngeneic mouse model and ex vivo in a leiomyoma invasion model. We also studied the role of different tumour
microenvironments on the expression and post-translational regulation of uPAR in the AT84 cells. The main results were:

- Increased expression of uPAR did not promote aggressive tumour growth or metastasis in tongue or skin tumours in the mouse model for OSCC.
- Expression of uPAR was up-regulated in the tumour-stroma interface in the mouse model for tongue tumours, and in OSCC cells invading the tissue of the leiomyoma invasion model.
- OSCC cells expressing high levels of uPAR \textit{in vivo} and \textit{ex vivo} showed increased activity of gelatinolytic enzymes.
- Soluble factors derived from the tumour microenvironment of the \textit{ex vivo} leiomyoma tissue induced increased expression and altered glycosylation and cleavage of uPAR.

\textbf{Paper III}

\textbf{Transforming Growth Factor-\(\beta\)1 (TGF-\(\beta\)1) Regulates Cleavage of the Urokinase Plasminogen Activator Receptor (uPAR) through Increased Expression of Plasminogen Activator Inhibitor-1 (PAI-1)}


Manuscript.

In this paper we studied how uPAR cleavage was regulated in the OSCC AT84 cells, both by the tumour microenvironment factor TGF-\(\beta\)1, the plasma constituent plasminogen and the uPA secreted by the cells. The main findings were:

- TGF-\(\beta\)1 increased the ratio of full-length uPAR versus cleaved uPAR.
- TGF-\(\beta\)1 reduced uPAR cleavage through induction of PAI-1 expression.
- The amount of cleaved uPAR vs full-length uPAR regulated intracellular signalling and cell migration and invasion.
4. General discussion

The main focus of my work has been on OSCC, with emphasis on the role of the plasminogen activation system, and more specifically uPAR, in the malignant progression of OSCC. The findings in each individual paper are discussed within the respective papers. In the first section a selection of methods are discussed (methodological considerations), and in the second part (discussion of results) the major issues and highlights from the three papers will be discussed.

4.1 Methodological considerations

In this section, advantages and limitations of a selection of methods relevant for the project will be discussed.

4.1.1 Prognostic biomarkers

A patient diagnosed with OSCC today is treated according to a strategy based on the primary tumour size, growth pattern and its spread. The ability to give cancer patients even better treatment according to their needs requires reliable prognostic- and predictive biomarkers. Whereas a prognostic marker indicates the patients’ prognosis, a predictive marker indicates the outcome of a given treatment. Even after decades of research within this field, one important question still remains “will this patient need additional treatment, after the initial (often surgical) intervention?” [295]. In paper I, we present uPAR and PAI-1 as potential prognostic markers, and show that low expression was associated with lower disease specific death [49].

There are several approaches to identifying new tumour biomarkers. In addition to protein based methods such as immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA), RNA and DNA based techniques are also used and have gained increasing interest [296]. However, implementing RNA and DNA based techniques to screen patient material in the clinic would involve changing the current diagnostic system. To analyse biopsies for RNA and microRNA (miRNA), high-quality tissue samples are required; not formalin-fixed paraffin embedded (FFPE) samples, as is the standard today. Collection and
analysis of high-quality samples might therefore require both a change in the way samples are preserved and require specialized centres to perform the analysis [297]. There are clear benefits to the use of IHC-based techniques to identify tumour biomarkers on FFPE samples, and it was therefore our method of choice. Morphology-based diagnosis and IHC-based markers are already standard procedure in clinical practice today. Furthermore, FFPE samples preserve the tissue architecture, which enables location-specific detection of the biomarkers, such as the presence of a marker at the tumour invasive front. We did however use tissue microarrays (TMA) in our study [49], where the tumour invasive front is not necessarily included. Nevertheless, TMAs might actually represent a more objective and quantifiable method than when using whole tissue sections, as discussed below [298].

**Patient cohort and study design.** In this study, we used a North Norwegian cohort consisting of 160 patients with primary SCC of the oral cavity and hypopharynx. When screening for biomarkers using patient material, it therefore is vital to know whether our patient cohort, and hence results obtained using the cohort, is representative and applicable to other populations. It is also of interest to know whether results obtained from other cohorts is applicable to our cohort, the North Norwegian population. This is especially interesting as the incidence of OSCC shows large geographical variations, due to culture-associated risk factors such as tobacco use and alcohol consumption [299]. In a recently published paper, the North Norwegian cohort used in paper I was found to be comparable to other European cohorts in most aspects such as tumour differentiation, location and patient age at diagnosis [300].

In paper I, 45 patients of the original cohort of 160 were excluded from the study. These patients had SCCs originating from the oropharynx, had verrucous tumours, or received radiotherapy prior to surgery. Up to 90% of cancers arising in the oropharynx are infected with high-risk HPV [12]. Increasing knowledge about HPV-induced oral cancer shows that these tumours display distinct molecular characteristics and develop through different carcinogenic pathways. They also present with largely better prognosis, and it is therefore suggested that these tumours are considered as a separate entity [1,301]. Verrucous tumours were also excluded due to the fact that these tumours are slow growing, with pushing borders, rarely metastasise and has a 5-year survival rate of 85-95% [4]. Patients that received radiotherapy were also excluded as these tumours may differ significantly from
the original primary tumour [302]. We believe that excluding these patients from our study on biomarkers makes the cohort more homogeneous, excluding obvious factors that may influence prognosis.

**Tissue microarray (TMA).** The use of TMA involves the punching of small cores, typically 0.6 mm in diameter, of representative tumour tissue. A number of cores are inserted into a recipient block, and tissue sections from the block can be used for IHC (figure 12). Using TMA to screen for potential biomarkers is time- and cost-efficient, as several hundred patients can be analysed on the same section. This also ensures identical experimental conditions for a large number of samples. For TMA to be beneficial, the cores must be taken from representative areas within the tissue. However, OSCC is histologically heterogeneous and shows a large variation in degree of differentiation within the same tumour [303-305]. Furthermore, the invasive tumour front has been suggested as the most important area for determining prognosis in oral cancer [14,16,33]. These areas may not be represented in a TMA since it may be technically difficult to sample cores from the invasive front. Challenges encountered when grading histopathological features such as the invasive front in whole tissue sections include inter- and intraobserver variability [306]. High inter- and intraobserver variability is not acceptable for a biomarker. A selection of biomarkers have been tested on TMA and compared to whole sections, where TMAs were found to be representative and reliable [305,307]. For heterogeneously expressed markers, TMA has even been suggested as a more objective and quantifiable method than when analysing whole tissue sections [298]. To enable accurate assessment of biomarkers, it was found that 3-4 cores [308] of 0.6 mm from each tumour were sufficient [309]. The use of TMA to screen for potential biomarkers in OSCC has been validated by others and found to be satisfactory and efficient [305,307,308]. The TMA used in paper I was based on eight cores from each tumour. We experienced a loss of 16.5% of the cores due to technical issues during preparation, or lack of tumour tissue within the core. This is in line with previous reports, and gives a reliable number of cores for evaluation [298,308]. The interobserver variability of tumour staining was tested and shown to be acceptable [49]. Taken together, we concluded that the use of TMA as a method to screen for biomarkers using immunohistochemistry on OSCC tissue was suitable and reliable.
General discussion

Figure 12: Making tissue microarrays. Tissue cores are taken from selected regions of the original tumour tissue in the donor block, and transferred to a recipient block. The recipient block containing several cores per patient, and harbouring tissue from numerous patients. Sections taken from the recipient block may then be analysed for potential biomarkers.

Immunohistochemistry (IHC). There are several ways to screen for biomarkers, where IHC is a method often used. IHC is relatively rapid to perform and there is no need for expensive equipment. Furthermore, if using TMAs, it can also be relatively cost-efficient. In addition, IHC is routinely used in all clinical pathology departments, making new IHC markers easier to implement. However, there are important methodological considerations, such as validation of antibodies, proper positive and negative controls, pre-staining procedures (fixation, storage temperature and time, epitope retrieval etc.), an appropriate scoring protocol, and standardized procedures [39,310].

Pre-staining conditions are seldom, if ever, reported in biomarker studies. Yet, how the tissue is handled before staining (e.g. delay in fixation and temperature), how long it is fixed, which fixative, how and for how long the tissue blocks are stored can have an impact on the result, and its reproducibility [39,311]. Especially the intensity of tissue staining can be affected in archival material, which is a challenge as many scoring methods are based on the quantity and intensity of the stain [39,310].

Validation and titration of antibodies are necessary steps when using IHC as a method to find new biomarkers [312]. In addition, including the correct positive and negative controls is important [39,310,313]. Validation of an antibody is a process that involves testing whether the antibody is specific for the target protein, selective and reproducible. Even though no common IHC guidelines exist, recommendations have been published, including how to
validate antibodies [312-314]. Western blot (WB) is often used as an initial step in the verification of specificity, and should preferably give one band of the anticipated molecular weight. Lysates of cells overexpressing the epitope or cells that have been stimulated to induce its expression are appropriate positive controls in WB. Likewise, lysates from cells where the expression of the epitope has been knocked-down should be used as a negative control. Testing the antibody on WB secures that it is specific for its antigen in WB, but gives little extra information, and it therefore needs to be tested in IHC. Excellent negative controls in IHC include cells or tissue from knockout mice not expressing the target protein, however such tissues are not always available. Negative controls also include isotype controls, where an irrelevant immunoglobulin of the same type that does not recognise the epitope is used. This will show any unspecific binding of both the isotype primary antibody and the secondary antibody. Tissues and cells known to express the target protein functions well as positive controls, though for new and untested antibodies this may not be known. A good positive control is an internal control, e.g. a cell type within the stained tissue known to express the epitope [313]. A reproducible antibody should give similar results across different antibody lots and time, and similar results should be obtained using another antibody for the same target protein. Another method used to ensure the specificity of the antibody involves pre-absorbing the antibody to its target, often a peptide or a recombinant protein before IHC analysis [312].

In paper I and II, even though the antibodies used were not fully validated, the antibodies were extensively tested, and the specificity was verified using positive and negative controls (tissue/cells expressing or not expressing the target protein), and pre-absorption controls. In paper I, we used a mouse monoclonal anti-uPAR antibody (#3936) purchased from Sekisui (previously American Diagnostica). According to the company datasheet, the antibody was produced using soluble uPAR isolated from phospholipase C treated U937 cell as immunizing antigen and purified from ascites fluid by protein G affinity chromatography. Ferrier et al. have previously tested this antibody for use in IHC [315 2421]. They reported that the antibody did not correctly stain controls, but did not specifying the staining pattern any further. The control that were used was rat tumours established from uPAR-positive human melanoma cells, as positive controls, and cytospins from uPAR-negative human breast carcinoma cells, as negative controls. The antibody was not tested on human tumour tissue.
Enzymatic pronase E treatment and microwave treatment were both tested as methods for antigen retrieval [315]. In contrast, we report in paper I, a satisfactory staining of normal buccal mucosa tissue and tumour tissue. The heat-induced antigen retrieval (HIER) method used in paper I was somewhat similar to that reported by Ferrier et al., however there were differences that may explain discrepancies in our results. We boiled our sections in 10 mM citrate buffer, whereas Ferrier and colleagues used 50 mM. While they used PBS containing 1% bovine serum albumin for antibody dilution, we followed a staining protocol specifically developed by the supplier for the anti-uPAR antibody (#3936, see company datasheet online at http://www.sekisuidiagnostics.com/products/205-murine-mab-against-human-upar). To further optimize the staining protocol and to reduce background staining, a high salt concentration was used in the assay buffers [49]. Although the antibody has not been fully validated using all the suggested steps by Bordeaux et al., both we and others have tested its specificity by various methods. Constantini et al. tested the antibody in IHC on normal breast tissue and breast carcinoma tissue, including negative control where samples were pre-incubated with non-immune mouse serum [316]. Furthermore, the performance of the antibody has been compared with another uPAR antibody (#399), and was found to stain satisfactory [317]. The antibody was also pre-absorbed with recombinant native soluble uPAR [318], or suPAR shed from U937 cells using phospholipase C [316] and used for IHC on tumour tissue, where there was a reduction in staining. We also tested the specificity of the antibody; first by WB on lysates from GD25 cells overexpressing human uPAR and U937 cells stimulated with PMA. In both samples, the antibody only detected a band of the expected size of uPAR. A time-dependent increase in the intensity of the band was observed with PMA stimulation (paper I, figure S1). The band was absent in non-PMA-stimulated U937 cells and in GD25 cells not expressing uPAR (only empty vector). In addition, we performed IHC using our protocol with the antibody pre-absorbed with recombinant human uPAR and found a significantly reduced staining of OSCC tumour tissue [49]. Thus, in our hands, the #3936 antibody performed satisfactory, although further tests are needed to fully validate the antibody.

In paper I we used a scoring protocol that combines the staining intensity (0 = none, 1 = weak, 2 = moderate, 3 = strong) and proportion of cells stained (0 = none, 1 = <10%, 2 = 10-50%, 3 = 51-80%, 4 = >80%). Based on our scoring protocol (value range: 0-12), tumours with
a scoring index (SI) above 5.63 were termed high uPAR expressing tumours. The average SI of this group was 8.22. This equals to a staining intensity of moderate to strong, and a percentage of positively staining cells between 10 and 80%. Illemann et al. reports that less than 1% of tumour cells were positive for uPAR in the tumour core of colorectal cancers, while at the invasive front 5-10% of the tumour cells were uPAR positive [319]. The number of positively stained tumour cells is very different between this study and our study, most likely due to differences between cancer types and scoring protocols.

Publication bias. In 2013 almost 18.000 publications on cancer biomarkers were registered in Pubmed (figure 13), and out of these a little more than 3.000 were on prognostic cancer biomarkers. A common goal of biomarker studies is the finding of markers that can aid clinicians in treatment stratification. In an analysis of 1500 reports on biomarker studies, 95% of the publications reported a statistically significant finding, clearly showing that there is a publication bias [320]. Furthermore, it has been states that as much as 75% of reported findings in biomarker studies are not reproducible [321]. However, only a very small fraction of these biomarkers get implemented into clinical practice [322]. The National Cancer Institute (NCI) reports the use of 31 tumour markers on their homepage (http://www.cancer.gov/cancertopics/factsheet/detection/tumor-markers), where uPA and PAI-1 are established biomarkers for breast cancer to “determine aggressiveness of cancer and guide treatment”. Tumour biomarkers not only states how aggressive the cancer is, but may also help determine the presence or lack of a tumour, guide treatment, predict treatment outcome, and tell us something about the prognosis. The poor reproducibility may partly result from the lack of study transparency. Information about the patient cohort, procedures used, controls included and validation of the antibodies are often lacking. Such a transparency is important to enable critical review and reproducibility of the experimental setup. Consequences of publishing non-reproducible data include waste of time, money and valuable patient material. Guidelines have now been published to aid publicists and researchers in reporting such necessary information [323-325]. The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) checklist was published simultaneously in seven renowned journals in 2005 in order to reach a broad spectrum of readers. The REMARK checklist gives an overview of information that should be included when publishing results on prognostic tumour markers. Another aspect of publication bias is
that journals emphasise on novel and important findings, and shows a lack of interest in publishing negative results or validation of previous studies [39,326]. The space limits of many journals might also push the authors to exclude important information. With open access publishing it may now become easier for authors to adhere to these guidelines with no limit on the amount of information that can be published. Paper I was published in the Public Library of Science (PLoS) ONE, an open access online journal, where we aimed at adhering to the REMARK guidelines.

Figure 13: Publications on cancer biomarkers. The search term “cancer biomarker” was used in Pubmed to find publications on cancer biomarkers. The first publications registered in Pubmed were from 1963. Each bar shows the number of publications per year until 2014. The search term includes both predictive and prognostic biomarkers.

4.1.2 Model systems in cancer research

There are many different model systems used in cancer research. These include different in vitro assays for cultured cells, ex vivo models and in vivo animal models. A selection of model systems relevant to this project are discussed below.

In vitro assays of migration and invasion. Cell migration and invasion takes place during physiological processes such as embryogenesis, wound healing and immune cells trafficking [327], but also during cancer invasion and metastasis [328]. To understand and prevent cancer invasion and metastasis it is necessary to understand the underlying basic principles
involved in migration and invasion. By using \textit{in vitro} migration and invasion assays, it is possible to study the mechanisms involved in more detail. However, the question remains how well the assay resembles real \textit{in vivo} situations. \textit{In vitro} assays are relatively easy to use and results are more reproducible compared to \textit{in vivo} assays. \textit{In vitro} assays are also less costly than animal experiments, and raise less ethical concerns since use of animals in research should be kept to a minimum [329].

Many different assays can be used to analyse migration and invasion \textit{in vitro}, reviewed by Kramer et al. [329]. The transwell migration assay (Boyden chamber) is one of the most frequently used assays to analyse cell migration. Most transwell migration assays involve the removal of cells from the top chamber (e.g. from the top part of a filter), and quantifying the cells that have migrated to the attractant in the bottom chamber. The classical transwell migration assay is therefore an endpoint assay that requires optimization for every cell line used [329]. In paper III, a modernised version of the transwell migration assay was used: The xCELLigence system (ACEA Biosciences. Inc.) performs real-time cell analysis (RTCA) of migration by measurement of impedance created by the cells that reach the microelectrode sensors on the lower side of a microporous membrane. The created impedance gives an arbitrary “cell index” value. There is no need to find a suitable endpoint, only an optimal cell concentration. There is no need to stain cells or remove non-migrated cells. Drawbacks however include difficulty in separating proliferation from migration, and excluding altered morphology and increased adherence from cell proliferation, as all aspects are measured as impedance.

Another assay used to study migration and invasion is vertical gel 3D migration/invasion assays [329]. Cells are then typically seeded on top of a collagen gel interspersed with fibroblasts, mimicking the \textit{in vivo} setting, and it was termed the organotypic skin model [330]. An alternative when studying oral cancer could be the use of a version of this 3D model; an \textit{in vitro} model of the oral mucosa [331,332]. The authors present the model as a good method to study cancer progression that will ultimately reduce the need for animal experiments. The model requires oral fibroblasts and keratinocytes cultured on an artificial matrix (e.g. Matrigel or collagen). Despite being physiologically relevant, these types of organotypic models represent a simplistic view of the tumour microenvironment lacking
several types of stromal cells, vasculature, a fully dysplastic stroma and is somewhat more labour-intensive than the leiomyoma invasion model which will be discussed next [333,334].

In paper II, we have used a model where the in vivo setting is more closely replicated through the use of an ex vivo leiomyoma invasion model [334]. The leiomyoma tissue is of endometrial origin and does not contain any living stromal cells that are known to influence tumour progression. However, the intact tissue contains many of the components known to be present in the tumour microenvironment such as collagens I, III and IV and laminin. Soluble factors present in the tissue have been shown to induce invasion, and the tissue of the leiomyoma mimics the hypoxic tumour microenvironment. Cells otherwise unable to invade the classical collagen gel has been shown to efficiently invade the leiomyoma tissue [334,335]. Taken together, the leiomyoma invasion model proved a valid model to study invasion of the AT84 cells in vitro.

Animal models of oral cancer. Even though no in vivo animal model is perfect for its purpose, it is generally agreed that the use of animals in cancer research is inevitable. Animal models used to study oral cancer include transplanted tumours, chemically induced tumours [336-339], xenograft tumours [340], and spontaneous tumours [341,342]. Chemically induced oral tumours resemble human oral cancer as many human OSCCs are also chemically induced through tobacco and alcohol consumption [7]. The chemically induced hamster cheek pouch model reflects human oral cancer in many aspects, often displaying p53 mutations and activation mutations of Ras [343]. However, inducing oral tumours chemically is labour-intensive, time consuming, requires exposure to carcinogens, and extensive animal handling which is potentially stressful for the animals [344]. Transplanted oral tumours include neoplastic cells injected into either immunosuppressed mice (used for xenografts) or immunocompetent mice (used for syngeneic grafts) [345]. Xenograft models open for the use of human neoplastic cells, however, these tumours seldom metastasise, and the lack of an intact immune system prevents evaluation of the immune cells’ role in tumour progression [344]. Furthermore, factors involved in tumour progression can be species specific, where some of these factors are provided by the host tumour stroma. This may explain why several anti-cancer compounds have shown promising results in xenograft tumours, but been ineffective in humans [345]. In vivo growth of tumour cells in a syngeneic mouse model constitutes a more realistic model, where the immune
system is intact [340,341,346-348]. The AT84 cells used in paper II [180], were derived from a spontaneous OSCC in C3H mice [341]. C3H mice accepted the tumour cells, which grew rapidly into large tongue tumour with as little as 10,000 cells injected [180]. Previous comparison of oral versus subcutaneous tumours using the same model system, revealed that oral tumours grew faster and metastasised to the lung while flank injected tumour cells did not [346]. We did not find any metastasis to lungs, liver or lymph nodes. However, Lou et al. injected tumour cells transcutaneously into the floor of the mouth where intravascular injections may have caused the metastasis, thereby bypassing the normal process of metastasis. We injected tumour cells directly into the tongue, which can explain the discrepancy of the findings. In addition, when using animal models it must be kept in mind that different tissues may influence on the tumour development. Metastasis occurs to a lesser extent when tumour cells are injected into another tissue than its origin. The observation that orthotopic (into the tissue of origin) injection of tumour cells, more often mimics progression of the human cancer better than heterotopic (into another tissue than the cells origin) injections, implies the importance of the tumour microenvironment [340,349,350]. A weakness of models using injected tumour cells is that these poorly reflect the early stages of tumour development. Another way to study the early stages of tumour development, is by the use of transgenic mice [344,351], however to our knowledge, no such model exists for OSCC.
4.2 Discussion of results

In this section, the major issues and highlights from the three papers will be discussed in context to each other and of previously published results within the field.

4.2.1 Prognostic biomarkers

The massive amount of publications on biomarkers (figure 12) signifies the need for better prognostic and predictive markers, allowing for more personalized treatment. Whereas the traditional view has been “one drug fits all”, the view now is that each patient should receive the optimal treatment through personalized medicine [297]. In light of this, the use of tumour biomarkers becomes important, especially to enable stratification of patients that will respond differently to the same treatment, even though they are diagnosed with tumours of the same TNM-stage [10]. The goal of paper I was therefore to find biomarkers that could aid clinicians in their decision-making regarding the extent of treatment for patients with T1N0 tumours.

Several studies on tumour biomarkers using IHC have been performed on OSCC, where EGFR, p53 and ki67 have been repeatedly suggested as potential prognostic markers together with many more [25,27,48]. However, a recent critical review has deemed EGFR, p53 and Ki67 as poor prognostic markers for OSCC [39]. This is in line with our findings, where Ki67 did not correlate with survival [49]. The need for new biomarkers is reflected by the ever-increasing mass of publications on the topic (figure 12). It was therefore our aim to determine whether factors of the PA system could be used as prognostic biomarkers for OSCC using IHC. Interestingly, p53 which is often mutated in OSCC, is a negative regulator of uPAR expression [352]. This suggests that uPAR may be up-regulated in OSCC, making uPAR an potential prognostic biomarker in OSCC.

Due to the PA systems role in tissue remodelling and wound healing, and its overexpression in many cancers [181,292,319,353,354], it has even been proposed as a target for cancer therapy [185]. In three separate studies, high levels of uPA and PAI-1 in primary tumours of the breast were significantly associated with increased risk of recurrence and worse survival [154,355,356]. These findings were later tested in a large study including 3424 breast cancer patients, where those expressing both uPA and PAI-1, where defined as high-risk patients and were found to benefit from adjuvant treatment [357]. uPA and PAI-1 are now approved
as prognostic markers for breast cancer. In OSCC, less is known about the role of the PA system, but it is thought to be important for invasion [264]. Previously published papers show that several of the components of the PA system are up-regulated in OSCC tumour tissue compared to normal tissue [53,55]. Elevated expression of uPA and PAI-1 was correlated to poor overall survival, though no reports were made on the disease specific survival [55]. The analyses were performed using ELISA on whole tissue samples, and distribution and expression patterns were therefore unknown. Hence, the expression of uPA, uPAR, PAI-1 and PAI-2 could originate from infiltrating stromal cells. Others have reported on the IHC staining pattern for uPAR, uPA, PAI-1 and PAI-2 in OSCC, where uPAR and PAI-1 expression has been reported at the tumour invasive front [52,54,183,280,281]. uPAR and uPA were furthermore reported to correlate with a more invasive behaviour [52,280], and worse overall survival [280].

Expression of uPAR may be up-regulated in either the cancer cells or the stromal cells (table 2), and often tumours show heavy infiltration of inflammatory cells and myofibroblasts [186,319,358-362]. Previous reports say that most of uPAR positivity in OSCC is found in stromal cells such as macrophages, fibroblasts and neutrophils [183,281], although more recent publications have reported that uPAR is expressed in tumour cells at the tumour core of oesophageal adenocarcinomas and colorectal cancer, and that the expression was associated with prognosis [319,354]. We report that low expression of uPAR and PAI-1 in cancer cells of early stage OSCC (T1N0) predicts lower disease specific death [49], which indicates that uPAR may play a role in early events of OSCC progression. This is in line with results obtained by Lindberg et al., who concluded that expression of uPAR and PAI-1 were indicative of early invasion [281]. The number of patients belonging to the T1N0 group in our study was small (N=27). Based on this, we present uPAR and PAI-1 as possible biomarkers, but further work on a larger patient cohort must be performed in order to strengthen these results. As already mentioned above, uPAR staining has been reported at the invasive front of OSCC [183,281], while we report in paper I that most of the uPAR staining was towards the centre of the tumour islands [49]. This might reflect the use of TMA as a method, as the cores do not necessarily represent the tumour invasive front. Whole tissue sections will in the future have to be stained to analyse the distribution of uPAR staining in the tumours.
Taken together, the results reported in paper I are in line with previous reports, indicating that uPAR and PAI-1 are involved in tumour progression of OSCC.

As has been reported for other cancers, soluble uPAR forms may be detected by ELISA based methods instead of IHC [283,288,289]. This allows for use of blood/plasma and urine as samples for detection of biomarkers. Collection of such samples can be done with little effort, and hospitals and clinics are already well equipped to collect and handle these samples. Collecting blood and urine samples are in addition non-invasive for the patient. The development of an ELISA based method to measure the presence of suPAR in blood showed that patients with either breast-, colon- or ovarian cancer had elevated suPAR levels compared to healthy individuals [285,293]. It was suggested as a method to assess prognosis and detect recurrence. Increased pericellular proteolytic activity of the tumour cells and/or stromal cells leads to shedding and cleavage of uPAR. Measuring uPAR fragments in blood/plasma may therefore reflect aggressive tumour behaviour, and maybe even angiogenesis as the fragments are shed from the tumour and into the bloodstream. High levels of suPAR and cleaved uPAR in blood/plasma has now been correlated to prognosis for several types of cancer: breast cancer [286], non-small lung cancer [288], small cell lung cancer [290], prostate cancer [289] colorectal cancer [292]. Increased levels of suPAR have also been detected in plasma from patients with HNSCC, although due to the short period of follow-up of the patients, no association with prognosis was determined [279]. ELISA-based techniques are quantitative, and the need for subjective assessment by a pathologist is not necessary. However, no information about the tissue complexity is gained using ELISA-based methods. The use of an ELISA-based method for our cohort of OSCC patients was not possible, as no blood and urine samples from these patients exist.

4.2.3 The tumour microenvironment

In paper II, we report that several different types of tumour microenvironments are involved in the up-regulation of uPAR expression [180]. Others have previously reported similar findings, where uPAR was shown to be expressed at the tumour invasive front of human colon adenocarcinomas [362], colorectal cancer [319], oesophageal adenocarcinoma [354], and OSCC [281]. In our mouse model for OSCC, uPAR was up-regulated at the tumour-stroma interface of tongue tumours in cells with initially low endogenous uPAR expression (paper II). The stroma of the skin also induced expression of uPAR, when cells were injected
subcutaneously. To counteract the endogenous up-regulation, cells were transfected with shRNA targeted for Plaur mRNA. Cells stably expressing the Plaur-targeting shRNA were then used to develop new tongue tumours, which showed reduced levels of uPAR (paper II, figure 4a and e). However, when these uPAR knock-down cells were invading the neoplastic tissue of the leiomyoma, uPAR expression was up-regulated already after 7 days in the cells invading deep into the tissue (paper II, figure 5). The leiomyoma tissue, which is a neoplastic but non-malignant tissue, shares many of the traits of the tumour microenvironment [334]. Furthermore, the leiomyoma tissue was shown to induce secretion of MMP-2 and MMP-9 in the invading OSCC cells, HSC-3 [334]. The leiomyoma also mimics the hypoxic tumour microenvironment, inducing invasion. It was furthermore shown that soluble factors from the leiomyoma tissue were important for invasion [335]. This shows that different tissues can modulate the expression of uPAR in different ways.

Interestingly, leiomyoma tumours express TGF-β1 [363], and we reported that factors present in the leiomyoma conditioned medium (LCM) increased expression and glycosylation of uPAR, and possibly effected the cleavage (paper II and III). In paper III, we aimed at investigating whether TGF-β1 was involved in regulating uPAR expression, glycosylation and cleavage. By stimulating cultured AT84-uPAR cells with TGF-β1, the amount of full-length uPAR was increased through increased expression of PAI-1 (paper III). It is however uncertain whether TGF-β1 had an effect on the glycosylation of uPAR (paper III). Others have reported that TGF-β1 induces uPAR expression in cell culture [188]. Yet, TGF-β1 did not induce a statistically significant increase of the Plaur mRNA levels in AT84-EV and AT84-uPAR cells after 24 hours of stimulation in culture (paper III). Further research must be done to map whether TGF-β1 is involved in the increased expression of uPAR in the mouse tongue and skin tumours, or whether other stromal derived factors are the central players. Skin and tongue tumours did not display a desmoplastic stromal environment, which is one of the features of carcinomas overexpressing TGF-β [72,364,365]. However, several cell types that are often present in the tumour microenvironment produce TGF-β, such as fibroblasts, platelets, macrophages, lymphocytes, (epithelial cells) and tumour cells [69,72,89,366]. As described in the introduction, TGF-β1 is secreted in a latent form that is sequestered in the ECM [76]. There are many known activators of TGF-β such as integrins (αVβ6, αVβ8) and proteases (plasmin, MMP-2, MMP-9, and BMP1) together with several more factors nicely
summarized by Robertson and Rifkin [76-78]. Through activation, TGF-β is released from the ECM (see figure 11), and can convey its functions through binding to its cell surface receptor(s). When uPAR is expressed, the cells can activate uPA and hence plasmin, and plasmin can activate and release TGF-β from the ECM. The EV1, EV2, uPAR1 and uPAR2 cells all secreted HMW-uPA, and even though they were borderline negative for plasmin(ogen) (see figure S1 in paper II), plasminogen may be supplied through the bloodstream to the tongue- and skin tumours (paper II). We do not yet know whether the AT84 cells produce TGF-β1, or whether stromal cells in the tongue or skin expressed it. Interestingly, TGF-β1 induces expression of PAI-1 in many cells including AT84 cells [367-369]. We observed that high levels of PAI-1 and uPAR in early stage human OSCC was associated with increased disease specific death. In paper III, we observed that cells expressing high levels of uPAR also secreted increased levels of PAI-1, compared to AT84-EV cells (paper III, figure 4A), and stimulating cells with TGF-β1 increased the levels further. It has however not been determined whether TGF-β1 is also involved in increasing PAI-1 or uPAR expression in our North Norwegian cohort of OSCC patients. One Brazilian study analysed 72 HNSCC tumours [370]. The results were not correlated to survival, but elevated levels of TGF-β1, uPA and PAI-1 mRNA was found in oral cavity tumours compared to normal oral mucosa. Interestingly, they found a small subgroup of pN0 patients that showed elevated levels of TGF-β1 [370].

Another interesting observation was the fact that together with increased uPAR expression, cells with up-regulated uPAR showed increased activity of gelatinolytic enzymes (paper II, figure 7, 8 and S8). In addition, when uPAR was knocked down using shRNA, the gelatinolytic activity was reduced in tongue tumours (paper II, figure 7). Plasmin can degrade gelatin and has the ability to activate several different types of MMPs [225]. As seen when using in situ zymography [371], the gelatinolytic activity is not only present on the cell surface, but is also intracellular. The activity seen in the invading EV and uPAR cells (see paper II, figure 8) could originate from intracellular proteases or recycled proteases [372]. More recently, MMPs have been shown to have functions also intracellularly [373, 374]. As was shown in figure 8 in paper II, the cells invading deep into the leiomyoma tissue, with up-regulated uPAR expression (EV1-sh), did not show reduced gelatinolytic activity in the presence of the MMP-inhibitor EDTA. This shows that MMPs were not responsible for the gelatinolytic activity
seen in these cells. The increased activity could also originate from serine proteases such as plasmin, elastase and cathepsins that are located either pericellular or in the extracellular environment. Taken together, either cells invading deep into the leiomyoma tissue with increased uPAR expression, or cells overexpressing uPAR showed increased gelatinolytic activity. This shows that either stromal induced expression or overexpression of uPAR increases the cells ability to activate gelatinolytic enzymes.

Increased proteolytic activity potentially enhances the invasive and metastatic ability of the tumour cells. Interestingly, TGF-β increased the amount of full-length uPAR on the AT84-uPAR cells (paper III, figure 3). It is tempting to speculate that increased amount of full-length uPAR gives the cells increased ability to activate proteolytic enzymes and signal through its partners. At the same time, an increase in PAI-1 expression was observed when cells were treated with TGF-β1 (paper III, figure 4). PAI-1 induces turnover of the uPAR/uPA/PAI-1/LRP1 (+/-integrins) complex, and has been shown to increase detachment of cells from the matrix protein vitronectin [250,375]. Through the cycles of attachment and detachment to the ECM; PAI-1 regulates migration [160,252,376,377]. Increased uPAR levels (and more full-length uPAR vs cleaved uPAR), could potentially increase the invasive and metastatic abilities of the cancer cells and thus induce tumour progression [225,378]. Nevertheless, in the syngeneic mouse model for OSCC used in paper II, we found no metastasis to lymph nodes, livers, lungs or mandibles from neither high- nor low uPAR expressing tumours. None of the tumours displayed an infiltrative growth pattern, but were instead rounded with a pushing-boarder type growth pattern (paper II, figure 2c and d, figure 4d). Even with as little as 10,000 cells injected, the mice were euthanized at day 14 due to rapid tumour growth, thus maybe not allowing the time needed for metastasis to establish. Others have also suggested that the expression of uPAR is associated with the early events of tumour development, and suggested it as a marker for onset of invasion. Publications on premalignant lesions of oesophageal carcinomas showed that these lesions were uPAR-negative until early stromal invasion occurred [354]. Similarly, Lindberg et al. concluded that increased expression of uPAR was as a sign of early invasion in OSCC [281]. Although the AT84 tumours in the C3H mouse model did not show aggressive behaviour, the tumours showed increased uPAR expression at the tumour-stroma interface; that might mark the transition to a more malignant tumour. The induced uPAR expression of the
tongue tumours, and in cells invading the leiomyoma tissue (paper II), could therefore reflect the T1N0-group of patients observed in paper I. The T1N0-group with high uPAR expression was associated with higher disease specific death than the in the group with low uPAR expression (paper I).

The levels of cleaved uPAR vs. full-length uPAR in the tumour tissue has not been determined. The distribution of the uPAR forms on the cells, and within the tumour would have been interesting to analyse. However, to the best of my knowledge, no antibody exists that can recognise uPAR (II-III) without also recognising full-length uPAR. To what extent uPAR is shedded from the tumour cells is also of interest. As reported, many cancers shed both suPAR and suPAR (II-III) into the blood stream [286,287]. SuPAR (II-III), harbouring the chemotactic peptide, is reported to be involved in homing of hematopoietic stem cells (HSC) from the bone marrow [379]. This uPAR-peptide is also involved in basophil chemotaxis [232] and monocyte-macrophage recruitment during inflammation [233,380]. The amount of infiltrating stromal cells was not evaluated in the EV and uPAR-expressing tumours (paper II). Using the C3H mouse model to study immune cell infiltration and the role of these cells in tumour progression would be beneficial; as the C3H mouse has an intact immune system.
5. Conclusions and future perspectives

Our findings provide support for the involvement of the PA system in the progression of OSCC. Low expression of uPAR and PAI-1 in early stage OSCC was associated with low disease specific death, suggesting that PAI-1 and uPAR play a role in the early stages of OSCC progression. Thus, PAI-1 and uPAR were proposed as possible prognostic markers for patients with T1N0 tumours. Furthermore, in vivo studies indicated a stromal involvement in the induction of uPAR expression, which simultaneously enhanced the activity of gelatinolytic enzymes, processes thought to be important for invasion and metastasis. However, in the in vivo syngeneic mouse model that was used, cells with high levels of uPAR did not show signs of aggressive tumour growth and metastasis. Tumour growth patterns resembled more that of early stage OSCC. Nevertheless, soluble factors from the neoplastic tissue of the leiomyoma was shown to increase expression, alter glycosylation and cleavage of uPAR. Finally, the stromal associated factor TGF-β1 was found to reduce uPAR cleavage, resulting in the presence of more full-length uPAR on mouse OSCC cells, through increased expression of PAI-1. The amount of full-length uPAR versus cleaved uPAR on the cells was shown to alter cell signalling through ERK phosphorylation, and uPA-mediated cleavage of uPAR was shown to induce migration and invasion.

Further studies are needed to unravel the specific role of uPAR and PAI-1 in the early events of OSCC development and to establish the role of TGF-β1 in this process. TGF-β1 clearly has role in the regulation of uPAR and PAI-1 through expression and control of uPAR cleavage in the AT84 cells. Whether this hold true for human OSCC cells needs to be established. Through the use of an in vitro model of the oral mucosa more insight may be gained into the role of uPAR, PAI-1 and TGF-β1 in the early events in OSCC development.

Tongue- and skin tumours and leiomyoma invading cells showed a stromal induced up-regulated uPAR expression. Simultaneously, an increase in activity of gelatinolytic enzymes was seen. It was however not assessed whether TGF-β1 and PAI-1 where present in the tumour tissues. Future research should be aimed at gaining insight into how also these factors are involved in regulating proteolytic activity in the tumours. Furthermore,
gelatinolytic enzymes are a large group of proteolytic proteins, and the objective is to map which proteolytic enzymes are involved.

The tumour microenvironment is often infiltrated with a diverse mix of stromal cells, including fibroblasts and immune cells. It is now recognised that such cells are involved in regulating the progression of tumour growth in many cancer types. The inherent chemotactic peptide located within uPAR is an attractant for immune cells through its interaction with the fMLF-receptors (FPR, FPRL1 and FPRL2). With this in mind, could uPAR function as a “calling signal” for certain immune cells, attracting these to the tumour tissue? In this aspect, regulation of uPAR cleavage becomes important. Does TGF-β1, or other stromal factors, regulate uPAR cleavage and thereby its many functions in human tumours? If not, how is cleavage regulated? The syngeneic mouse model we have used will enable insight into these aspects as it has an intact immune system.

The ultimate goal is to enable better treatment, or even curing patients with OSCC. It therefore becomes important to find a practical target for therapy, such as inhibiting certain functions of a protein. Understanding all aspects of how a therapy-targeted protein functions is vital. Treatment targeted for the function of a protein should preferably give little of no side-effects. If certain aspects of how a protein works is unknown, treatment targeted for this protein becomes a risk. uPAR is a protein with a broad spectrum of functions from proliferation, dormancy, migration, adhesion and invasion. uPAR targeted therapy then seems risky. However, uPAR knock-out mice are viable and fertile indicating that uPAR is not essential for life, and may therefore present an interesting target for therapy.
6. References


References


309. Jones S, Prasad ML (2012) Comparative evaluation of high-throughput small-core (0.6-mm) and large-core (2-mm) thyroid tissue microarray: is larger better? Arch Pathol Lab Med 136: 199-203.


