New molecular biomarker discovery for diagnosis and prognosis in oral and oropharyngeal cancer

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CHAPTER 1

General introduction

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General introduction

Head-neck cancer

Head-neck cancer is a broad term referring to the heterogeneous group of malignant neoplasms arising in the head-neck region. This would include rare tumours such as orbital and ear tumours and more frequent tumours such as thyroid and skin tumours. Commonly however, the term head-neck cancer refers to cancers of the upper aerodigestive tract, which arise from the epithelial lining of the oral cavity, pharynx and larynx. Over 95% of these tumours are squamous cell carcinomas (head-neck squamous cell carcinomas: HNSCC)[1]. These tumours largely have a common aetiology. Up to 75% of HNSCC are caused by use of tobacco with or without drinking alcohol[2], with the remainder of cases being attributed to dietary factors[3], infection with the human papilloma virus (HPV)[4] or considered idiopathic. Tobacco and alcohol use (termed 'classical risk factors') cause damage to the total aerodigestive tract in a pathological process called 'field cancerization'[5]. This process accounts for the high rate (~17%) of second primary tumours in HNSCC[6]. This overview focuses on two subgroups of HNSCC (figure 1.1): the tumours arising in the oral cavity (oral squamous cell carcinomas, OSCC) and the tumours arising in the oral part of the pharynx (oropharyngeal squamous cell carcinomas, OpSCC).

Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is the most common tumour of the upper aerodigestive tract, with an estimated worldwide annual incidence of 265,000 cases[7]. In recent years ~1,000 cases were diagnosed annually in the Netherlands[8]. The incidence has been increasing slowly but steadily over the last two decades (figure 1.2). The 1.6:1 male predominance reflects the distribution of smoking between both sexes (table 1.1)[9]. Although the incidence of OSCC in the Netherlands approaches the average worldwide incidence, internationally incidence varies widely. In countries such as India and Pakistan oral cancer may contribute up to 25% of all new cancer cases[10], mostly because of the widespread use of chewing tobacco in various forms.
OSCC comprises tumours of the floor-of-mouth, anterior tongue, retromolar trigone, and gingiva. Most patients present to the dentist or general practitioner with a painful ulcer or a swelling[11]. Pre-malignant conditions such as leukoplakia may also be present in up to 50% of patients[12].
For the local treatment of the primary OSCC, according to several national and international treatment guidelines, surgery is generally preferred[13-15]. For certain early-stage OSCC, radiotherapy alone may result in comparable local control. Surgery however, has the additional benefit of obtaining tumour tissue and subsequently being able to perform histopathological examination of the tumour. Tumour features, such as size, infiltration depth, grade, perineural- & lymphovascular invasion may then be assessed, as well as the presence of tumour cells in the resection margins (radicality of resection). When several adverse features are present adjuvant treatment, such as re-resection, radiation or chemoradiation therapy may be given[13-15]. When optimal local treatment is provided, the two most important factors for regional and adjuvant treatment and prognosis, are patient age and the presence of regional metastases, with the primary tumour characteristics of grade and T status being of less importance[16].

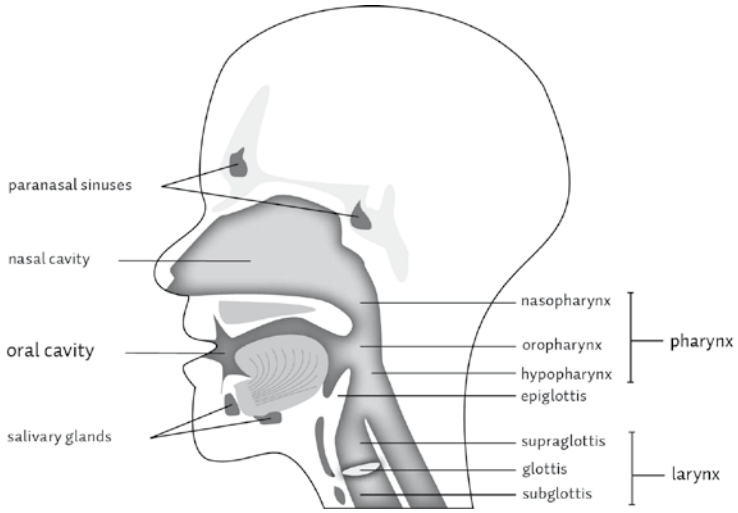

Figure 1.1. Common tumour localizations in the head-neck area.
Adapted from Gibcus, 2008, with permission.

Oropharyngeal squamous cell carcinoma

Oropharyngeal squamous cell carcinoma (OpSCC) occurs with an estimated worldwide annual incidence of 136,000 cases[7], approximately half of the OSCC incidence (table 1.1 and figure 1.2). Although the classical risk factors also seemed to apply to OpSCC, the main risk factors for OpSCC have changed over the last decade. Currently there is a lot of attention for the human papilloma virus (HPV) as a causative factor for the development of OpSCC[4]. Studies report 40-70% of OpSCC positive for high-risk (oncogenic) HPV types. This percentage varies heavily between different populations, tumour sublocalizations and study periods[17]. Because HPV DNA is prevalent in the oral cavity of at least 7% of healthy individuals at any given time[18], a combination of molecular tests is needed to detect only cases with active HPV infection in the tumour tissue[19,20]. Although HPV-positive tumours respond significantly better to therapy, to date clinical management for this subgroup has not changed[21]. Therefore, the most important factor in treatment choice and prognosis in both HPV-positive and HPV-negative OpSCC, as in OSCC, is the presence of metastases in the lymph nodes of the neck[22].

Assessment of the nodal status

Oral and oropharyngeal squamous cell carcinomas (OOSCC) metastasize largely according to anatomical patterns to the draining lymph nodes in the neck (figure 1.3). When all OOSCC are considered, these regional metastases occur in ~50% of all patients[23-25]. Regional metastases significantly affect survival; 5-year survival for patients with a localized OOSCC is 60-75%, however when suffering from a regionally metastasized tumour this rate drops to 40-55%[23,26].
Patients presenting with distant metastasis of OOSCC are rare, occurring in ~2% of cases[27]. Pa-

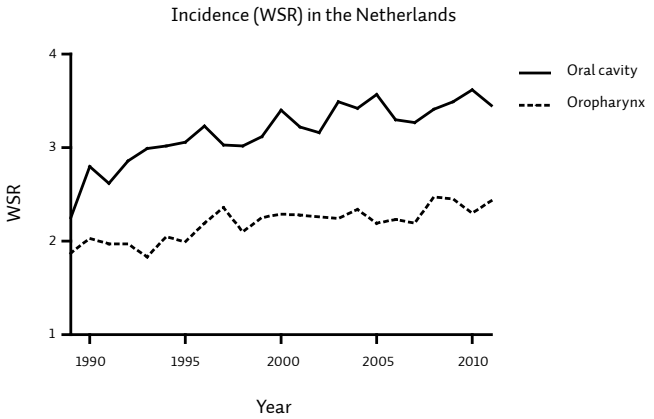

Figure 1.2. Incidence of oral cavity and oropharyngeal tumours in the Netherlands 1989-2011.
WSR: world standardized rate. Graph based on data from the Dutch cancer Registry[8].

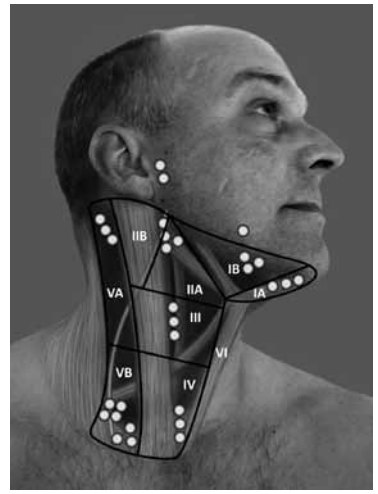

Figure 1.3. Lymph nodes and levels of the neck.
Adapted from Stegenga et al., 2013[106], with permission.

tients with distant metastasis from OOSCC are considered incurable[28]. Patients with regional metastasis are considered curable, and adequate diagnosis of the presence of lymph node metastases in the neck is of the utmost importance to determine an adequate treatment strategy.
The clinical assessment of nodal status is summarized in the cN status (table 1.2)[29]. Current techniques to assess the clinical nodal status in patients with OOSCC include palpation, imaging with CT, MRI or ultrasound, and ultrasound-guided fine needle cytology. Palpation (manual examination of the neck area for enlarged lymph nodes; figure 1.3), even by experienced head-neck surgeons has low sensitivity (70%) and specificity (60%)[30]. Although most modern imaging modalities have better specificity of 80-90%, mean sensitivity of CT, MRI, PET and ultrasound is generally still 60-70%[31], mainly due to inability to detect metastases < 3 mm in diameter, termed micrometastases[32]. >60% of the metastases occurring in HNSCC are micrometastases[33]. In ~16% of metastasized tumours a micrometastasis is the only metastasis present[34], therefore great efforts have been invested in improving the detection of metastases in general and micrometastases in particular.
Sentinel lymph node biopsy (SNB) is a technique in which the surgeon tries to identify the first draining lymph node(s) of a tumour, in which metastatic cells should occur first. Depending on the lymph drainage pattern one or several sentinel lymph nodes are detected, excised, and examined peroperatively. Only when metastatic cells are present in the sentinel node, treatment for the neck is recommended. Although commonly used for breast cancer and melanoma, the value of SNB in OOSCC is still being evaluated[35]. SNB in OOSCC has a reported sensitivity of up to 95% in the research setting, when using serial sectioning and immunohistochemistry to detect metastatic tumour cells in the sentinel lymph node[35]. However, these adjuvant histopathological techniques are not possible when performing intra-operative frozen-section

SNB in the clinical setting, to aid the head-neck surgeon during the operation in the decision to treat the neck. In this case, in the most relevant group of T1-2cNo tumours, sensitivity rates are comparable to most imaging modalities: 50-70%[36,37]. Moreover, in ~10% of cases SNB cannot be performed because no sentinel node is identified[38].
Current assessment of the nodal status in OOSCC patients, even when several techniques are combined is still imperfect.

The problem

There is general agreement that patients diagnosed with lymph node metastases (cN+) should be treated by removing all lymph nodes (level I-V, figure 1.3) from the affected side of the neck with postoperative radiotherapy in case of multiple or large metastases and chemo-radiation in case of irradical surgery or extranodular spread[13-15]. However, upon histopathological examination of the dissected lymph nodes by the pathologist (resulting in a pathological pN stage; table 1.2), in 10 to 20% of the patients diagnosed cN+, no lymph node metastases are found, referred to as pNo[33].
Conversely, when patients are diagnosed without clinical lymph node metastases in the neck (cNo), there is still a chance of 25-50% for the presence of occult metastases, because of imperfect diagnostic tools[39-41]. Occult metastases are missed during palpation of the neck and their size is below the detection levels of imaging techniques. Therefore, when the chance for occult metastases is thought to be greater than 20% (based mainly on T stage and localization of the primary tumour), an elective (supra-omohyoidal) neck dissection is performed[42], to acquire a pathological staging of the neck (figure 1.4). Of these patients 70-80% do not show lymph node

Table 1.1. Estimated annual incidence data of head neck tumours worldwide in 2008.

	Men				Women				Men & Women			
	WW		NL		WW		NL		WW		NL	
Tumour location	**N**	**WSR**	**N**	**WSR**	**N**	**WSR**	**N**	**WSR**	**N**	**WSR**	**N**	**WSR**
Oral cavity+lip	170496	5.2	689	5.2	92524	2.5	466	3.1	263020	3.8	1155	4.1
Oropharynx	108588	3.4	477	3.7	28034	0.8	164	1.3	136622	2.0	641	2.5
Nasopharynx	57852	1.7	101	0.7	26589	0.8	76	0.5	84441	1.2	177	0.6
Larynx	129651	4.1	608	4.4	21026	0.6	117	0.8	150677	2.2	725	2.6
Total	**466587**		**1875**		**168173**		**823**		**634760**		**2698**	

WSR: world standardized rate; WW: worldwide; NL: Netherlands. Table based on data from international and national cancer registries[8,107].

metastases (pNo) and received an unnecessary elective neck dissection, with associated shoulder and neck morbidity[43,44]. Of the patients considered to be low risk (<20%) for occult metastases, and consequently do not receive an elective neck dissection, 25-30% develop clinically detectable metastases during follow-up[45,46]. Unfortunately salvage surgery for regional recurrence is not feasible in more than a third of cases because of extent of the recurrence or performance status of the patient[47]. When performed, salvage surgery has a high complication rate of ~30% and disappointing outcomes, with survival rates of only 6-25%[47-49]. Costs for salvage surgery for regional recurrence have been estimated at $87-94,000 in the US[48]. For comparison, costs for the treatment of a local recurrence are less than half of that[48]. Therefore, management of the clinically negative neck in patients with OOSCC is a major dilemma in head-neck oncology.

Biomarkers for the prediction of nodal status

A tumour biomarker is an indicator of a clinical state, and may correlate to the biological behaviour of a tumour. Based on the hypothesis that presence or absence of certain characteristics in the primary tumour confers the ability to metastasize, many biomarkers have been studied for their association with the nodal status in OOSCC. Certain histological biomarkers such as T status[34], perineural invasion[34,50], lymph-angioinvasion[34,51], depth of invasion[34,52,53], degree of differentiation[34,53,54] and pattern of invasion[34,51,53,54] are reported to be associated with the presence of nodal metastasis for several decades. These biomarkers generally have not been analyzed by rigorous statistical methods, to determine Odds Ratios and independent predictive values[34,50,52-54]. Moreover, assessment of histopathological biomarkers, usually combined in grading systems, may show large inter- and intra-observer variability when reported[55-57]. Therefore, there is a need for objective biomarkers with an independent predictive value for the nodal status. Despite their limitations and because of a lack of better biomarkers, assessment of histopathological biomarkers by a head-neck pathologist has become standard procedure[13,14], and

Table 1.2. TNM classification for carcinomas of the oral cavity and oropharynx.

T stage	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour 2 cm or less in greatest diameter
T2	Tumour more than 2 cm but not more than 4 cm in greatest diameter
T3	Tumour more than 4 cm in greatest diameter
T4a (oral cavity)	Tumour invades through cortical bone, into deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face
T4a (oropharynx)	Tumour invades any of the following: larynx, deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), medial pterygoid, hard palate, and mandible
T4b (oral cavity)	Tumour invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery
T4b (oropharynx)	Tumour invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base, or encases the carotid artery
N stage	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
M stage	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

According to the International Agency for Research on Cancer, 2008[29].

some have become criteria for giving adjuvant radiotherapy or chemoradiation[13,14,58] ('adverse features'; figure 1.4).

Immunohistochemical studies of protein expression levels in tumours have yielded many candidate protein biomarkers with associations with nodal status (table 1.3; reviewed by Takes et al.[59] and Walk & Weed[60]). However these studies are not easy to compare, because of inclusion of differing tumour localizations and stages, outcome parameters, antibodies, staining evaluation systems et cetera[61]. Moreover, predictive values of these markers are generally poor[62].

Table 1.3. Biomarkers associated with nodal metastasis in HNSCC.

Cell cycle	Cyclins
	EGFR
	p53
	p21
	Ki-67
	Bcl-2
	Survivin
	H/K/N-Ras
	Sigma
	CEP55
	NBS1
Apoptosis	FADD
	Caspases
	RSK2
Cell adhesion	CD44
	Syndecan-1
	E-selectin
	E-cadherin
	EpCAM
	Claudins
	FAK
	α-catenin
	β-catenin
	Snail
	RhoC
	Connexins
	Twist
Invasion	MMP-2, -3, -9 & -14
	TIMPs
	Cortactin
	Maspin
	c-met
	HGF
Angiogenesis	HIF-1 alpha
	CA IX
	VEGFA-D
Other processes	CXCR4
	CCR7
	NFκB

Updated table from Takes et al., 2008[59].

One reason for the poor predictive power of single biomarkers is that metastasis is a multistep process, involving loss of cell-cell adhesion, lysis of extracellular matrix, cell motility, invasion in the lymph vessel, extravasation at the site of the future metastasis, and upregulation of cell-cell adhesion, amongst others, to develop into a metastatic tumour[63,64]. Many proteins have to cooperate in a concerted mechanism to result in only one step of the process and finally result in metastatic lymph nodes. Indeed, microarray expression studies revealed specific gene sets, composed of 46 to 500 genes[65,66] that are significantly differentially expressed in metastasized tumours ('metastatic signatures'). However, when comparing various microarray studies the composition of these signatures varies enormously and only a small number of genes are reported in multiple signatures[66,67]. When validation of a signature is performed, predictive values are disappointing, with an accuracy of 62%, compared to 80% for current clinical assessment[68]. Thus, also the combination of a large number of genetic markers does not result in a clinically relevant predictive test probably because of inter-tumour heterogeneity on the genetic level.

Other clues that combinations of specific markers might have the best association with the multistep process of metastasis come from biological studies. An example is the role of the epithelial cell adhesion molecule (EpCAM) in metastasis. EpCAM is a cell-cell adhesion molecule, and as such has been assessed for associations with lymph node metastases in HNSCC in several studies[69-71]. It may abrogate E-cadherin induced cell-cell adhesion[72]. In biologic studies it was found that EpCAM associates with claudin-7 in a complex[73], which blocks EpCAM's adhesive functions, and increases cell migration in scratch and transwell assays[73]. This interaction might explain the lack of association with lymph node metastases when EpCAM expression is assessed as single tumour biomarker in HNSCC. Analyzing the co-expression of EpCAM, E-cadherin and claudin-7 might provide a better predictor. Such studies have not yet been performed in HNSCC.

In addition to studying protein or mRNA expression levels to find tumour biomarkers for the prediction of nodal status in OOSCC, DNA methylation might be a mechanism to identify such markers. DNA hypermethylation is one of the most important mechanisms for the regulation of gene expression, both in physiological and in pathological conditions[74]. In contrast to DNA mutations, which result in definitive changes in DNA sequence, DNA methylation is a form of epigenetic regulation, where the genetic sequence is not altered, but methyl groups are added to CG dinucleotides present in the promoter region of a gene, leading to transcriptional repression and decreased expression of the associated protein[75]. DNA methylation is reversible, and hypomethylation leads to reactivation of gene transcription and increased expression of the associated protein[75]. Because of its dynamic nature, methylation is a promising candidate mechanism for the dynamic gene regulation during the multistep metastatic progression of OOSCC cells[76]. Although different cancer types are epigenetically diverse[77], currently only a relatively small set of frequently methylated genes in OOSCC are known, most not specific for this type of cancer. Only a few of these methylation markers have occasionally been associated with nodal status on relatively small patient groups[78,79]. Therefore, identification of new methylation markers in OOSCC is needed.

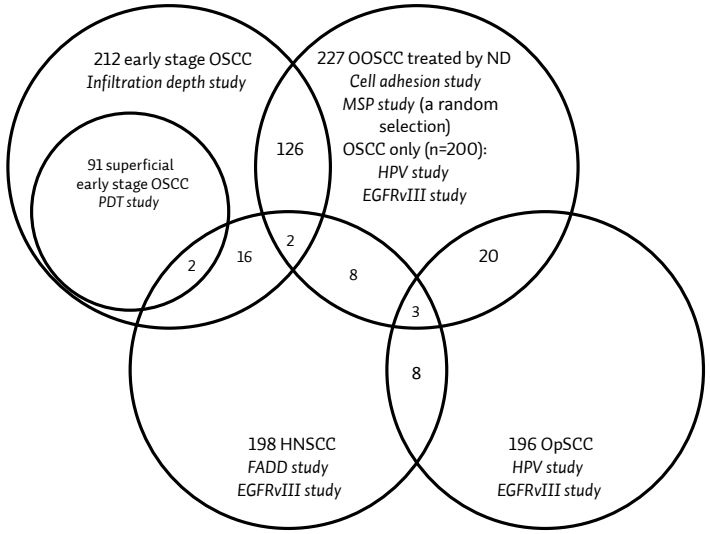

Figure 1.5. Venn diagram of patient series used in this project.
OSCC: oral squamous cell carcinomas; OpSCC: oropharyngeal squamous cell carcinomas; OOSCC: oral & oropharyngeal squamous cell carcinomas; HNSCC: head-neck squamous cell carcinomas.

Biomarkers for the prediction of treatment response

Currently, the head-neck oncologist has several possible treatment modalities available such as surgery, radiotherapy, chemotherapy, photodynamic therapy (a minimally invasive therapy where a photosensitizer is intravenously injected and activated locally by illuminating the tumour at a specific wavelength) and biologics (targeted therapy). To be able to give (combination) therapy that is optimally suited to a specific patient and a specific tumour, it is important to identify biomarkers for the prediction of treatment response. These prognostic biomarkers are assessed for their ability to predict the disease-specific and disease-free survival and may identify patients most likely to benefit from specific adjuvant therapy[80].

There is a certain overlap between markers that predict nodal status en those that predict treatment response. Markers like cyclin D1[81] and p53[82] have been associated with nodal metastasis in OOSCC. However, adequate multiple regression analysis reveals that these markers are not independent when including primary tumour characteristics such as differentiation of growth pattern[83]. Association of these markers with nodal status is based on their role in the cell cycle resulting in a more aggressive tumour phenotype, but not based on a direct effect on the metastatic process. Conversely, the presence of lymph node metastasis also has an important effect on disease-specific survival[16]. These possible interactions should be taken into account when analyzing biomarkers for the prediction of treatment response.

HPV can be regarded as a biomarker for treatment response in OOSCC[22]. HPV-positive tumours develop metastases at rates comparable to HPV-negative tumours[22,84], but have a significantly

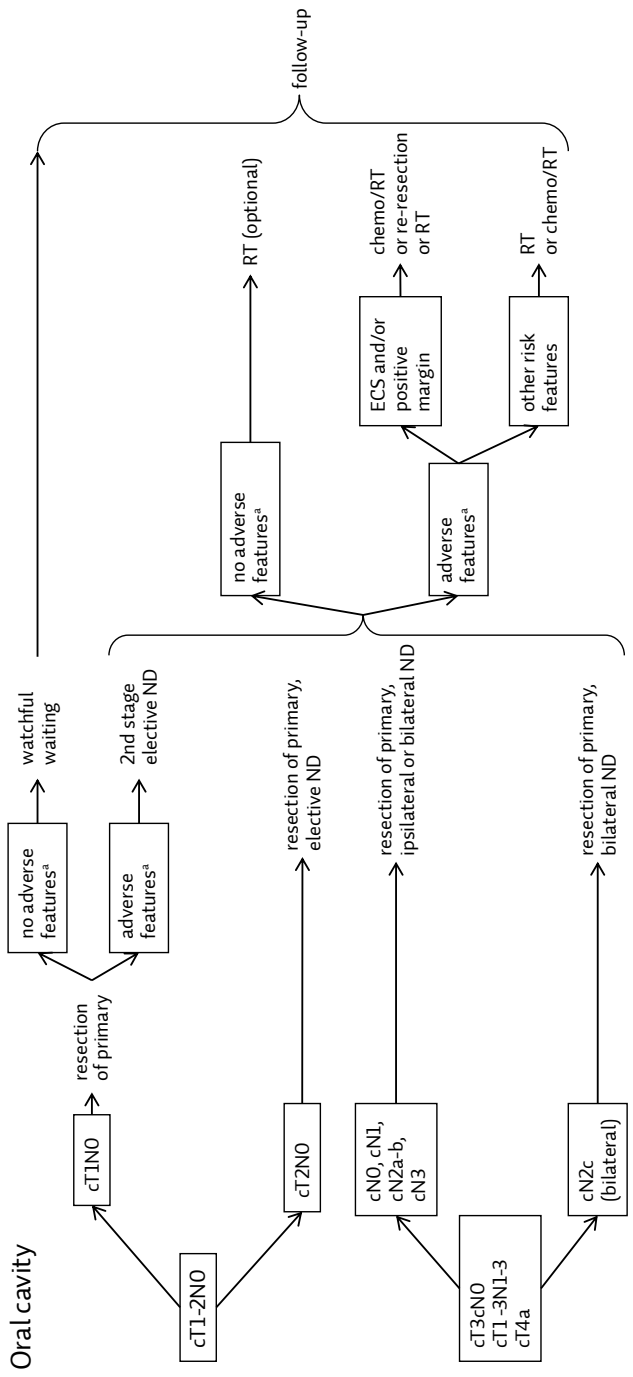

Figure 1.4. Example of a flowchart for current neck therapy of oral cavity carcinoma.
ND: neck dissection; RT: radiotherapy; ECS: extracapsular spread; [a]adverse features: extracapsular spread, positive margins, pT3 or pT4 primary, pN2-3 nodal disease, perineural invasion, lymphangioinvasion. Adapted from NCCN guidelines[14].

Table 1.4. Definitions used in this thesis.

Parameter	Definition
No nodal metastasis (N0)	No metastatic cells present in neck dissection (either SOHND[a] or mRND[b]) specimen, as determined by head-neck pathologist during routine histopathological assessment. Or, if no neck dissection has been performed, no development of regional metastasis during at least two years of follow-up.
Nodal metastasis (N+)	Metastatic cells present in neck dissection (either SOHND or mRND) specimen, as determined by head-neck pathologist during routine histopathological assessment. Or, if no neck dissection has been performed, development of regional metastasis within two years of follow-up.
Disease-specific survival	Time from first treatment till last follow-up (censored) or disease specific death (death from disease or from treatment, event).
Disease-free survival	Time from first treatment till last follow-up (censored) or disease recurrence (event).
Local recurrence	A tumour that conforms to all of the following criteria: a new malignancy diagnosed after a previous tumour (index tumour); of the same morphological classification as the index tumour; diagnosed within three years after the index tumour; at the same location[c] as the index tumour or in the resection area of the index tumour.
Second (third, fourth) primary tumour	A tumour diagnosed after the index tumour that does not conform to one or more of the criteria for local recurrence and that is not a metastasis.
Regional recurrence	A tumour arising in or from the lymph nodes in the neck, after the patient has been treated.
Distant recurrence	A tumour arising elsewhere in the body after treatment, and which is considered to be a metastasis of the index tumour.

[a]supra-omohyoidal neck dissection, an elective procedure removing the lymph nodes from level I-III (fig 1.3); [b]modified radical neck dissection, removing the lymph nodes from levels I-V (fig 1.3); [c]This definition harbours some subjectivity. In literature sometimes only tumours within an area of 1.5-2 cm from the former location of the index tumour are considered. However, there is no clear evidence for this distance.

better disease-free and disease-specific survival. This better prognosis seems to be attributable to a limited number of cellular pathways that are dysregulated by HPV, such as Rb/p53, wnt-signalling, PI3K[85], resulting in a higher radiosensitivity in HPV-positive tumours[86,87].

Certain early stage OOSCC may be treated primarily with radiotherapy alone. As adjuvant treatment, radiotherapy is frequently used in all OOSCC[13-15]. Because of the possibility to adjust dose and fractionation scheme, radiation therapy is easy to customize based on prognostic markers. Various markers have been assessed for their predictive value for treatment response in radiotherapy, of which hypoxia/angiogenesis and proliferation/apoptosis related markers are the most extensively studied[88]. Because of the lack of translational studies, currently no marker is routinely used in the clinical setting[88].

Recently, molecular therapeutics have been developed that target specific molecular pathways in the tumour cell ('targeted therapy'). These drugs block a specific receptor (eg. monoclonal antibodies) or a downstream signal transducer (eg. tyrosine-kinase inhibitors)[89]. Obviously these

Table 1.5. Detailed description of the various patient series used in this thesis.

Series	Used in study	Number of cases	Tumour location (ICD-O-3 code)	Period of diagnosis	Selection criteria
Early stage OSCC	Infiltration depth study	212	Oral (C02.0-6.9)	1997-2008	cT1-2pN0, treated in UMCG by resection of primary tumour, without prior head-neck or systemic oncological treatment
-Subset: superficial early stage OSCC	PDT study	91	Oral (C02.0-6.9)	1997-2008	Subset: cases with ≤5 mm infiltration depth from early stage OSCC series
OOSCC	Adhesion study MSP study	227	Oral & oropharyngeal (C00.3-6.9, 9.0-10.9)	1997-2008	Treated in UMCG by resection of primary tumour and a neck dissection, without prior head-neck or systemic oncological treatment. For the MSP study a random selection from this series (n=70) was used.
-Subset: OSCC	HPV study EGFRvIII study	200	Oral (C02.0-6.9)	1997-2008	Subset: all oral tumours from the 227 OOSCC series
OpSCC	HPV study EGFRvIII study	196	Oropharyngeal sub-sites: base-of-tongue (C01.9) and tonsil (C09)	1997-2012	Diagnosed in UMCG
Series previously composed by the department of Radiotherapy UMCG:					
HNSCC	FADD study EGFRvIII study	198	Oral, oropharyngeal, hypopharyngeal and laryngeal	1993-2003	Treated in UMCG by resection of primary tumour and postoperative radiotherapy

drugs work only when the specific pathway is hyperactivated, and the tumour has become dependent on this specific pathway. Therefore, to have the greatest benefit of targeted therapy, the tumour should be assessed for the presence of hyperactivated pathways for which targeted therapeutics are available[90,91]. In some more common types of cancer (eg. lung cancer, breast cancer) data on the relative presence of specific hyperactivated pathways, and the effect of targeted therapy of these pathways has become widely available. For OOSCC less data is available, however, a few phase III trials using targeted therapy have been performed[92-95]. Most trials have been performed in patients with metastatic or recurrent disease, targeting the Epidermal Growth Factor Receptor (EGFR), which is present in >95% of included HNSCC patients[96-99] and some guidelines have added anti-EGFR therapy to radiation and chemoradiation protocols in treatment of advanced OOSCC[14,15]. However, these trials show relatively low response rates of 10-16% to cetuximab (an EGFR inhibitor) irrespective of use as single agent or in combination with chemotherapy[96-99]. Many mutations of the *EGFR* gene have been reported both in the tyrosine kinase domain in lung tumours[100] and in the extracellular domain in glioblastomas[101]. The presence of *EGFR* mutations may account for response or resistance to EGFR-targeted therapy and therefore assessment of *EGFR* mutational status may be essential to select patients that will benefit from EGFR-targeted therapy[102]. In HNSCC the presence of a specific activating *EGFR* mutation[103], called EGFRvIII mutation, has been reported to cause resistance to EGFR-targeted therapy[104]. Although targeted therapy is becoming available for HNSCC, there is still a need for optimization of currently used (EGFR-) targeted therapy as well for the identification of new targets[105].

Scope of this thesis

Goal of this thesis is to find new molecular biomarkers in the primary tumour that have predictive value for the nodal status and for prognosis in patients with oral & oropharyngeal squamous cell carcinoma, to improve regional staging and treatment selection based on current clinical and histopathological characteristics.

To be able to identify new biomarkers and assess the predictive values of these markers in the clinical setting, tumour tissue and associated clinicopathological and follow-up data of a sufficiently large group of patients are necessary. For the project 'New molecular biomarker discovery for diagnosis and prognosis in oral & oropharyngeal cancer' a large patient database was constructed which contains clinicopathological and follow-up data of several patient cohorts including in total over 600 patients who developed more than 700 OOSCC between January 1st 1997 and December 31st 2012, and of which tumour tissue was available in the archives of the department of Pathology of the University Medical Centre Groningen. To be able to compare the predictive values of newly identified biomarkers with current clinicopathological assessment, over 200 clinicopathological variables per tumour were extracted from the hospital files and registered in the database. Patient follow-up data was also registered and clinical outcomes were defined (table 1.4). From this database several patient selections were made for the various studies (table 1.5 and figure 1.5). These well-defined patient cohorts and extensive data registered in our database enables us to address clinically relevant research questions on the predictive and prognostic values of biomarkers in OOSCC.

Infiltration depth has predictive value for the nodal status in OOSCC, however no clinically relevant cut-off has been established. In **chapter 2** we measured infiltration depth on a homogenous group of pT1-2 OSCC and performed rigorous statistical tests to find the most optimal cut-off for performing a neck dissection. In **chapter 3**, the surgically treated tumours with ≤ 5 mm infiltration depth were compared with a group of tumours of comparable depth that were treated with photodynamic therapy for response to therapy and disease-free and overall survival. This way we were able to define the patient group that might benefit from photodynamic therapy.

EpCAM, a cell adhesion molecule, has been studied extensively for its role in metastasis. It might prevent metastasis as adhesion molecule, but might also promote metastasis because it abrogates another important adhesion molecule, E-cadherin. The role of EpCAM in human carcinogenesis was reviewed in **chapter 4**. **Chapter 5** describes an analysis of the co-expression of E-cadherin, EpCAM and claudin-7, three functionally related cell adhesion proteins, for their predictive value for determining the nodal status. For this purpose, tissue microarrays (TMAs) were generated of the tumour front and tumour centre of 227 OOSCC, to be able to assess these markers in both tumour sublocalizations.

The *FADD* gene is located in a region which is frequently amplified in HNSCC. The protein expression of FADD in a group of generally advanced HNSCC was analyzed for its predictive value for nodal status and distant metastasis-free interval in **chapter 6**.

HPV is a known predictor for less recurrences and longer survival in OpSCC. In **chapter 7** the prevalence of high-risk HPV in OOSCC in the Northern Netherlands was analyzed using a validated triple detection algorithm, based on detecting not only the presence of HPV DNA, but also p16 immunohistochemistry and HPV-in situ hybridization to detect active HPV infection in the tumour tissue. The incidence of HPV-associated OpSCC over a period of 16 years (1997-2012) was determined and compared to that in the rest of the Netherlands.

DNA methylation of several genes has been associated with nodal status. **Chapter 8** describes the selection and testing of 28 new candidate methylation markers for their predictive value for the nodal status on a group of 70 OOSCC.

EGFRvIII is the most common form of mutant EGFR and is associated with a more aggressive phenotype, and resistance to chemo and radiation therapy. In **chapter 9** the prevalence of this mutant EGFR was determined on a large group of tumours in order to decide whether EGFRvIII could be used as a prognostic marker in patients with OOSCC.