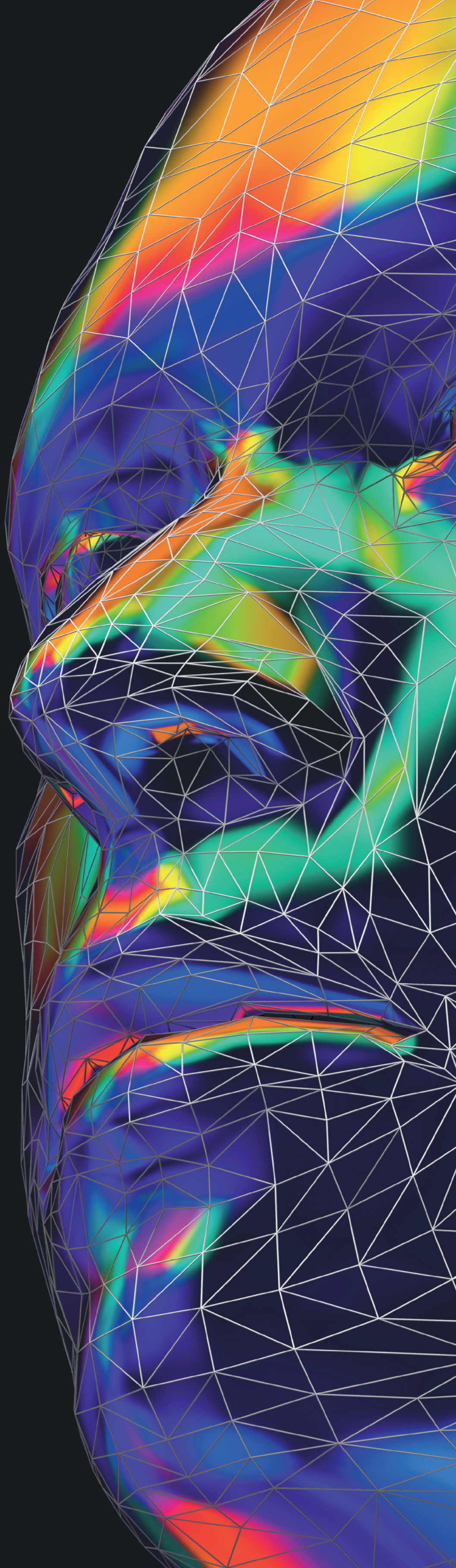
ROELAND W.H. SMITS

Resection Margins in Oral Cancer Surgery: Room for Improvement



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Resection Margins in Oral Cancer Surgery: Room for improvement

Resectie marges voor mondholte kanker chirurgie: ruimte voor verbetering

Proefschrift

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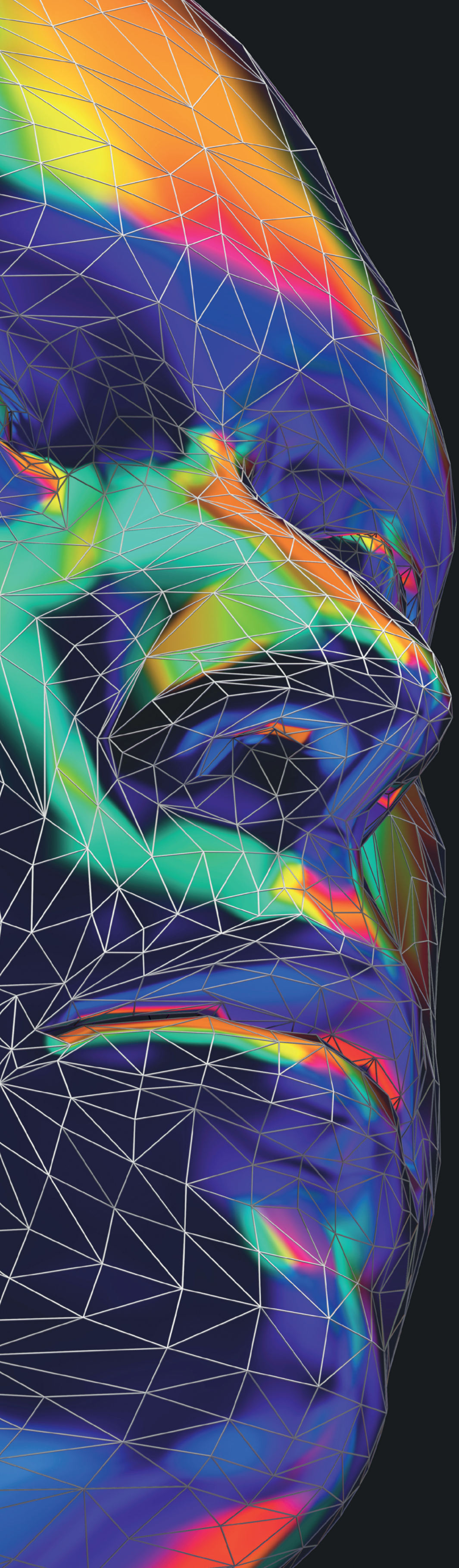

I

General Introduction

CHAPTER 1: GENERAL INTRODUCTION

Oral cavity anatomy

The oral cavity includes the lips, the inside lining of the lips, the buccal mucosa, the alveolar process, the retromolar trigone, the front two-thirds of the tongue, the floor of the mouth and the hard palate (figure 1). (1)

The oral cavity is the first part of the aero-digestive system, comprising numerous anatomical structures that work together in order to perform several functions. It is a very complex part of the head and neck comprising nerves (motor and sensory), mucosa and muscles. (2)

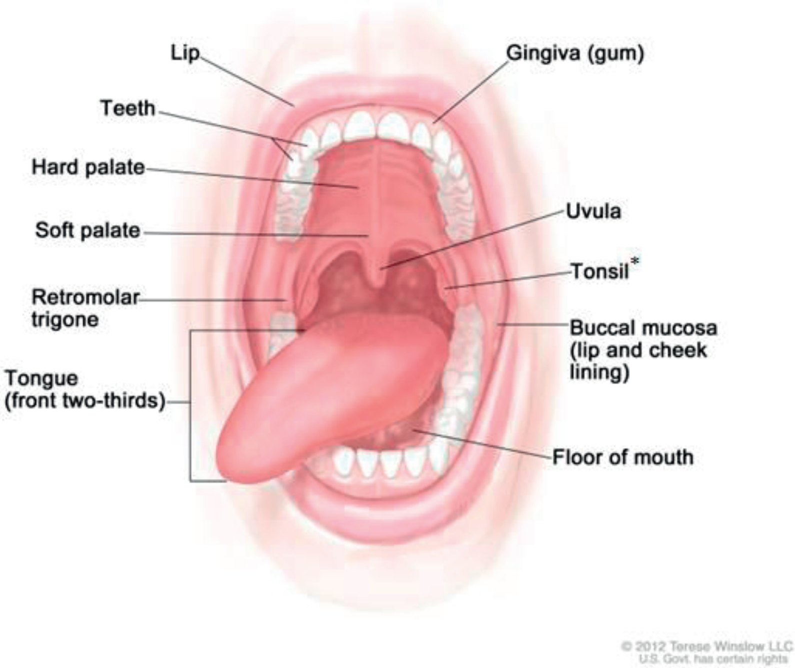

Figure 1. Anatomy of the oral cavity. Permission to use figure by Terese Winslow
* tonsils belong to oropharynx.

Oral cancer

Epidemiology

The estimated worldwide incidence of oral cavity cancer is 350,000, with a male:female ratio of approximately 2:1. (3) During the last 2-3 decades the 5-year survival rate was around 50%

in Europe. Upon histological examination, more than 90% of all oral cavity cancer presents squamous cell carcinoma (SCC). (4)

In Europe, the tongue and floor of mouth (FOM) are the most common locations for oral cavity squamous cell carcinoma (OCSCC), comprising over 70% of all OCSCC. (5)

This opposed to India and surrounding countries where the buccal mucosa is traditionally one of the most common locations for OCSCC, because of the strong association with betel nut chewing. Mainly because of both betel nut - and tobacco chewing the incidence of OCSCC is higher in Southern Asia than in other parts of the world. (6-9)

Overall, the most important risk factor for the development of OCSCC is tobacco smoking or chewing. Alcohol consumption is of less importance but has a synergistic effect with tobacco. (10-12)

Histology

SCC is characterized by squamous differentiation, often seen as keratinization with pearl formation, and invasive growth with disruption of the basement membrane. SCC is graded into well-, moderately- and poorly-differentiated, as seen in figure 2. Angiolymphatic and perineural invasion may be present.

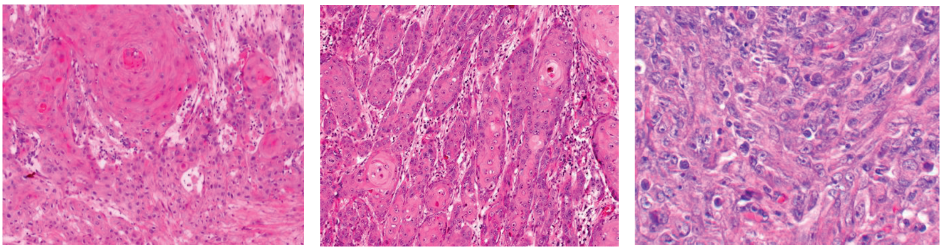

Figure 2. Different grades of squamous cell carcinoma. Adapted from pathologyoutlines.com, with permission of Alcides Chaux, M.D. and Antonio Cubilla, M.D.

- On the left well differentiated SCC: tumor nests are composed of neoplastic cells with minimal basal / parabasal atypia, retained squamous maturation with gradual keratinization and well-defined cellular borders.
- In the middle moderately differentiated SCC: almost all neoplastic cells show evident nuclear atypia with pleomorphism, prominent nucleoli and irregular nuclear membranes, but squamous maturation and keratin pearl formation are retained.
- On the right poorly differentiated SCC: note the overt nuclear atypia with nuclear pleomorphism and high mitotic/apoptotic rate. Squamous nests and keratin pearls are not evident.

TNM Classification

The TNM classification plays an important role in clinical care and cancer registry.

It delineates anatomic tumor characteristics, where "T" describes the extent of the tumor, "N" refers to absence or presence and extent of regional lymph node(s), and "M" depicts the absence or presence of distant metastasis. (13)

The majority of OCSCC patients present with an early stage (T1-T2) carcinoma. (5)

Treatment

The clinical TNM (cTNM) classification is an internationally accepted classification of the size and extent of the histopathological proven tumor (T1-T4), the presence of nodal metastasis (N0-N3) and the presence of distant metastasis (M0-M1). (14, 15) Patient characteristics relevant for treatment choice include age, comorbidity and the patient acceptance to undergo treatment.

The tumor site, its TNM classification, patient characteristics, and any previous treatment are important determinants of the choice of treatment of OCSCC. (16) Surgery is the mainstay of treatment for most OCSCC cases with adequate margins as the main goal, together with preservation of function. The primary site of the tumor will indicate the type and extent of surgery. In case of bone involvement, the affected bone should be removed. In case of previous surgery the earlier histopathological report is important to give directions for a new treatment. For instance, in case of suspected residual tumor after surgery the exact location and extent of the tumor-positive resection margin should be used to plan second surgery. Also, if radiotherapy was a (part of) previous treatment, this will usually not be used again in treatment of OCSCC. An exemption is when the earlier radiated areas of the head and neck are not overlapping with the newly planned areas for radiotherapy.

Whereas complete cure may be accomplished by surgery, achieving adequate tumor resection with acceptable remaining function and appearance is often difficult in the complex region of head and neck.

Surgical resection margins

We classify surgical resection margins (defined as the smallest distance between tumor and resection surface) histopathologically as clear: >5 mm, close: 1 to 5 mm, and positive: <1 mm. (17) Clear margins are regarded as adequate, close and positive margins as inadequate. Adequate tumor resection with acceptable remaining function and appearance is the main goal.

Unfortunately, in the literature, a range of definitions is used to for a "clear margin" in OCSCC, based on tumor free margins varying between 2mm and 10mm. (18-20) In addition, some centers use terminologies such as "tumor-free" and "tumor-negative" for describing a surgical result, instead of quantifying resection margins. A survey among members of the American Head and Neck Society in 2005 showed that 64% of its members felt that a surgical margin of >5 mm can be considered a clear margin. (19) Meanwhile, 11% of the respondents felt that a gross margin of 1 cm should be considered clear. Lack of universal definition on resection margins hampers comparison of the surgical results and of patient outcome between different centers.

Achieving adequate resection margins is crucial for patient outcome, as inadequate surgical resection necessitates re-operation or adjuvant (chemo)radio-therapy leading to a significantly worse local control, negatively affecting patient prognosis. (21-24) Several studies showed higher local recurrence rate for inadequate resection margins compared to adequate resection margins. (25-27) Sutton *et al.* not only showed a significantly higher local recurrence rate for inadequate margins, but also found a corresponding decrease in 5-year survival from 78% for adequate resection margins to 11-47% for inadequate resection margins. (28)

This indicates that all effort should be focused on achieving adequate resection margins, yet adequate resection margins are only reported in 17-48% of cases. (16, 22, 29)

How can we increase the number of adequate resection margins?

Although the resection margin is not the only parameter of influence to the tumor recurrence rate and to patient survival, it is the only factor which can be altered by the surgeon and the pathologist.

Preoperative planning

The first step towards adequate resection of OCSCC is optimal preoperative planning. Full clinical examination of the patient is essential to collect all necessary information of the tumor. Depending on the tumor site, extent of the tumor, and possible bone invasion the surgeon may choose to plan the surgery without additional diagnostic steps, for instance in case of a well accessible cT1N0M0 tumor of the side of the tongue. Yet, in most cases additional information is required. In case of a large tumor or inability to judge the extent of the tumor additional radiologic imaging is required.

The standard preoperative imaging modalities include magnetic resonance (MR) and computed tomography (CT). Contrast-enhanced MR is superior to CT when it comes to soft tissue characterization in oral cancer. (30) Adding CT in case of suspected bone invasion increases specificity. (31) Using both MR and CT may increase specificity, because both hard and soft tissue can be examined. (32) In some cases, positron emission tomography (PET) is used for further evaluation of tumor extent or regional or distant metastasis. (33, 34) Panoramic radiography (OPT) can also be used to evaluate potential bone invasion.

Yet, even when combining multiple imaging modalities, the determination of tumor extent remains very difficult. For instance, even when combining CT, MR and PET, sensitivity in detection of bone invasion of OCSCC is only 83.3%. (35) This implies that the remaining 16.7% of patients will be planned for local excision instead of marginal or segmental resection of the mandible.

All in all, assessing tumor extent by preoperative imaging is not always sufficient.

Intraoperative assessment of resection margins

Traditionally, intraoperative assessment of resection margins is usually based on histopathological evaluation by means of the so-called frozen section procedure. In the frozen section procedure the surgeon takes tissue samples from either random locations or suspicious locations in the surgical wound bed for microscopic evaluation by a pathologist. This is also known as the "defect driven approach" (figure 3A). This type of intraoperative assessment has a sensitivity for detecting inadequate resection margins as low as 34.3%. (20, 36, 37) This is understandable because usually only a small portion of resection margin is examined (approximately 0.1% to

1%). Moreover, often only the mucosa is sampled leaving the underlying soft tissue uninspected. (38) The anatomical location of soft tissue margins sometimes makes it difficult to take obtain tissue for a proper frozen section, while the mucosal surface is easier to reach for frozen section. Remarkably, up to 87% of inadequate margins are found in the submucosal soft tissue layers. (16) This is explained by the often irregular submucosal expansion of the tumor below the mucosa. In the majority of cases the small number of frozen section biopsies that can be processed leads to sampling error, i.e. the histopathological assessment of these frozen section cannot be considered to be representative of the complete resection surface. (24, 39-44)

Because of its limitations the defect driven approach is being advocated less and less.

Instead, more evidence in favor of the use of the "specimen driven approach" is available in recent literature. (18, 24, 40, 44-46) In the specimen driven approach, the resection margin is evaluated on the resection specimen, as displayed in figure 3B.

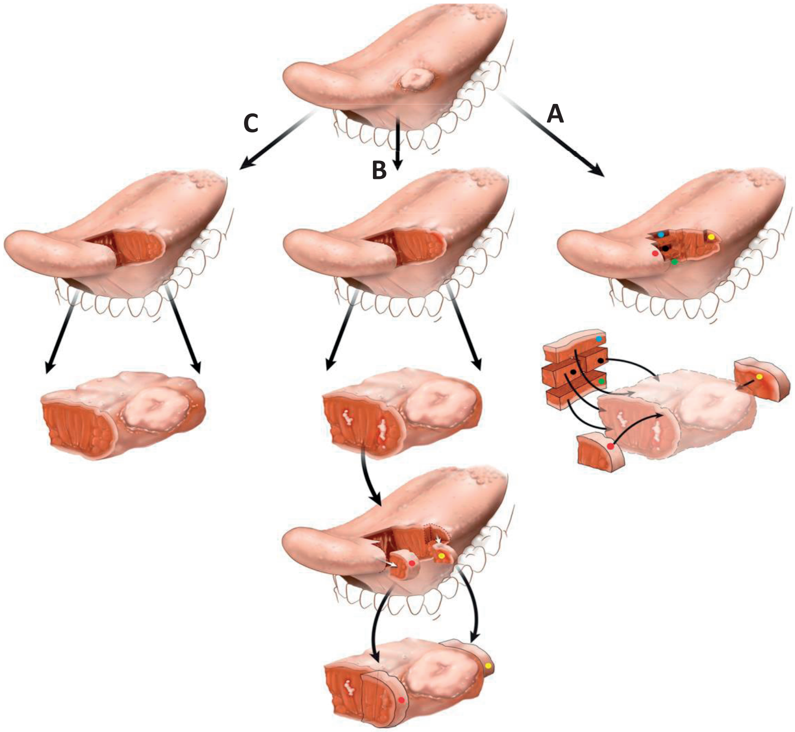

Figure 3. Schematic representation of a resection of a tumor of the side of the tongue. In '"**C**", no intraoperative assessment was used. Instead the complete resection specimen is send for routine histopathology. In "**B**", margins were examined from the resection specimen and found to be inadequate (i.e. specimen driven). The surgeon revised margins by obtaining additional tissue (red and yellow dots) from the tumor bed. In the bottom picture the additional tissue is projected on the resection specimen. In "**A**", 5 margins are primarily sampled from the tumor bed (*red, green, yellow, blue, and black dots*), without preceding examination of the resection specimen by the pathologist (i.e. defect driven).
Reproduced with permission from S. Chiosea MD, University of Pittsburgh Medical Center and Laura Pliego, Medical Illustrator.

In this way, the surgeon and the pathologist can inspect the resection specimen together and decide where to take frozen sections, if needed. Often, the surgeon will specify areas of concern. In evaluating and cutting the specimen, the pathologist can choose additional sites to sample if they appear close to the tumor. (40)

With the specimen driven approach, a sensitivity for tumor deposits of up to 83% is described. (45)

However, this type of intraoperative assessment, requiring a dedicated team of specialists, poses a logistic challenge, and is laborious. Therefore, it is not realistic to expect that this approach can be widely adopted to become part of standard care.

That is why an objective, fast and less laborious method is needed for real-time assessment of complete resection margins.

Optical techniques for intraoperative assessment of margins

Most exploratory research with optical techniques has focused on in vivo delineation of the tumor at the mucosal surface, prior to surgery. However, assessment of only mucosal tumor resection margins is of very little value because inadequate margins mostly occur in the deeper (submucosal) soft tissue layers as discussed above. (16) Various intraoperative methods like cytology, ultrasonography and optical techniques are being explored for use in OCSCC surgery, and were reviewed in 2014 by Ravi *et al.* (47)

Optical techniques like high-resolution micro-endoscopy (HRME), optical coherence tomography (OCT), fluorescence spectroscopy, elastic scattering spectroscopy and Raman spectroscopy show promise because of their ease of use, relatively low cost and potentially high operating speed. (48-52)

In the remaining part of the introduction I will focus on Raman Spectroscopy, as this technique is the primary technique discusses and applied throughout this thesis. The remaining techniques will be discussed in chapter 7.

Raman Spectroscopy

Raman effect

Light can be scattered either elastically or inelastically as it interacts with the molecules in a sample (figure 4). The majority of light is scattered elastically, known as Rayleigh scattering, and does not involve an exchange in energy between the incident light and the molecules within the sample. The Rayleigh scattered light therefore has the same frequency as that of the incident light. Inelastically scattered light, known as Raman scattering, involves an energy exchange between the incident light and the molecules within a sample. The Raman scattered light has a different frequency than the incident light.

The energy exchange causes a change in the vibrational state of the molecule. The energy required to alter a vibrational state of molecule is specific for the molecule, and can also be influenced by interaction with surrounding molecules. The number of vibrational states of a molecule is proportional to the number of atoms in the molecule (3N-6, with N the number of atoms). A Raman spectrum of a molecule shows the intensity of the Raman scattered photons as a function of the energy level, and therefore shows a number of peaks that are all associated with a particular molecular vibration. The energy level is expressed as a Raman shift, which is proportional to the difference in energy of the incident photon and the emitted photon. The Raman shift is measured in relative wavenumbers (expressed in cm^{-1}), that are proportional to the reciprocal of the wavelength. The Raman spectroscopic information is mainly contained in two spectral regions: the 400 - 2000 cm^{-1} spectral interval, often referred to as the fingerprint region, which contains the majority of possible vibrations, and the 2000 - 4000 cm^{-1} region, or high wavenumber region (HWVN), which contains the CH-, OH-, and NH-stretching vibrations. Figure 5 shows examples of Raman spectra from both regions for a number of different molecules.

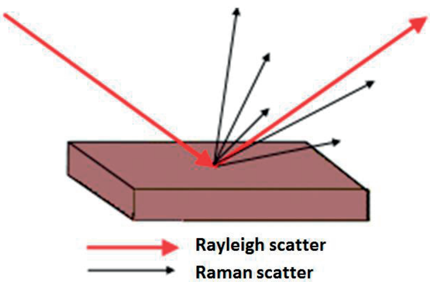

Figure 4. Schematic representation of interaction between light and a sample. The thick (red) arrow represents the fraction of light which is elastically scattered with an identical wavelength (Rayleigh scattering). The thin black arrows represent a smaller fraction of light which is scattered at different wavelengths due to inelastic light scattering (Raman scattering), in which energy is exchanged between an incident photon and molecules of the sample.

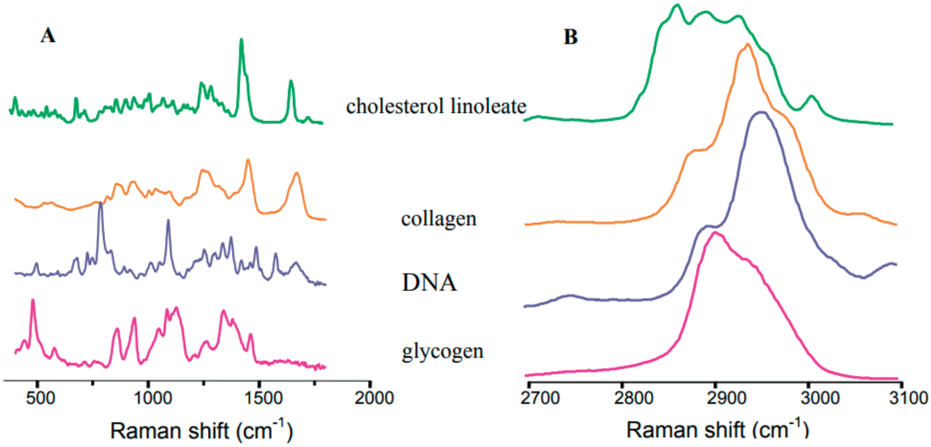

Figure 5. Raman spectra of pure chemical compounds, obtained in fingerprint region (A) and in high wave number region (B). (53)

Raman spectroscopic analysis of OCSCC

An individual peak in a Raman spectrum corresponds to a specific molecular vibration. Depending on their size, molecules can have many different Raman-active vibrational modes and as a consequence, the Raman spectrum of a molecule is highly specific for that particular molecule. Raman spectra of cells are very complex because all molecules contribute to the overall Raman spectrum of the cell. However, based on the known Raman spectra of pure compounds, in many cases peaks in a spectrum of a cell or tissue can be assigned to particular molecular constituents. Therefore, it is possible to acquire detailed qualitative and quantitative information regarding the molecular composition of tissues on the basis of their Raman spectrum. (54, 55) Different tissues will differ in their overall molecular composition and consequently their Raman spectra will also be different. Pathological changes results in changes in molecular composition, and will be reflected in the Raman spectra, enabling the use of Raman spectroscopy as diagnostic tool.

Raman spectroscopy is suited for intraoperative use because it is nondestructive, fast and does not need labelling or pretreatment. Also, the fact that it can be used with fiber-optic probes makes it especially suitable for use in the often narrow oral cavity.

A growing number of studies reported on Raman spectral differences between normal tissue and OCSCC. (56-62) Cals *et al.* developed an annotated database of Raman spectral characteristics of individual histopathological structures in oral tissue, by *in vitro* mapping of frozen tissue sections. (63) This annotated database was used to develop a classification model to differentiate between tongue squamous cell carcinoma and non-tumorous tissue. (64)

OUTLINE OF THIS THESIS

First, a "baseline measurement" of the performance in achieving adequate soft tissue resection margins (Chapter 2) and bone resection margins was performed (Chapter 3). Both chapters give an extensive review of literature regarding resection margins for OCSCC. We suggest ways to improve the rate of adequate resection margins. Awareness of the importance of achieving adequate resection margins and thereby altering surgical approach seems crucial. Also, pre- and postoperative evaluation of diagnostics in a multidisciplinary approach is important. The use of preoperative imaging to determine tumor extent is being discussed, especially in case of suspected bone invasion.

Yet, the most effective way to improve surgical results seems optimization of intraoperative assessment of the resection specimen. In chapter 4 the comprehensive intraoperative assessment of resection margins with a specimen driven assessment is compared with the defect driven approach using the frozen section procedure which was used more often in the past. With comprehensive intraoperative assessment of resection margins a major improvement of the rate of adequate margins is achievable. Unfortunately, this approach has limitations, which are also discussed in chapter 4.

Therefore, other ways to perform intraoperative assessment are investigated. We show that Raman spectroscopy is a technique that can accurately differentiate between oral tissue types *in vitro* (Chapter 5), and between oral squamous cell carcinoma and healthy surrounding tissue *ex vivo* (Chapter 6).

The discussion in Chapter 7 gives an overview of the hurdles when trying to achieve adequate resection margins. It also gives directions on how to overcome these hurdles.

In this thesis we have highlighted the number of inadequate resection margins for OCSCC and its impact on patient outcome. We elaborate on potential ways to improve the percentage of adequate resection margins by intraoperative assessment of resection margins. Furthermore, we show that Raman spectroscopy is an objective and fast technique that can potentially support the surgeon in achieving adequate tumor resection.

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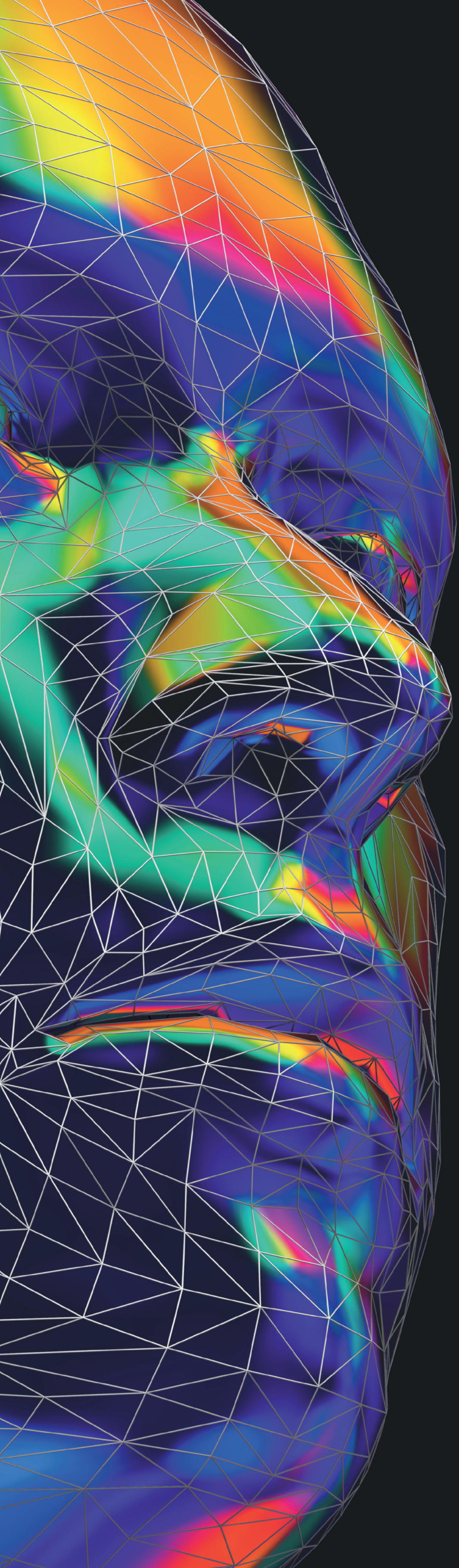

2

Resection margins in oral cancer surgery: room for improvement

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ABSTRACT

The aim of this review was to identify publications on resection margins in oral cancer surgery, and compare these with the results from two Dutch academic medical centers. Eight publications were considered relevant for this study, reporting 30 to 65 percent inadequate resection margins (i.e. positive- and close margins), compared to 85 percent in Dutch centers. Yet, clinical outcome in terms of overall survival and recurrence seemed comparable.

The misleading difference is caused by lack of unanimous margin definition and differences in surgico-pathological approaches. This prevents comparison between the centers. Data from Dutch centers showed that inadequate resection margins have significantly negative effect on local recurrence, regional recurrence, distant metastasis and overall survival.

These results confirm the need for improvement in oral cancer surgery. We underline the need for consistent protocols and optimization of frozen section procedure. We comment on development of optical techniques for intra-operative assessment of resection margins.

INTRODUCTION

Despite progress in surgical and reconstructive techniques, clinical outcomes for patients with oral cavity squamous cell carcinoma (OCSCC) is still unsatisfactory[1]. Because a significant number of these tumors are at an advanced stage at the time of diagnosis, five-year survival rates of about 50% are generally reported[2-4]. Adequate surgical resection (i.e. clear margins) is crucial for local control and prognosis[5-6]. At the same time, there is no universal definition of resection margins, with definition of clear margins varying between 2mm and 10mm[7- 9]. In addition, some centers use descriptive terminologies such as "tumor-free", "tumor-negative", "the tumor does not reach the resection margin", "resection margin shows no malignancy", instead of quantifying resection margins. The same is true regarding the definition of close and positive margins. Lack of universal definition on resection margins hampers comparison of surgical results and of patient outcome between different centers. In addition, this ambiguity bias the choice of adjuvant therapy.

The aim of this study was to estimate the status of OCSCC resection margins in two Dutch academic centers and to compare these with a review of relevant literature in order to assess the room for improvement in OCSCC surgery. In the discussion we suggest measures and methods that could potentially improve surgical resections in OCSCC.

MATERIAL AND METHODS

Retrospective study from two Dutch academic centers

At the Erasmus University Medical Center (Erasmus MC) records of weekly multidisciplinary oncology conferences between October 2010 and December 2012 of the department of oto-rhinolaryngology were examined, to identify all surgically treated cases of OCSCC.

A database was created containing entries regarding age, gender, medical history, localization of tumor and date of surgery. Data on tumor characteristics and resection margins were obtained.

At the Leiden University Medical Center (LUMC) data of patients who were surgically treated for OCSCC between 1990 and 2005 were extracted from the hospital-based cancer registry system (ONCDOC; established in 1969)[11]. Histopathological reports (in line with the WHO criteria[12]) were obtained for all selected patients. Reports that could not be interpreted unambiguously were excluded. Data on resection margins were obtained from the histopathological reports, in the same way as described above for Erasmus MC.

At Erasmus MC and LUMC, OCSCC surgical specimens were dissected by a dedicated pathologist. The resection margins were systematically evaluated, mucosal and deep. According to our protocol, tumor specimens are cut into sections of 2-3mm thick. Tissue, including tumor and its relationship with all resection margins (in respect to all directions), was then widely sampled (one block per 4-6 mm).

At these two hospitals, the histopathologically assessed resection margins were defined (according to the Royal College of Pathologists) as clear: smallest distance between tumor and resection border >5mm, close: smallest distance between tumor and resection border 1-5mm and positive: distance from tumor to resection border <1 mm[10]. Clear margins are regarded adequate, close and positive margins inadequate.

The data were analyzed with SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA: IBM Corp.). For both centers, data on local and regional recurrence, adjuvant radiotherapy, metastasis and overall survival were collected as was the time in months until these events occurred. For each center, descriptive statistics, Chi-Square tests and independent sample t-tests were used to compare mean time until event in the group with an adequate resection margin with the group with an inadequate resection margin. P-values <0.05 were considered to be statistically significant. Overall survival was also analyzed on combined data using the likelihood ratio test in Cox survival analysis.

Literature

Search strategy and selection of articles

A computerized search was carried out of the Medline-, Embase- and the Cochrane Collaboration databases, for publications reporting on OCSCC surgery, and outcome in terms of surgical margins. The date of the final search was 31 March 2014. The search filter contained synonyms and derivatives for the keywords: "oral", "cancer", "surgery", "resection margins" and " resection borders", and searched the title and abstract fields. Papers that were cited in these publications were sought manually to complete the compilation.

Our search strategy was compiled using the STARLITE mnemonic described in the proposed "standards for reporting literature searches"[13]. Articles were selected using the following inclusion criteria: patients surgically treated after 1990, appropriate description of tumor resection margins for oral cavity squamous cell carcinoma specifically. Articles on tongue SCC surgery, including surgery for the base of the tongue, or articles where it remained unclear which delimitations were used for the oral cavity were excluded. Also, articles that only focused on single sub-site within the oral cavity, or articles limited to early stage cancer were discarded. Subsequently, remaining articles were obtained in full text and reviewed to yield the final selection. Commentaries and letters to the editor were not selected. Furthermore, articles on a non-human study population, and articles not written in English were discarded.

Data on local and regional recurrence and overall survival was extracted from the studies if available. No statistical analysis was carried out on the literature data, because of lack of information on follow up and survival data for individual cases.

RESULTS

LUMC and Erasmus MC data

Data from Erasmus MC and LUMC were compared to the literature review (tables 1-3). The clinicopathological characteristics of all patients are shown in table 1. Tumor subsites with corresponding tumor classifications are displayed in table 2. Table 3 shows resection margin status in respect to local and regional recurrence, and to overall 5 years survival. When available, the definition of margins is also displayed, showing differences between the institutions.

Table 1
Clinicopathological characteristics

Study	Gender Male/Female (%)	Age (years)	pT3-pT4 (%)	pN1-pN2 (%)
Erasmus MC (this study)	69/31	64.4 (range 16-93)	47	33
LUMC (this study)	52/48	63.5 (range 27-95)	37	42
Kreppel et al.[14]	66/34	60.6 (SD 11.6)	54	46
Hoffmannova et al.[15]	78/22	59.4 (range 39-88)	51	48
Rogers et al.[16]	62/38	62 (average)	40	37
Woolgar et al.[17]	63/37	60 (range 30-88)	47	39
Koo et al.[18]	75/25	55.3 (range 20-77)	34	37
Priya et al.[8]	77/23	50 (range 19-80)	45	unknown
Liao et al.[19]	92/6	49 (range 25-83)	59	38
Kademani et al.[20]	48/52	66 (SD 14)	32	22

At the Erasmus MC, 174 patients were treated surgically for OCSCC between 2010 and 2012.

As shown in table 1, the mean age was 64.4 years, the male/female ratio 69/31%, T1-T2/T3-T4 rate 53%/47%. Upon histopathological examination, 33% of patients had positive lymph nodes in the neck dissection specimen. Distribution of tumor sites in respect to tumor classification is presented in table 2. Summary of data from two Dutch centers and from literature is given in table 3. The clinicopathological characteristics of Erasmus MC data were comparable with those from LUMC and the literature. At Erasmus MC (according to the definition of margins by Royal College of Pathologists[10], clear resection margins were found in 15% of cases, close in 42% and tumor positive margins in 43% of cases. The total of inadequate resection margins (close and positive) was 85%. Early OCSCC (T classification T1-T2) showed higher numbers of adequate resection margins, with 22.6% clear margins, 41.7% close and 35.7% positive margins. For advanced OCSCC (T classification T3-T4) clear margins were found in 5.1%, close in 42.3% and positive in 52.6%.

At the LUMC 117 histopathological reports on resection margins were available for patients who received surgical therapy for OCSCC between 1990 and 2005. The mean age was 63.5 years, male/female ratio 52/48%, T1-T2/T3-T4 rate 63%/37%. Positive lymph nodes in the neck dissection specimen were found in 42% of patients (table 1). Table 2 shows tumor classification per site.

Table 2
Tumor site and respective tumor stage

Location	Erasmus MC			LUMC			Literature average [8, 14-20]
	Percentage of total (%)	**pT1/T2 (%)**	**pT3/T4 (%)**	**Percentage of total (%)**	**pT1/T2 (%)**	**pT3/T4 (%)**	**Percentage of total (%)**
Tongue	41	62	38	43	84	16	38
Floor of mouth	32	53	47	27	62	38	25
Alveolar process	11	33	67	13	13	87	16
Retromolar trigone	8	31	69	12	40	60	12
Cheek	7	36	64	4	60	40	5
Hard palate	1	100	0	1	50	50	4

Comparable with the Erasmus MC, at the LUMC clear resection margins were found in 15%, close in 45% and tumor positive margins in 40% of cases. The total of inadequate resection margins adds up to 85%. Advanced tumor stage was associated with higher number of inadequate resection margins, as was observed in the Erasmus MC. In the T1-T2 group 23.9% had clear-, 47.9% close- and 28.2% positive margins. For T classifications T3-T4, margin percentages were respectively 2.3%, 42.9% and 54.8%.

As at the Erasmus MC, a significantly higher number of LUMC patients received adjuvant radiotherapy in the group with inadequate margins, compared with the group with adequate resection margins. Of the 18 patients with clear margins 28% received adjuvant radiotherapy, compared to 46% of the 52 patients with close margins and 81% of the 47 patients with positive resection margins ($p=0.009$).

For the two Dutch centers, the influence of adequate versus inadequate resection margin on clinical outcome, including local- and regional recurrence, metastasis and overall survival, is shown in table 4. It is clear that all these events are more frequent in the inadequate margin group. It must be noted that four patients developed both a local and a regional recurrent lesion during follow up time. Since there were no cases of metastasis in the group with adequate resection margins in the Erasmus MC and no cases of local recurrence in the group with adequate resection margins in the LUMC, we used independent sample t-tests to analyze differences between mean follow-up until event. Data on follow up time (and therefore censoring) was normally distributed between the two resection margin groups. For the Erasmus MC population, significant positive impact of adequate resection margin compared to the inadequate resection margin was seen in case of, local recurrence, regional recurrence, metastasis and death. At the LUMC mean time until event was significantly longer for the adequate resection margin group in case of local recurrence.

The estimated overall survival in the Erasmus MC and the LUMC is illustrated by figure 1. For the clarity of presentation we made two separate lines, using the cumulative estimated survival rates per month, calculated with the Cox regression analysis, adjusted for adequacy of resection margins. Erasmus MC data showed that survival decreased in the group of patients with inadequate resection margins with a hazard ratio of 5.7 (p=0.016). LUMC data showed a decrease of survival in the group with inadequate resection margins with a hazard ratio of 2.0 (p=0.07).

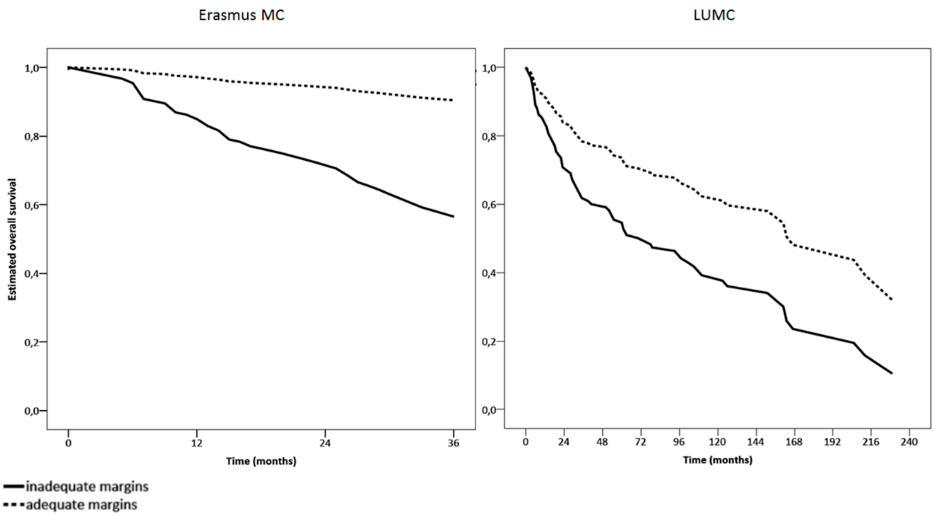

Figure 1

Review of literature

Using the inclusion criteria described in the methods section, eight studies remained after further exclusion of those that did not specifically report on oral cavity squamous cell carcinoma[8,14-20].

The eight studies included in this review reported on a total of 2557 patients. Data on patient and tumor characteristics were extracted and displayed in table 1.

Table 2 shows the average distribution of tumors among the subsites within the oral cavity, as mentioned in the eight studies, compared to the distribution in the Erasmus MC and LUMC.

Data on resection margin status, local recurrence and overall survival for each study are shown in table 3. The exact information on dissection and sampling protocol could not always be extracted from the literature reports included in our review. Moreover, margin definition differed per study or was not mentioned. Margin status was not complete for all studies or was not known at all for some studies. There was striking difference in resection margin status in the studies reviewed. For instance, percentage of clear margins varies from unknown (Kreppel *et al.*) to 85% (Kademani *et al.*) when clear margin is defined as "≥2 mm".

Kreppel *et al*. report on 26.8% of positive surgical margins. In this study positive margins were defined as "when vital tumor cells were found in the surgical margins", close and clear margins were not mentioned.

The study by Hoffmanova *et al.* shows in 19% of resections positive margins, defined as "extending the margin of the tumour". In this study, close margins were defined as "<5 mm" and were found in 11% of cases; clear margins were described as ">5 mm". In the article from Koo *et al*. positive resection margins were found in 7% of cases, but no information on what they considered as "positive margin" was given. Neither data, nor descriptions of close and clear resection margins were given.

Similar to data on resection margins, clinical outcome varied among the studies, with local recurrence reported in 10%-35% and regional recurrence in 7%-13% of cases. Overall 5-years survival varied from 26%-71%. No data on adjuvant radiotherapy could be extracted from these studies.

As mentioned in the Methods, no statistical analysis was carried out on the literature data, because of lack of information on follow up and survival data for individual cases.

Table 3
Summary of data from two Dutch centers and from literature

Study	No. of patients	Clear margins (%)	Close margins (%)	Positive margins (%)	Inadequate margins (%)	Recurrence (%)		Overall 5-years survival (%)
						Local	Regional	
Erasmus MC	174	15 (>5 mm)	42 (1-5 mm)	43 (<1 mm)	85	7*	9*	70 (2-years)
LUMC	117	15 (>5 mm)	45 (1-5 mm)	40 (<1 mm)	85	11	15	57
Kreppel et al.[14]	183	unknown	unknown	27	unknown	unknown	unknown	56
Hoffmannova et al.[15]	147	70 (>5 mm)**	11 (<5 mm)**	19	30	35	10	26
Rogers et al.[16]	489	48 (>5 mm)**	35 (<5 mm)**	17	52	10	7	56
Woolgar et al.[17]	253	35 (>5 mm)	44 (1-5 mm)	21 (<1 mm)	65	unknown	unknown	unknown
Koo et al.[18]	127	unknown	unknown	7	unknown	15	13	71
Priya et al.[8]	306	62 (≥5 mm)	33 (1-5 mm)	5 (<1 mm)	38	15	5	unknown
Liao et al.[19]	827	57 (>7 mm)	43 (≤7 mm)	NS	43	13	31	67 (6-years)
Kademani et al.[20]	225	85 (≥ 2 mm)	15 (<2 mm)	NS	15	16	(loco-regional)	56

* after average of 25 months follow-up time
** clear resection margins stated as ">5mm", close margins as "<5mm", margins of 5 mm therefore not classified
Abbreviations:
NS= not specified

Table 4
Clinical outcome in Erasmus MC and LUMC population

Event	Erasmus MC					LUMC				
	Adequate margins (>5 mm)		Inadequate margins (≤5 mm)			Adequate margins (>5 mm)		Inadequate margins (≤5 mm)		
	No. of patients (%)	Mean time until event (months)	No. of patients (%)	Mean time until event (months)	P (independent sample t-test)	No. of patients (%)	Mean time until event (months)	No. of patients (%)	Mean time until event (months)	P (independent sample t-test)
Local recurrence	2 (17)	9	10 (83)	12	0.015	0 (0)	NA	13 (100)	19	0.015
Regional recurrence	1 (7)	30	14 (93)	12	0.002	2 (14)	10	12 (86)	12	0.103
Metastasis	0 (0)	NA	15 (100)	11	0.001	2 (9)	12	21 (91)	23	0.06
Death	2 (4)	25	51 (96)	15	0.002	8 (11)	69	68 (89)	54	0.06

Abbreviations:
NA: Not applicable

DISCUSSION

There has been much discussion on the potential impact of surgical margins in OCSCC on clinical outcome. Our results confirm the general conclusion that inadequate margins have an adverse effect. Of course, other factors, such as tumor site, T/N/M Classification, patient age, comorbidity and tumor histological characteristics affect the clinical outcome too.

Of these, however, it is only the resection margin that usually can be controlled by the surgeon and the pathologist.

To assess the room for improvement, we performed a "zero measurement" on surgical results at two academic centers and compared those with reports from literature. The number of inadequate margins at the Erasmus MC and LUMC were comparable to each other (85%). Contrary, literature reports included in our study showed great spread in the incidence of inadequate margins, varying from 30% to 65%. In spite of a strikingly higher percentage of inadequate margins (85%) at the two Dutch medical centers, robust outcome measures such as overall survival (57%) and local- and regional recurrence were comparable to those from literature. We think that the major reason for this discrepancy lies in divergent practical procedures and definitions of resection margins. Our institutional protocol, for specimen dissection and sampling for microscopic evaluation, is probably more comprehensive compared to the different institutes. The comparison of our data with the literature was even more hampered by the fact that some studies did not define resection margins.

Among different centers or study groups, the tumor size and stage seem to have no obvious impact on adequacy of safety margins; this may be due to the ambiguity of definition of adequate safety margin or difference of pathological interpretation between different centers. However, within a center, large tumor size (T3/T4) and advanced tumor stage (Stage III/IV) may lead to a higher percentage of inadequate margins since clear definition of safety margin and identical pathological diagnostic criteria may be adapted. This may explain why early OCSCC (T1-T2) showed higher numbers of adequate resection margins (22.6%) than advanced OCSCC (T3-T4) in the study of Erasmus MC (5.1%).

The impact of a resection margin on patient outcome depends on T classification and the potential presence of tumor positive lymph nodes. For instance, Barry *et al.* reported that the resection margin does not influence the local recurrence rate in case of T1-T2 oral cancer [21]. In case of positive lymph nodes (without extracapsular spread) they did find a significant effect of the resection margin on local recurrence. In case of positive lymph nodes with extracapsular spread, the recurrence rates were high, independent of the resection margin. This is in accordance with the results of Shaw *et al.* who showed that positive lymph nodes with extracapsular spread are the most significant factor for local recurrence [22].

On the other hand, some authors advocate margins of over 1 cm for early tongue cancer [23]. This once again emphasizes the versatility and contradictions of the discussion about resection margins.

To estimate the impact of inadequate resection margins on clinical outcome of our patient groups, we used the follow-up data of the Erasmus MC and LUMC. For these centers, local recurrence, regional recurrence, metastasis and death occurred more frequent in the patient group with inadequate margin. For the Erasmus MC, inadequate resection margin had a significant negative effect on all these events. For the LUMC, a significant negative effect was demonstrated on local recurrence, and, a trend that approached significance could be seen for metastasis and survival. These results were not different from those reported in literature. Therefore, we hypothesize that our higher incidence of inadequate margins is caused by the scrutiny of our pathologists rather than by poor skills of our surgeons.

All in all, our results clearly underline the fact that inadequate resection of oral cancer has a negative effect on clinical outcome.

The number of inadequate resection margins could be reduced by following the current recommendations more strictly.

The first step towards improvement of resection margins in oral cancer surgery would be the universal definition of resection margins. We propose the guidelines by the Royal College of Pathologists, which we use at our institutes[10]. This will enable clear comparison of resection margin status between different institutes.

Next, multidisciplinary meetings should always take place. These meetings not only play a role in improving patient outcome, but also help to educate health professionals, assure quality improvement, develop protocols and contribute to innovation and research [24]. Multiple studies reported an association between multidisciplinary counselling and improved survival, as summarized in the systematic review by Hong *et al.* [25]. Nguyen *et al.* stated that following the recommendations of a multidisciplinary tumor board provides the optimal care for patients with locally advanced head and neck cancer [26]. Usually during these meetings all cases should be discussed preoperatively and difficult cases postoperatively as well.

We want to emphasize that not only complex cases, but all cases should be discussed postoperatively. This way, the corresponding surgical and pathological reports can be discussed, reducing the risk on misinterpretations by either surgeon or pathologist. During these "postoperative meetings" the surgeon has the opportunity to explain where an extra resection was taken, and the pathologist can, by means of both macroscopic and microscopic images, provide the explanation on the exact localisation and extent of the inadequate resection for each individual case.

Based on the finding of this study we implemented a weekly discussion of all patients postoperatively. To best of our knowledge, discussing every single case in a multidisciplinary setting postoperatively has not yet been described in literature, or been incorporated in a protocol. Moreover, pathologists at Erasmus MC have adopted extended evaluation and reporting on resection margins, including the exact margin and its extent (e.g. "*resection margin posterior is 2,1mm, over a distance of 2cm*"). This working method goes beyond the methods described in the guidelines by the Royal College of Pathologists [10]. Postoperative multidisciplinary discussion and detailed pathologic workup gives the advantage of collecting data prospectively. By doing so, we will be able to better understand the possible cause of an inadequate resection and its consequences.

Intra-operative assessment of resection margins has been proven valuable in determining the course of oncologic surgery. A current frozen section practice is to sample the selected resection margin areas that are suspicious for cancer. This method is accepted to such an extent that the American Society of Clinical Oncology recommends using frozen sections in any surgical [27]. The limitation of frozen section is the fact that only a small portion of resection margin is examined (approximately 0.1% to 1%). Yet this procedure is time consuming and [28,29]. Evaluating only a few percent of resection margin can lead to underestimation.

To improve the status of resection margins in the Erasmus MC we now perform the best practice in our hands by extensive intraoperative macroscopic examination of resection margins and more frequent frozen sections.

Ideally, only by evaluation of complete resection margin we can objectively measure the impact of resection margins on clinical outcome. To achieve this, the entire resection surface should be histopathologically evaluated during the operation by means of frozen section procedure, like in Mohs micrographic surgery for non-melanoma skin cancers [30]. Unfortunately, this method would be even more laborious and costly for HNSCC than for skin.

Therefore, objective, fast and less costly tools are needed for real-time assessment of complete resection margins. Sophisticated optical techniques are offering the opportunity for real-time guidance of surgical and pathological procedures. Optical techniques such as Raman spectroscopy and (auto)fluorescence imaging show much promise for assessment of resection margins during OCSCC surgery[31-32]. Recently Cals *et al.* developed an annotated database of Raman spectral characteristics of individual histopathological structures in oral tissue, by *in vitro* mapping of frozen tissue sections[33]. With this work a base was created for future *in vivo* image-guided Raman tissue characterization of oral cavity. Bergholt *et al.* at the National University of Singapore already showed that transnasal, image-guided Raman endoscopy can be used *in vivo* for tissue characterization in the nasopharynx and larynx[34]. By using this technique it may prove possible to assess complete resection margin, rapidly and accurately, leading to an "optimal radical resection" where the balance between the width of resection margin and spearing of healthy tissue will be achieved. This would minimize morbidity by avoiding the resection margins of up to 2 cm, or even "*as wide as possible*" [8,35].

At Erasmus MC we are now developing a method for OCSCC surgery guidance based on Raman spectroscopy, with future perspective of achieving optimal resection margins, and thereby improvement of patient outcome.

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Evaluation of bone resection margins of segmental mandibulectomy for oral squamous cell carcinoma

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ABSTRACT

Resection margins are frequently studied in oral squamous cell carcinoma patients and are accepted as a constant prognostic factor. While most evidence is based on soft tissue margins, reported data for bone resection margins is scarce.

The aim of this retrospective study was to determine the room for improvement of surgical margins in bone resections for oral squamous cell carcinoma. We recorded the status of bone resection margins and their impact on survival in patients with segmental mandibulectomy for oral squamous cell carcinoma.

Medical records between 2000 and 2012 were retrieved. Tumour-positive bone resection margins were found in 21% (of 127 patients). Overall 5-year survival was significantly lower in this group ($p<0.005$). This means that there is a need for intraoperative feedback on the status of bone resection margins, enabling immediate extra resection where necessary. Although the problem of lack of intraoperative methods for evaluation of bone tissue has been addressed by many authors, there is no reliable method for widespread use. Future research should focus for an objective, accurate and fast intraoperative assessment of entire bone resection margin to optimize patient outcome.

INTRODUCTION

For oral cavity squamous cell carcinoma (OCSCC) surgery remains the treatment of choice with the goal of complete removal of tumour with adequate margins[1,2]. In the case of mandibular invasion by OCSCC the affected part of the bone is resected. The extent of mandibular resection depends on the degree of bone invasion, but should be as limited as possible in order to preserve function. In general, a segmental mandibulectomy is indicated where there is a large tumour with bone infiltration or where the inferior alveolar nerve is involved. Also, in case the mandibular resection would result in a remnant that does not have adequate functional strength a segmental mandibulectomy is preferred over a marginal resection. Moreover, circumferential tumour enlargement is also an indication for segmental mandibulectomy. With segmental mandibulectomy the continuity of the mandible is lost, with negative effects on mastication, speech, swallowing and aesthetics[3,4]. To limit these adverse effects it is preferable to perform immediate bone reconstruction, often with a free flap[5-7].

The magnitude of bone resection is based on preoperative imaging and visual inspection. The standard preoperative imaging modalities include magnetic resonance (MR), computed tomography (CT) and panoramic radiography (OPT). Additional positron emission tomography (PET)-CT and single-photon emission CT (SPECT) can improve diagnostic accuracy. However, despite advanced imaging techniques, estimation of bone involvement by carcinoma can still be inaccurate possibly leading to inadequate resection margins[8]. Moreover, preoperative imaging can lead to overestimation of the extent of bone invasion leading to resection of healthy bone.

Ideally there should be intraoperative feedback on bone resection margin status to improve surgical results, but unfortunately there is no routine method to evaluate bone margins during surgery. Frank tumour in the bone marrow or n. alveolaris inferior can be established by simple visual inspection and if necessary proved by (cyto)histology. However, cytology method is limited because it does not provide information about bone cortex. Regarding the involvement of the inferior alveolar nerve it is general practice that the surgeon is asking for frozen section. Yet negative frozen section of inferior alveolar nerve is not ensuring complete tumour-negative bone resection margin.

The problem are those cases where there is no frank involvement of the bone marrow or n. alveolaris inferior and where visual inspection is not providing confident information for the surgeon. Moreover, cortical invasion can be difficult to establish. In these cases there is no established method to intraoperatively assess the bone resection margin.

In current practice, the status of bone resection margins is only known after several weeks, because of the specific requirements for preparation of bone tissue[9]. If final pathology shows tumour-positive bone resection margins a re-resection should be considered. However, after several weeks the surgical defect has healed making re-resection very undesirable[5,6,10].

In contrast to soft tissue resection margins, the incidence of inadequate bone resection margins has not often been reported. Moreover, there is poor literature on the effect of inadequate bone resection margins on patient outcome[11-13].

The goal of this retrospective study was to assess the room for improvement of surgical margins in bone resections for oral squamous cell carcinoma. We estimated the status of surgical margins in patients with OCSCC who underwent segmental mandibulectomy, at Erasmus MC, University Medical Center, Rotterdam, the Netherlands. In addition, the association between tumour-positive bone resection margins and survival was investigated.

MATERIAL AND METHODS

Retrospective data selection

With approval of our medical ethical committee (MEC 2017-412), the pathology reports for all patients who underwent segmental mandibulectomy for OCSCC in the Erasmus MC, University Medical Center Rotterdam, the Netherlands, between January 2000 and December 2012 were scrutinized. Patients were included for further analysis if mandibular invasion of OCSCC was confirmed by the final pathology. In our institute the histopathological guidelines of the Royal College of Pathologists are strictly followed[1]. Accordingly, for the soft tissues the exact distance from invasive carcinoma to surgical margin is one of the core data items that should be included in histopathological reports. This distance can be subdivided in three groups: clear margins (smallest distance between tumour and bone resection surface >5 mm), close margins (smallest distance between tumour and resection surface 1-5 mm) and tumour-positive margins (distance from tumour to resection surface <1 mm).

With respect to bone resection margins this guideline specifies that "if bone invasion is present, the presence or absence of carcinoma at the bone margins should be recorded".

Using the electronic patient files, a database was created containing entries on age, gender, preoperative imaging, comorbidity, and other tumour characteristics (i.e. perineural growth in general, without specification of the inferior alveolar nerve; spidery growth, angioinvasion, bone invasion) and bone margin status. Also, soft tissue resection margin status was extracted from the pathological reports. Both clinical TNM (cTNM) and pathological TNM (pTNM) were calculated[14]. The presence of one or more different comorbid ailments was coded for all patients using Adult Comorbidity Evaluation-27 (ACE-27)[15]. The ACE-27 grades specific comorbid conditions in different organ systems into one of three levels of comorbidity, and is commonly used in head and neck cancer literature. The overall comorbidity score is graded in four levels, none, mild, moderate or severe and is based on the highest ranked single ailment. Patients with two or more moderate ailments in different organ systems or disease groupings are graded as severe.

Statistical analysis

The data were analyzed with IBM SPSS Statistics, version 21.0 for Windows. For statistical processing, two variables were converted to dichotomous values. This was the case for comorbidity and lymph node status. ACE27 score 0 or 1 was noted as 'low level of co-morbidity', ACE27 score 2 or 3 was noted as 'high level of co-morbidity'[16]. Descriptive statistics, student T-tests and χ^2 tests were used to compare clear versus tumour-positive margins in bone resections with respect to patient characteristics and tumour characteristics. P-values <0.05 were considered to be statistically significant.

Multivariate analysis using logistic regression methods was performed to determine patient characteristics and tumour characteristics that are independently and significantly associated with the presence of clear versus tumour-positive margins in bone resections. For each characteristic an odds Ratio (OR), as measure of association between exposure and outcome, was calculated. An OR of <1 or 1.0 represents no predictive stratification; a value of >1 reflects increased risk of tumour-positive bone resection margins. A Log Rank test and a Kaplan Meier curve was used to compare survival of patients with tumour-negative bone resection margins versus survival of patients with tumour-positive bone resection margins. Furthermore, a Cox proportional hazards regression analysis on all data was done, after checking the proportional hazards assumption for each variable, and adjusting the survival analysis for age, gender, N status, soft tissue margin status, comorbidity and perineural growth.

RESULTS

In the selected period 158 patients underwent segmental mandibulectomy for OCSCC in our institute. We excluded 4 cases where no information on mandibular bone involvement and/or bone resection margins was available, and 27 cases without histopathologically confirmed bone invasion.

In 127 patients bone invasion was confirmed by histological examination and the status of resection margin was available. This group was further analyzed. Mean age was 62 years, and the male/female ratio was 58%/42%. Of the 127 patients, 119 had bone invasion by OCSCC based on preoperative CT and/or MR imaging, as shown in table 1. In the 8 remaining cases there was no bone invasion on preoperative imaging, but the decision for segmental mandibulectomy was made intraoperatively based on significant periosteal tumour extension around the mandible upon visual inspection and periosteal stripping during tumour removal. In these cases a marginal resection of the mandible would have resulted in insufficient remaining strength of the mandible.

Tumour-negative margins were found in 100 cases (78,8%). Tumour-positive bone margins were found in 27 cases (21,2%).

Hundred sixteen patients (91%) were treated with postoperative radiotherapy.

Table 1 - Patient characteristics of included patients.

	Patients N=127	%
Age	62,2 (mean)	10,7 (SD)
Gender		
male	74	58,3
female	53	41,7
Tumour location		
Alveolar process	62	48,8
Cheek	3	2,4
Floor of mouth	44	34,6
Lip	2	1,6
Retromolar trigone	13	10,2
Tongue	3	2,4
Clinical tumour stage (cT)		
T1	0	0
T2	4	3,2
T3	4	3,2
T4	119	93,6
Clinical lymph node status (cN)		
N0	84	66,1
N1	12	9,4
N2	30	23,6
N3	1	0,8
Preoperative imaging		
CT	77	60,6
MRI	37	29,1
Both	7	5,5
None	6	4,7
Comorbidity (ACE 27)		
none	35	27,6
mild	38	29,9
moderate	29	22,8
severe	25	19,7
Soft tissue margin status		
negative	81	63,8
positive	46	36,2
Bone margin status		
negative	100	78,7
positive	27	21,3

Table 1 - Patient characteristics of included patients. (*continued*)

	Patients N=127	%
Perineural growth		
no	59	46,5
yes	49	38,5
unknown	19	15,0
Angio-invasive growth		
no	82	64,6
yes	25	19,7
unknown	20	15,7
Spidery growth		
no	10	7,9
yes	86	67,7
unknown	31	24,4
PORT		
no	11	8,7
yes	116	91,3
Deceased		
yes	74	58,3
no	53	41,7

Statistical analysis

Table 2 shows the results of univariate analysis in regard to the distribution of all patient- and tumour characteristics between the two groups: tumour-negative and tumour-positive bone resection margins group.

Pathological lymph node (pN) status, soft tissue margin status and perineural growth differed significantly between the two groups. Because pN status has no influence on tumour resection margins it is not regarded to be associated with margin status and therefore no further analysis was performed. For soft tissue margin status and perineural growth multivariate logistic regression analysis was performed. For perineural growth a significant association was found with an OR of 4.188 (95% CI 1.527-11.484, p=0.005). Soft tissue resection margin status did not contribute independently to the bone margin status (OR 1.998, 95% CI 0.736-5.422, p=0.174).

Patients with tumour-positive bone resection margins had a significantly lower overall 5-year survival than patients with tumour-negative bone resection margins (23.0% versus 35.3%, p<0.005). However, other factors such as age or levels of comorbidity, could be contributing to a lower survival rate. Therefore a Cox regression analysis was performed, after checking the proportional hazards assumption for each variable, and adjusting the survival analysis for age, gender, pN status, comorbidity, soft tissue margin status and perineural growth. Figure 1 shows the outcome of this analysis. Survival for patients with tumour-positive bone resection margins was still decreased (Hazard ratio 2.36 (95% CI 1.204 – 4.628, p=0.012).

Table 2. A two-tailed student T-test was used for the univariate analysis of age (continuous variable) and two-tailed χ^2 tests were used to compare patient characteristics, tumour characteristics and all other categorical variables for clear versus tumour-positive bone resection margins

	Tumour-negative bone resection margins	**Tumour-positive bone resection margins**	***p-value (univariate)***
	N=100	N=27	
Age (years)	62 (SD=11)	63 (SD=11)	*0,622*
Gender			*0,747*
male	59	15	
female	41	12	
Primary tumour location			*0,370*
Alveolar process	52	10	
Cheek	3	0	
0, Floor of mouth	34	10	
Lip	1	1	
Retromolar trigone	8	5	
Tongue	2	1	
Lymph node status (pN)			*0,017*
N0	52	20	
N1	17	4	
N2	31	2	
N3	0	1	
Preoperative imaging			*0,560*
CT	61	16	
MRI	28	9	
Both	5	2	
None	6	0	
Comorbidity			*0,884*
none	29	6	
mild	29	9	
moderate	22	7	
severe	20	5	
Soft tissue margin status	69	12	*0,024*
negative	31	15	
positive			
Perineural growth			*0,004*
no	52	7	
yes	32	17	
Angio-invasive growth			*0,446*
no	65	17	
yes	18	7	

Table 2. A two-tailed student T-test was used for the univariate analysis of age (continuous variable) and two-tailed χ^2 tests were used to compare patient characteristics, tumour characteristics and all other categorical variables for clear versus tumour-positive bone resection margins (*continued*)

	Tumour-negative bone resection margins	Tumour-positive bone resection margins	*p-value (univariate)*
Spidery growth			*0,247*
no	9	1	
yes	63	23	
PORT			*0,200*
no	7	4	
yes	93	23	
Time between scan and surgery(days)	32,6 (SD=15,1)	39,1 (SD= 28,7)	*0,111*
Comorbidity			*0,820*
none or mild	58	15	
moderate or severe	42	12	

Virtually all patients (91%) with and without tumour-positive bone resection margins received postoperative radiotherapy. Accordingly, statistical analysis of the effect of postoperative radiotherapy on survival was considered not meaningful.

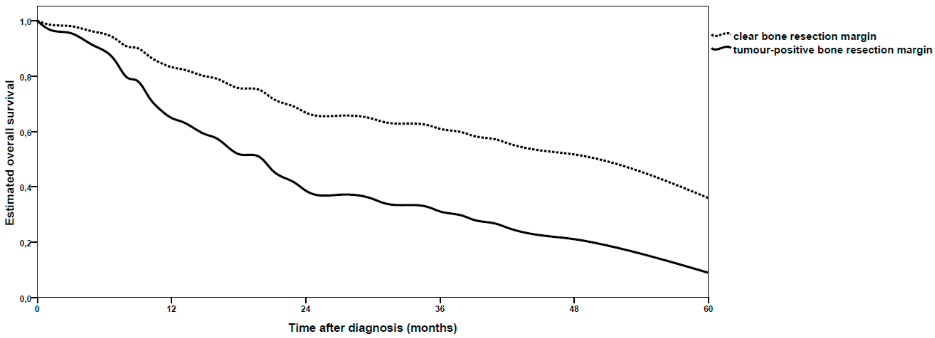

Figure 1. Estimated overall survival of patients with a tumour-negative bone resection margin (interrupted line) vs. patients with a tumour-positive bone resection margin (continuous line).

DISCUSSION

Despite the fact that the impact of surgical margins in OCSCC has been largely discussed, it is accepted that resection margins are a constant prognostic factor and major determinant for postoperative adjuvant therapy, which can lead to more morbidity[2,17-21]. We show that tumour-positive bone resection margins have a significant effect on patient overall survival. Of course, other factors such as TNM classification, tumour site, tumour histological characteristics, patient age and comorbidity also affect the clinical outcome. Of these, however, it is usually only the resection margin that can be controlled by the surgeon and the pathologist.

While the most evidence is based on soft tissue margins, reported data for bone resection margins is scarce. To date, only a few studies have reported on bone resection margins in patients with OCSCC, with the number of tumour-positive bone resection margins varying between 2% and 20%[11-13,22]. In our study group 21% of patients had tumour-positive bone resection margins. For all 27 specimens with tumour-positive bone resection margins tumour in the bone margins was not detectable by visual inspection during surgery. Because there was no suspicion of tumour-positive margins no further evaluation, in terms of frozen section of the inferior alveolar nerve or cytology of the bone marrow, was performed. In 3 out of 27 patients with tumour-positive bone resection margins the positive margin referred to microscopical nerve infiltration either proximally or distally. The remaining 24 cases included only bone tissue involvement, either marrow or cortex. No re-resection was performed for tumour-positive bone resection margins. Based on the Dutch national guidelines all pT4 cases should be considered for adjuvant radiotherapy. Accordingly, adjuvant radiotherapy was used in almost all (91%) cases of our study population, including both tumour-negative and tumour-positive bone resection margins. Remaining cases included patients who either refused adjuvant treatment, deceased or suffered from major comorbidity. Because both, patients with tumour-positive bone margins and those with tumour-negative bone margins received adjuvant radiotherapy in our study population, we believe that adjuvant treatment did not cause the difference in survival between the two groups. There was no discernible improvement in survival for the tumour-positive bone margin patients with adjuvant radiotherapy which highlights the importance of bone resection margins. In literature evidence of benefits of adjuvant radiotherapy on survival is inconsistent[23-25].

Possible causes for tumour-positive bone resection margins are discussed by a number of authors[11-13,22]. One of the suspected possible causes is delay between preoperative imaging and surgery[8,26]. Feichtinger *et al.* showed that a short period of 2-7 days between preoperative imaging and surgery could improve surgical results[8].

In our study average time between the preoperative scan and the segmental mandibulectomy was 35 days. Despite this relative long period of time, we did not find a significant association with tumour-positive bone resection margins (table 2).

Preoperative imaging itself is another important factor influencing the status of bone resection margins. Our study showed that in 27/154 cases the mandibulectomy was performed while bone invasion could not be confirmed by final histopathological examination. Of these, in 25 of these cases the preoperative imaging showed signs of bone invasion and accordingly mandibulectomy was performed. In the remaining 2 cases preoperative imaging showed no bone invasion but decision to perform segmental mandibulectomy was made intraoperatively based on the judgment of surgeon. In this study population the choice for either using CT or MR could not always be clearly extracted from the electronic patient files.

In contrast to 16% (25/154) false positives found in our study, Hoffmannova *et al.* reported a more than threefold false positive rate (54%) when comparing bone invasion on preoperative imaging (CT) with final histopathology[27].

The main limitation of current study is its retrospective character, which makes the study susceptible to bias in data selection and analysis. We were unable to consequently extract working methods of surgeons and pathologists who handled the investigated patients. For example, motivation for using either CT or MR for preoperative imaging are unclear and inconsistent. There was a substantial difference in extensiveness and clarity of pathological reports over time and between the multiple pathologists involved.

Meanwhile, not all pathological specimens could be re-evaluated because they were no longer available.

The main conclusion of this study is the finding of tumour-positive bone resection margins in 21% of segmental mandibulectomies and that these patients had a significantly worse survival, even though almost all patients in this group received adjuvant radiotherapy. Furthermore a false positive rate of 16% for CT/MR imaging was found in determining bone involvement preoperatively.

All in all, this study demonstrates that there is room for improvement where bone margins are concerned. Currently we are using both CT and MR for optimal preoperative imaging. The radiologist will indicate the exact location and extend of the tumour. Also we perform comprehensive macroscopic intraoperative assessment of bone resection margins with pathologists and surgeons together. However, an objective, accurate and fast technique should be available for intraoperative assessment of bone resection margins that could also be used to confirm bone invasion intraoperatively.

At the Erasmus MC we are now developing a method for OCSCC surgery guidance based on Raman spectroscopic intraoperative assessment of resection margins[28,29].

We believe that, together with preoperative imaging, intraoperative assessment of bone resection margins might have a direct positive impact on the surgical results and finally on the patient outcome.

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Intraoperative assessment of surgical specimen improves resection margins in oral cavity squamous cell carcinoma

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ABSTRACT

Background: Inadequate resection margins in oral cavity squamous cell carcinoma have an adverse effect on patient outcome. Intraoperative assessment provides immediate feedback enabling the surgeon to achieve adequate resection margins. The goal of this study was to evaluate the value of specimen-driven intraoperative assessment by comparing the margin status in the period before and the period after the introduction of specimen-driven assessment as a standard of care (period 2010-2012 vs period 2013-2017).

Methods: A cohort of patients surgically treated for oral squamous cell carcinoma at the Erasmus MC Cancer Institute, Rotterdam, between 2010-2012 was studied retrospectively and compared to results of a prospectively collected cohort between 2013-2017. The frequency, type and results of intraoperative assessment of resection margins were analyzed.

Results: 174 patients were included from 2010-2012, 241 patients were included from 2013-2017. An increase in the frequency of specimen-driven assessment was seen between the two periods, from 5% in 2010-2012 to 34% in 2013-2017. When performing specimen-driven assessment, 16% tumor-positive resection margins were found in 2013-2017, compared to 43% tumor-positive resection margins overall in 2010-2012. We found a significant reduction of inadequate resection margins for specimen-driven intraoperative assessment ($p < 0.001$). Also, tumor recurrence significantly decreased, and disease-specific survival improved when performing specimen-driven intraoperative assessment.

Conclusions: Specimen-driven intraoperative assessment improves resection margins and consequently, the outcome of oral cancer patients. We advocate this method as standard of care.

INTRODUCTION

Patients with inadequate tumor resection margins often receive adjuvant treatment (radiotherapy, chemoradiation and/or re-operation), which leads to higher morbidity (65).

Moreover, inadequate resection margins in oral cavity squamous cell carcinoma (OCSCC) lead to a significantly worse clinical outcome (24, 66, 67).

In our previous retrospective study, we found inadequate resection margins (i.e., a distance of ≤5 mm from tumor border to resection surface) in 85% of OCSCC cases based on final histopathology (67). Equally low numbers of adequate OCSCC resections were reported by other authors (24, 66).

This illustrates that for the oral cavity, with its complex anatomy, inspection and palpation by the surgeon during the operation are often insufficient to warrant an adequate resection.

In order to control resection margins, intraoperative assessment by frozen section procedure is available. During this procedure, the surgeon samples tissue from seemingly the most suspicious areas in the wound bed (i.e., the defect-driven intraoperative assessment). For the detection of inadequate margins during OCSCC surgery, this defect-driven frozen section procedure has been shown to have low sensitivity (20, 36, 37, 68, 69). Moreover, this procedure is time-consuming and only a limited number of tissue samples can be examined, leading to sampling error, and resulting in underestimation of inadequate margins (39-42, 44, 70). Furthermore, the defect-driven frozen section procedure cannot provide the exact length of resection margins (in millimeters); it can only indicate the presence of tumor-positive margins.

To overcome these limitations, the specimen-driven intraoperative assessment, performed by the surgeon and pathologist together, has been advocated. This approach provides immediate feedback on whether an additional resection is needed. Recent studies show that this type of intraoperative assessment is superior to defect-driven assessment due to better visualization, less sampling error and it has been recommended in the latest AJCC guidelines (18, 24, 68, 71-75).

At our institute, this multidisciplinary approach has been introduced in 2013.

This study aimed to evaluate the value of specimen-driven intraoperative assessment by comparing the margin status in the period before and the period after the introduction of specimen-driven assessment (i.e., period 2010-2012 vs period 2013-2017).

MATERIAL AND METHODS

Patient Selection

The study was approved by the institutional Medical Ethics Committee (MEC-2015-150). All patients treated surgically for OCSCC in the period from October 2010 - October 2012 and September 2013 – January 2017 were selected for analysis.

The period from 2010-2012, when specimen-driven intraoperative assessment was not standard of care, has been described earlier (67).

Data collection

A database was created containing patient characteristics (i.e., age, gender, comorbidity, smoking habit), and tumor characteristics (i.e., subsite, pathological TNM classification, differentiation grade, perineural growth, pattern of invasion).

In addition, margin status was recorded, based on both; intraoperative assessment and final histopathology. The type of intraoperative assessment was recorded as defect-driven or specimen-driven. The margins were defined based on the guidelines of the Royal College of Pathologists: >5 mm as clear, 1-5 mm as close and <1 mm as tumor-positive (17). Clear margins are referred to as adequate, close and tumor-positive margins as inadequate. All cases were reviewed by one or two dedicated head and neck pathologists (S.K., V.N.H.).

Follow up data was collected from the patient files until 27-09-2019. Data on local recurrence, regional recurrence and distant metastasis were recorded. Mortality was also recorded, including the cause of death to calculate disease-specific survival (DSS).

Specimen-driven intraoperative assessment

Figure 1 shows an example of the specimen-driven intraoperative assessment of resection margins (IOARM) procedure. During operation, the surgeon places numbered tags in a pair-wise manner on both sides of the resection line, both superficially and deep in the wound bed (Figure 1.A). When the resection is completed, one tag of each pair remains attached to the specimen and the other tag stays in the wound bed. These tags are later used to relocate an inadequate margin in the wound bed. This relocation method was described in more detail by van Lanschot *et al.* (76).

Next, the specimen is taken to the pathology department for intraoperative assessment. The surgeon and the pathologist select an anatomical template that best illustrates the anatomical orientation of the resection specimen and wound bed (Figure 1.B). The pathologist and surgeon visually inspect and palpate the specimen to locate suspicious areas (i.e., areas on the resection surface that might have an inadequate margin). If a suspicious area is found, the pathologist makes one or more parallel (partial or complete) incisions, perpendicular to the tissue surface with a mutual distance of approximately 5mm (Figure 1.C). In most cases, this enables the

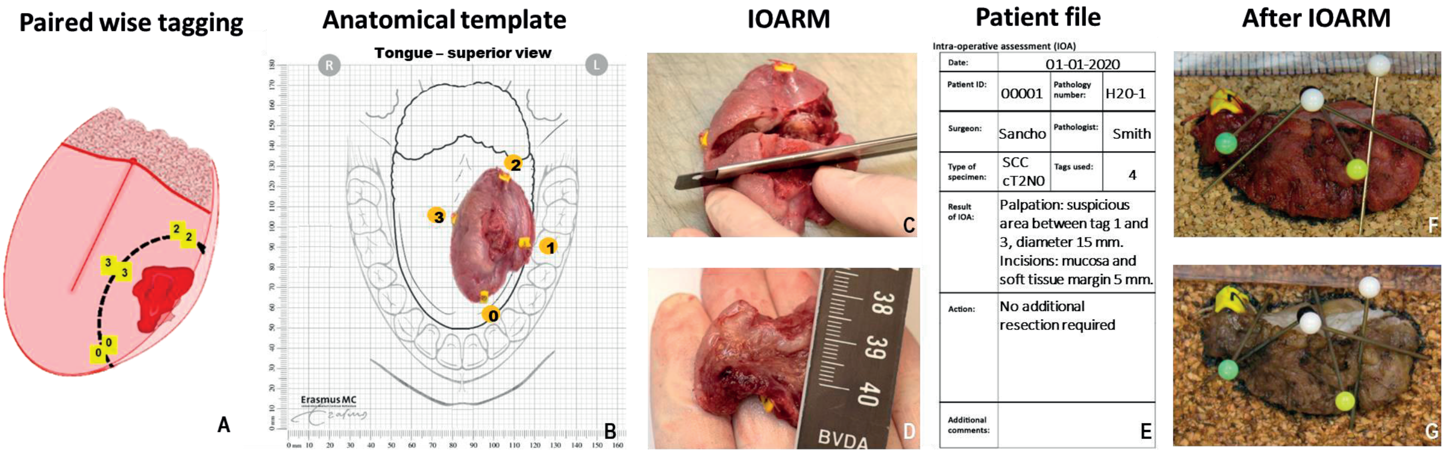

Figure 1.

A. Paired wise tagging on both sides of the resection line, performed during surgery (76).
B. Anatomical template, used to maintain orientation, tags are noted on the template.
C. Grossing of the tissue, perpendicular incisions must be 5-6mm from each other.
D. Measuring the margin with a ruler.
E. Patient file, used for patient information, reporting results and recommendations.
F. Cross section of fresh tissue placed against cork to maintain shape and orientation during fixation.
G. Cross section after fixation shows no shrinkage of tissue or change in shape.

visualization and measurement of the margin of healthy tissue on the cross-sectional side with a ruler (Figure 1.D).

If no inadequate margins are found, the surgeon can return to the operating room and close the wound. If an inadequate margin is detected on the specimen, the numbered tags enclosing such area are used by the surgeon to detect this area in the wound bed. It can then be determined if an additional resection is possible. The required thickness of the additional resection is indicated by the pathologist (in millimeters). For example, if the initial margin is 2 mm, the pathologist recommends an additional resection of tissue with at least 4 mm thickness to achieve a margin of more than 5 mm.

The whole specimen-driven IOARM process, including the conclusion and the recommendation for additional resection, is recorded and stored in the patient file (Figure 1.E).

Next, to maintain the anatomical orientation and shape of the specimen, tissue cross sections created for intraoperative assessment are placed between two pieces of cork at the original location in the specimen, and held in place by needles (Figure 1.F, 1.G) prior to formalin fixation.

After the intraoperative assessment, the resection specimen enters the routine procedure for the final pathological examination.

Statistical analysis

Differences in patient and tumor characteristics between the two periods (2010-2012 vs 2013-2017) were tested with t-test for continuous variables and with a chi-square test for categorical variables. Differences between the three intraoperative assessment types (i.e., 'no intraoperative assessment', 'defect-driven assessment' and 'specimen-driven assessment') were tested with a one-way ANOVA for continuous variables and with a chi-square test for categorical variables.

Differences in achieving adequate resection margins comparing IOARM groups were estimated with Poisson regression with robust standard errors. Crude relative risks (RR) for defect-driven assessment and specimen-driven assessment compared to no intraoperative assessment were estimated as well as RRs adjusted for gender, age, tumor size and location. Tumor subsites were: tongue, floor of mouth, alveolar process, retromolar trigone and palate. Because of the low number of patients with tumors located at the retromolar trigone and palate we decided to merge these two groups into the group 'other' for statistical analysis.

Time to local recurrence within three years after surgery was described with Kaplan-Meier estimations, and compared between groups based on margin status (i.e., >5 mm 'clear', 1-5 mm 'close' and <1 mm 'tumor-positive') with a logrank test for trend. For comparing time to all recurrence events (local recurrence, regional recurrence, distant metastasis) complete follow-up was analysed. For disease-specific survival, events within 2 months after surgery were omitted to exclude surgery-related mortality.

RESULTS

2010-2012

During this period, 174 patients were treated surgically for OCSCC at the Erasmus MC Cancer Institute. Patients and tumor characteristics are shown in Table 1.

IOARM was performed during 24 operations (14%), with defect-driven assessment in 16 cases (9%) and specimen-driven in 8 cases (5%) (Table 2).

Upon final histopathological evaluation, adequate resection margins were found in 15% of cases, close resection margins in 42%, and tumor-positive resection margins in 43% of cases. Resection margins status per subsite are shown in Table 3.

2013-2017

In this period, 241 patients were treated surgically for OCSCC at the Erasmus MC Cancer Institute. Patients and tumor characteristics are shown in Table 1.

Table 1. Patient characteristics

	2010-2012 n=174	2013-2017 n=241	p-value difference
Median age (range)	65 (16-93)	67 (24-95)	0.09
Male, %	68	53	0.002
pT1-pT2, %	53	71	< 0.001
Subsite, %			0.03*
- tongue	41	46	
- floor of mouth	27	22	
- alveolar process	27	17	
- cheek	5	8	
- lip	0	1	
- other	0	6	

* Difference tested after re-categorization to 'tongue', 'floor of mouth', 'mandible' and 'other'.

IOARM was performed in 146 cases (61%), as shown in Table 2.

Defect-driven intraoperative assessment was performed in 65 cases (27%), specimen-driven in 81 cases (34%).

Upon final histopathological evaluation, adequate resection margins were found in 32% of cases, close resection margins in 42%, and tumor-positive resection margins in 26% of cases. Resection margins status per subsite are shown in Table 3.

All cases, for both periods were subdivided into three IOARM groups; 1) no intraoperative assessment, 2) defect-driven assessment, and 3) specimen-driven assessment. The results are shown in Table 4.

Table 2. Frequency and type of intraoperative assessment of resection margins

Type of intraoperative assessment of resection margins	2010-2012 (n=174)	2013-2017 (n=241)
Defect-driven	**9%**	**27%**
Specimen-driven	**5%**	**34%**
Total	**14%**	**61%**

Table 3. Resection margin status per subsite based on final pathology

	Adequate		**Close**		**Tumor-positive**	
	2010-2012 n=26	2013-2017 n=78	2010-2012 n=73	2013-2017 n=101	2010-2012 n=75	2013-2017 n=62
- tongue	15 (21%)	51 (46%)	40 (56%)	47 (42%)	17 (23%)	13 (12%)
- floor of mouth	8 (16%)	11 (21%)	18 (36%)	24 (45%)	24 (48%)	18 (34%)
- alveolar process	1 (5%)	9 (22%)	7 (37%)	14 (34%)	11 (58%)	18 (44%)
- cheek	1 (8%)	3 (15%)	3 (25%)	10 (53%)	8 (67%)	6 (32%)
- lip	0 (0%)	3 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
- other	1 (5%)	1 (7%)	5 (24%)	6 (43%)	15 (71%)	7 (50%)

Table 4. Resection margin status in relation to IOA based on final pathology

	None		**Defect-driven**		**Specimen-driven**	
	2010-2012 n=150	2013-2017 n=95	2010-2012 n=16	2013-2017 n=65	2010-2012 n=8	2013-2017 n=81
-adequate	24 (16%)	16 (17%)	2 (12.5%)	15 (23%)	0 (0%)	47 (58%)
-close	62 (41%)	49 (52%)	6 (37.5%)	31 (48%)	3 (37.5%)	21 (26%)
-tumor-positive	64 (43%)	30 (31%)	8 (50%)	19 (29%)	5 (62.5%)	13 (16%)

Impact of intraoperative assessment

The impact of intraoperative assessment was investigated only from September 2013, when the comprehensive specimen-driven IOARM protocol was implemented.

Patient characteristics did not differ between the IOARM groups. When comparing tumor characteristics, significant differences were found for the subsite of the tumor, with the specimen-driven assessment group having more tumors located at the tongue, and fewer tumors located at the alveolar process and at the 'other' subsite (P = 0.05).

The crude relative risk of inadequate resection margins for defect-driven assessment compared to no intraoperative assessment was not significant (RR 0.93, 95% CI 0.79 to 1.09). Comparison between specimen-driven assessment and no intraoperative assessment was significant (RR 0.51, 95% CI 0.39 to 0.66). Adjusted RR of inadequate margins for defect-driven assessment was 0.93 (95% CI 0.79 to 1.09) and for specimen-driven 0.54 (95% CI 0.41 to 0.71). The results are listed in Table 5.

Table 5. Effect of intraoperative assessment on inadequate resection margins

		Unadjusted model			Adjusted model*		
		RR	95% CI	p-value	RR	95% CI	p-value
IOARM	None	ref		< 0.001	ref		< 0.001
	Defect-driven	0.93	0.79, 1.09		0.93	0.79, 1.09	
	Specimen-driven	0.51	0.39, 0.66		0.54	0.41, 0.71	

* adjusted for gender, age, tumor size and location

Specimen-driven intraoperative assessment

The accuracy of specimen-driven IOARM was calculated by comparison of margin status based on IOARM and that from final histopathology. This resulted in an overall accuracy of 63.1%.

Final margin status, with or without additional resection, is shown in figure 2.

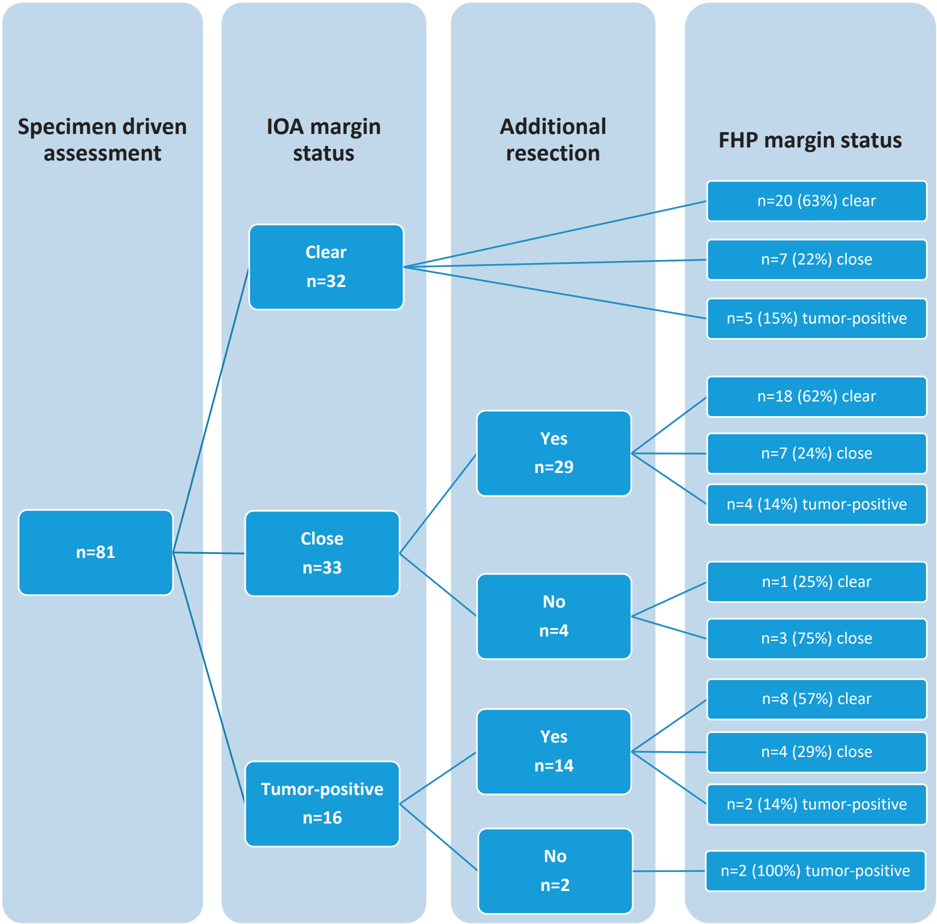

Figure 2. Comparison of margin status based on intraoperative assessment (IOA) and margin status based on final histopathology (FHP), including additional resection.

In 43 cases an additional resection was performed based on specimen-driven IOARM. In 30 cases additional resection resulted in improvement; 26 from close to clear margin, and 4 cases from positive to close margin. In the remaining 13 cases margins did not improve after additional resection.

In six cases inadequate margins were identified during IOARM but additional resection was not performed because of close proximity of vital structures.

Tumor recurrence rate and survival based on margin status

Local recurrence rate within three years was 4.5% for patients with clear resection margins, 10.6% in the group with close resection margins, and 18.5% in the group with tumor-positive resection margins (logrank test for trend P = 0.01). Kaplan Meier curves are shown in Figure 3.

The difference in occurrence of any recurrence (i.e., local, regional, distant) within 5 years was significant (logrank test for trend P = 0.001) between the three groups; 22.2% (clear), 38.3% (close) and 48.2% (tumor-positive). Kaplan Meier curves are shown in Figure 4.

For disease-specific survival these percentages after 5 years were 15.7% (clear), 20.9% (close) and 51.7% (tumor-positive) respectively (logrank test for trend P < 0.001). Pairwise comparison of clear resection margins and close resection margins showed no significant difference (P=0.60). However, when comparing clear resection margins with tumor-positive resection margins, and close resection margins with tumor-positive resection margins, there was a significant difference (both P<0.001). Kaplan Meier curves are shown in Figure 5.

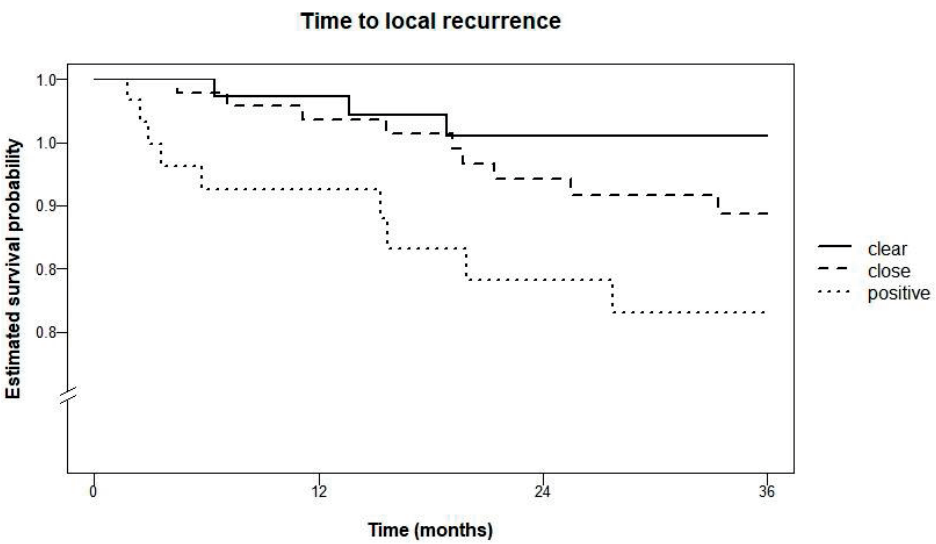

Figure 3. Kaplan Meier estimations of time to local recurrence in months.

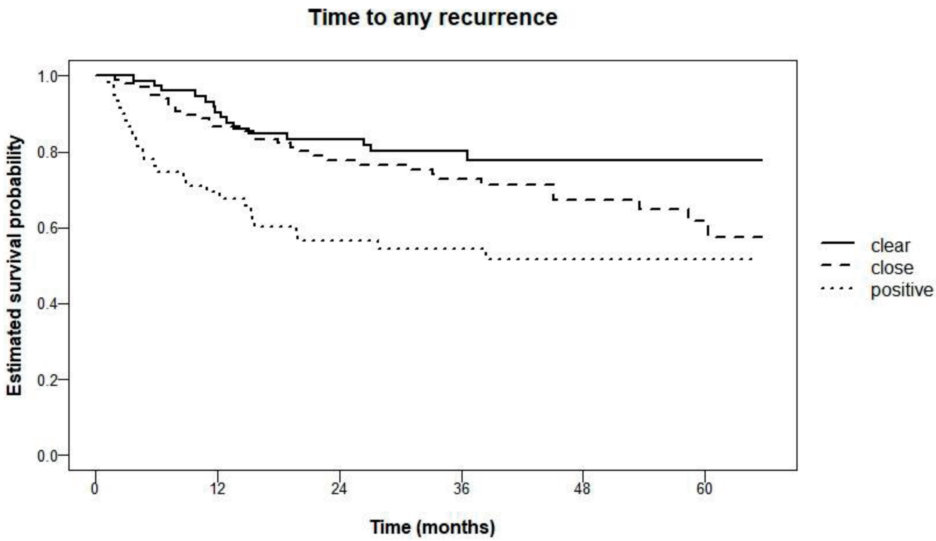

Figure 4. Kaplan Meier estimations of time to any recurrence (local, regional, distant metastasis) in months.

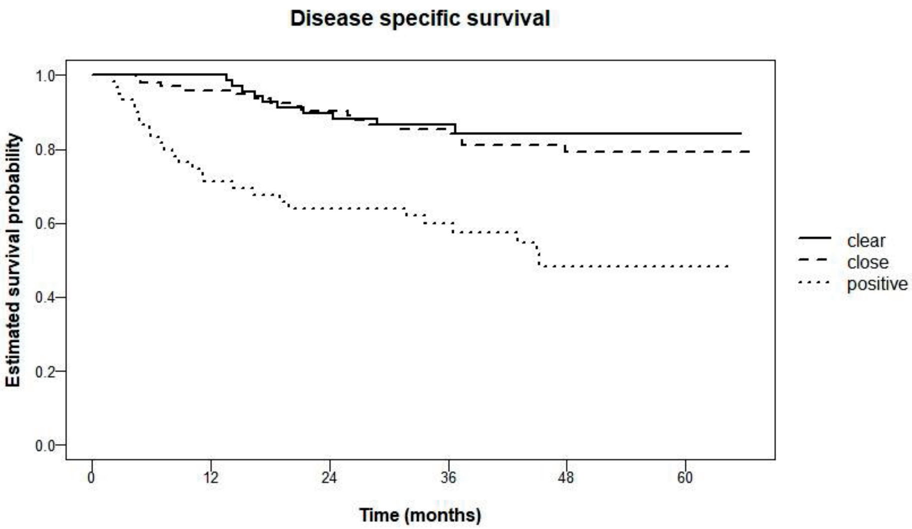

Figure 5. Kaplan Meier estimations of disease-specific survival in months.

DISCUSSION

Of all the prognostic factors (i.e., patient and tumor characteristics) in oncological patients, surgeons and pathologists can only influence the resection margins. Adequate resection of OCSCC, as for many other tumors, is sometimes hard to achieve because of a lack of reliable intraoperative guidance and the complex anatomy of the oral cavity. These are some of the explanations why multiple studies showed a high number of inadequate resection margins for OCSCC (24, 66).

To improve the status of resection margin at our institute, a comprehensive specimen-driven intraoperative assessment of resection margins has been implemented in September 2013. The procedure is performed by a dedicated team of head and neck surgeons and pathologists.

The frequency of intraoperative assessment increased from 14% for the period before 2013 compared to 61% in the period after 2013, irrespective of the assessment type. Moreover, since 2013, for OCSCC, specimen-driven intraoperative assessment was performed almost seven times more often compared to the period before 2013 (34% vs. 5%). Furthermore, we saw an increase of specimen-driven intraoperative assessment from 12% in 2013 to 54% in 2017.

Comparing the resection margin status of all cases from both periods (2010-2012 and 2013-2017), with or without intraoperative assessment, we found an increase of adequate margins from 15% to 32% and a decrease in tumor-positive resection margins from 43% to 26%. Further improvement was achieved when specimen-driven intraoperative assessment was performed: 58% adequate margins and only 16% tumor-positive margins were found after 2013. A decrease of tumor-positive margins was also seen when defect-driven intraoperative assessment was performed: from 50% to 29%. This can be explained by an increase of awareness of the head and neck surgeons who participated in this study. Since our retrospective study where we showed 85% inadequate margins overall, the head and neck surgeons confirmed that they started to be more aware of inadequate margins (3). This can explain the fact that tumor-positive resection margins decreased in all groups, even in the group without intraoperative assessment. The decrease of the number of tumor-positive margins was highest in the specimen-driven assessment group (62.5% to 16%).

The inadequate margins found when analysing specimen-driven intraoperative assessment from 2010-2012 are partly caused by the fact that we only started performing an extensive specimen-driven approach (as illustrated in this paper) in 2013. In the period 2010-2012 specimen-driven method was not optimal, and was only performed in eight cases, compared to 81 cases from 2013-2017.

As we have shown, adequate margins result in lower rates of local recurrence, regional recurrence, and distant metastasis. Also, disease-specific survival is significantly higher for patients with adequate margins. This is in accordance with other studies (18, 24, 68, 73, 74).

We therefore advocate specimen-driven assessment as standard of care during OCSCC surgery. This is in line with the latest guidelines of the AJCC (71).

There is a number of possible sources of bias in this study. During surgery, it can become evident that achieving adequate resection margins is virtually impossible due to close proximity of vital structures. Although preoperative planning is of essential importance, it unfortunately does not always reflect the intraoperative situation. Preoperative images are often made weeks prior to surgery and tumor may expand in the meantime. Because complete tumor resection (R0) remains the aim of surgery, most structures in the oral cavity can be sacrificed to obtain adequate margins. On contrary, doubt about tumor invasion in of for instance major head and neck nerves or the mandible, can pose surgeon to a difficult choice at that moment, when adequate margins are warranted.

Therefore, achieving adequate resection margins can be more difficult for some locations within the oral cavity. For tongue and lip it seems to be easier to achieve an adequate margin than, for instance, for hard palate or floor of mouth, as shown in Table 3. As there were significantly more tumors of the tongue in the specimen-driven assessment group, this could influence the results. Therefore, we have adjusted results for patient and tumor characteristics, including tumor subsite.

There are limitations of specimen-driven IOARM that need to be addressed. Grossing fresh tissue is counter-intuitive to pathologists because it is more difficult than grossing fixated tissue. Grossing fresh tissue might affect the anatomical orientation and shape of the specimen, which in turn might affect final pathology assessment (77, 78). Our specimen-driven IOARM protocol addresses this by digitally recording every step of the procedure, including the grossing of the specimen and its reconstruction on cork plates, for preservation of anatomical orientation and shape. We have not observed changes in shape or size (shrinkage) of cross sections after fixation, and we have not encountered a single case in which final pathology was affected in any way.

Performing the specimen-driven IOARM, as described here, takes additional time. We estimate that, on average, 30 minutes is needed including transfer of the specimen to the pathology department. In this time, sometimes the surgical procedure can be continued by performing a neck dissection, but in other cases the procedure has to be put on hold until results of IOARM are known.

Perhaps the most critical limitation of IOARM is that the method remains subjective and only a limited number of incisions can be placed on freshly resected specimen so as not to interfere with final histopathological evaluation. We found 63.1% overall accuracy of IOARM, which means that there is room for improvement.

A potential limitation of the current study is the fact that for close resection margins we use the definition of the Royal College of Pathologists, 1-5 mm. In recent years there has been much debate about the optimal resection margin for OCSCC (79). Several authors suggest that resection margins between 2-3 mm could be sufficient while not hampering patient outcome

(80-82). Still, no change of guidelines has been made, so for this study, we have chosen to stay with the 1-5 mm definition.

There is a learning curve to go through. For the pathologist, this learning curve comprises discriminating salivary gland tissue and scar tissue from tumor upon palpation and inspection, and to refine the procedure by microscopic evaluation of frozen sections. Another important aspect of the learning process is the meticulous handling of the tissue before fixation. However, the most important prerequisite is close coordination of logistics between surgeons and pathologists. Unfortunately, this will not be feasible for all clinical settings, so alternative methods or techniques should be investigated.

Based on the favourable results presented in this study, and despite its limitations and the additional effort, we strongly advocate the implementation of specimen-driven IOARM in OCSCC surgery.

At the Erasmus MC Cancer Institute, we are currently developing a method for OCSCC surgery guidance based on two optical techniques, fluorescence-guided surgery and Raman spectroscopy (83, 84). The combination of these techniques is being developed to allow for a rapid and accurate specimen-driven intraoperative assessment of all resection surfaces that will fit in the surgico-pathological workflow.

Only by intraoperative assessment of all resection margins, it will be possible to consistently obtain a high number of adequate margins and thereby improve the clinical outcome of OCSCC patients.

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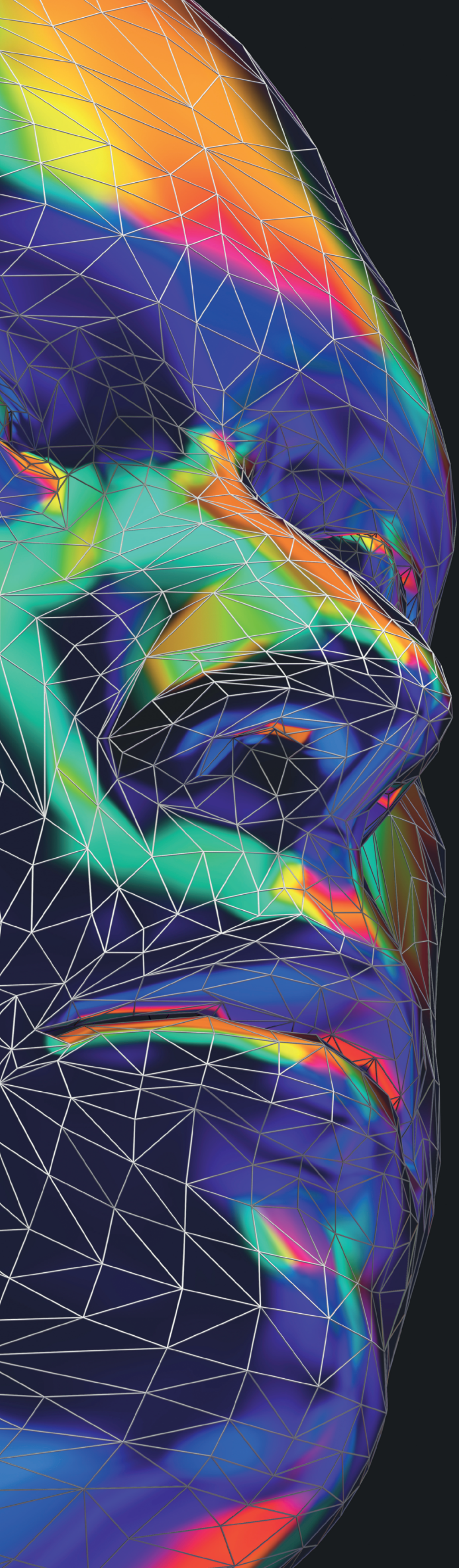

5

Discrimination between oral cancer and healthy tissue based on water content determined by Raman Spectroscopy

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ABSTRACT

Tumor-positive resection margins are a major problem in oral cancer surgery. High-wavenumber Raman spectroscopy is a reliable technique to determine the water content of tissues which may contribute to differentiate between tumor and healthy tissue. The aim of this study was to examine the use of Raman spectroscopy to differentiate tumor from surrounding healthy tissue in oral squamous cell carcinoma. From fourteen patients undergoing tongue resection for squamous cell carcinoma, the water content was determined at 170 locations on freshly excised tongue specimens using the Raman-bands of the OH-stretching vibrations (3350-3550cm^{-1}) and of the CH-stretching vibrations (2910-2965cm^{-1}). The results were correlated with histopathological assessment of hematoxylin and eosin stained thin tissue sections obtained from the Raman measurement locations. The water content values from squamous cell carcinoma measurements were significantly higher than from surrounding healthy tissue (p-value <0.0001). Tumor tissue could be detected with a sensitivity of 99% and a specificity of 92% using a cut-off water content value of 69%. Because the Raman measurements are fast and can be carried out on freshly excised tissue without any tissue preparation, this finding signifies an important step towards the development of an intra-operative tool for tumor resection guidance with the aim of enabling oncological radical surgery and improvement of patient outcome.

INTRODUCTION

The primary goal of oncological surgery is the complete removal of cancerous tissue. However, in practice this goal is often not achieved. A recent retrospective study of oral cavity squamous cell carcinoma (OCSCC) surgery showed tumor-positive resection margins in up to 43% of cases[1]. Tumor-positive resection margins are well known to lead to a significantly worse clinical outcome after surgery for oral cancer[2,3]. The most notable cause of tumor-positive resection margins is the surgeon's inability to reliably differentiate between tumor and healthy tissue by ocular inspection and by palpation. Intra-operative histopathological assessment of suspicious tissue by means of frozen-section procedures have been shown to be unreliable and time-consuming[4-6]. This is due to the fact that only a very small percentage of the resection margins can be investigated by this procedure and that the selection of apparently suspicious tissue still depends on the eyes and hands of the surgeon and/ or the pathologist. Intra-operative tools are needed which enable support to the surgeon in his assessment of the resection margin.

As early as 1971, water content was described as a possible discriminator between tumor and healthy tissue[7]. Subsequently, tissue water content has been explored using several techniques, such as magnetic resonance imaging (MRI)[8-15] and terahertz imaging[16]. Research on nuclear MRI showed that the main cause of the differences observed between the relaxation times of normal and malignant tissues is the higher water content in the latter[7,9,13-15]. MRI is a standard imaging modality for localization of a tumor in the oral cavity and for estimating its relation to surrounding tissues and size prior to surgery. However, intra-operative application of these imaging technologies is unfeasible in oral cancer surgery for practical and financial reasons[17].

Another promising technique is terahertz (THz) imaging, which is used to measure water concentration and water distribution in tissues. For breast cancer, cervical cancer, skin cancer and liver cirrhosis, high absorption coefficients were measured using this technique[16,18]. These high absorption coefficients reflect high water content. THz imaging is a surface and/or a sub-dermal technique. For measuring the skin this technique can be used quite easily and without extensive preparation. Unfortunately, this technique is still in its infancy with only a few studies describing small numbers of measurements[16]. Future research will determine whether THz imaging merits a place in the competitive world of cancer diagnostics.

García-Flores *et al.* (2011), using Fourier-Transform (FT) Raman Spectroscopy, demonstrated that cancerous tissue in mammary gland tumors in rats shows a higher signal intensity in the OH-stretching region of water (3100-3500 cm^{-1}) than normal mammary gland tissue. In that study a 1064nm laser was used for excitation and signal was detected in the 2800-3600cm^{-1} spectral region[20]. Carvalho *et al.* (2011), using FT Raman spectroscopy, showed that there are significant differences between oral inflammatory fibrous hyperplasia lesions and normal tissue with respect to the OH water bands[21]. Philipsen *et al.* (2013), using near infrared Fourier Transform (NIR-FT) Raman spectroscopy, showed that the diagnosis of malignant and nonmalignant skin lesions is also possible due to significant differences found in the bands around 3250cm^{-1}

(due to OH-stretching vibrations and N-H stretching vibrations) in combination with other specific proteins[22]. Confocal Raman imaging and infra-red (IR) spectroscopy have also indicated that the amount of water in breast cancer is remarkably higher than in non-cancerous tissue[23].

Abramczyk *et. al.* (2014) demonstrated that the vibration properties of water at the biological interfaces of human breast tissues, acquired from IR and Raman spectroscopy, are sensitive to the cellular environment of the human tissue. These differences in the vibrational properties allow distinguishing between malignant and normal human breast tissue[24].

Raman spectroscopy is very suitable for rapid quantitative (*in vivo*) determination of the water concentration of tissue[25,26]. In the current study, we have applied high-wavenumber Raman spectroscopy (HWNRS) to quantitate the water concentration in freshly excised tissue immediately after OCSCC surgery. The primary goal of this study was to assess the possibility to distinguish tumor from surrounding (healthy) tissue in fresh resection specimens immediately after the surgical procedure.

MATERIALS AND METHODS

Tissue Samples

Resection material of patients undergoing surgery for squamous cell carcinoma (SCC) of the tongue was examined. Tongue resection specimens from 14 patients were included in this study. The resections were part of the normal therapeutic surgical procedures performed at the Erasmus MC Cancer Institute, University Medical Center Rotterdam. Informed consent was obtained prior to the operation according to the protocol approved by the Medical Ethics Committee (MEC-2013-345) of the Erasmus MC Cancer Institute, University Medical Center Rotterdam. The maximum time allowed for the experimental procedure described below was fixed at 30 minutes, to keep tissue preservation optimal and to avoid interference with the routine histopathological examination of the resection specimen.

Measurement locations were chosen by the surgeon and the pathologist aiming to obtain spectra of both tumor and normal tissue. Every chosen location was digitally noted with a number on a photograph of the specimen (figure 1.3). Immediately after a Raman measurement, each measured position was marked with a colored pin (exactly at the place of laser spot) and with the respective digital number. In this way, it was possible to relocate measurement positions after routine histopathological handling of the resection specimen.

Raman Instrumentation

Raman measurements were obtained using a confocal Raman microscope (CRM), built in-house.

The instrument was placed in a room adjoining the examination room of the pathology department, enabling Raman measurements without delay. The setup consisted of a multichannel Raman Module (HPRM 2500, RiverD International B.V, the Netherlands), equipped with a

671nm laser (Crystal Laser, CL671-150-SO) and a Charge-Coupled Device (CCD-) camera, fitted with a back-illuminated deep depletion CCD-chip, which was thermo-electrically cooled to -65 °C (Andor iDus 401, DU401A BR-DD, Andor Technology Ltd., UK). The Raman Module was coupled to a microscope (Leica DM RXA2, Leica Microsystems Wetzlar GmbH, Germany)

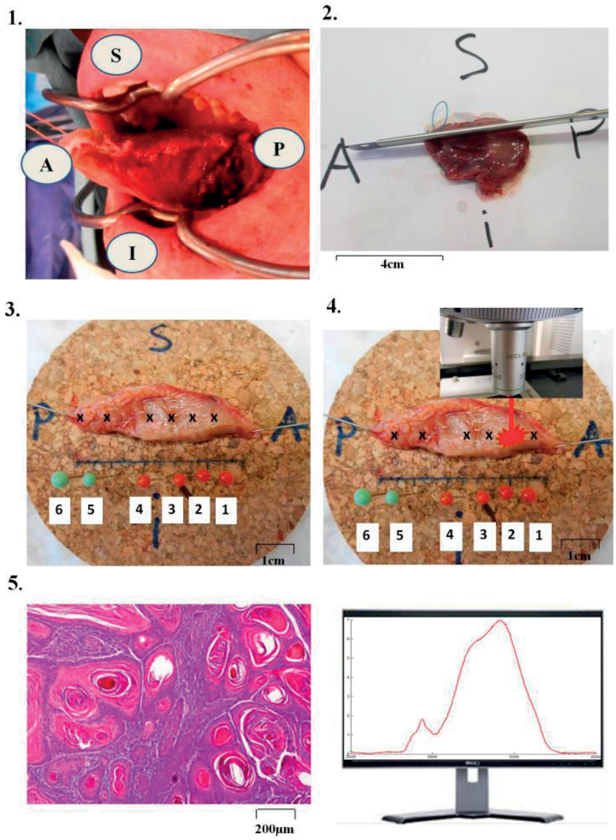

Figure 1. Overview of specimen handling and measurement protocol.

1. Resection of SCC of the tongue in the surgery room with the orientation shown (anterior (A), posterior (P), superior (S), inferior (I).
2 Immediately after operation, the resection specimen was transferred to the pathology department in close proximity to the operating room. Here, blood was rinsed from the surface of the tissue using physiological salt solution. Under supervision of the pathologist an incision was made through the tumor and the surrounding healthy tissue In this way both tumor and normal tongue tissue were made accessible for Raman measurements (see also figure 1.3).
3. Pins were used to fix the tongue specimen to a cork plate. The orientation of the specimen was indicated on the cork plate (Anterior (A), Posterior (P), Superior (S), Inferior (I)). Figures 1.3 and 1.4 also show the demarcation of the locations selected for Raman measurements by means of digital labels; red for locations presumed to be tumor and green for locations presumed to be surrounding normal tongue tissue.
4. Specimen is then transported to the adjoining Raman spectroscopy room. Raman spectra were obtained from the marked locations.
5. After the Raman measurements, the specimen was placed in formalin and processed for routine clinical histopathological evaluation as well as histopathological assessment of each of the locations marked for Raman measurements. These assessments were used for definitive histopathological annotation of the measured spectra.

and a computer-controlled sample stage (Leica DM STC). A 20x, 0.4 numerical aperture dry objective with a free working distance of 1.1 mm (N PLAN 11566026, Leica Microsystems B.V, the Netherlands) was used to focus 30-80 mW of laser light to a spot of 4μm in diameter. Spectral data were collected in the wavenumber interval from 2500 cm^{-1} to 4000 cm^{-1} with a resolution <5 cm^{-1}.

Measurement Protocol

Raman spectra were collected at the selected tissue locations. At each measurement location up to 30 measurements were carried out with a signal collection time of 1 second to assure high signal to noise, until all the selected positions were measured, or the maximum period of 30 minutes available for this study had passed. After performing the Raman measurements, the specimen was immediately immersed in formalin according to the standard protocols of the pathology department.

Histopathological Examination

Histopathological evaluation of the measurement locations (marked with pins) was performed by means of routine hematoxylin and eosin (H&E) stained thin tissue sections (figure 1.5). Each H&E-stained section was photographed and archived.

DATA ANALYSIS

Calibration and preprocessing of spectra:

After data acquisition, the spectra were calibrated to a relative wavenumber axis and corrected for the wavelength dependent detection efficiency of the setup according to the directions of the spectrometer supplier (RiverD International B.V., the Netherlands). Spectral preprocessing was applied comprising removal of cosmic ray events and subtraction of background signal generated in the optical path of the setup itself. After pre-processing an average spectrum was calculated for each measurement point.

Calculation of water concentration:

The ratio of the Raman bands at 3390cm^{-1} and 2935cm^{-1} was used to determine the concentration of water from the average spectra of each measurement point, according to the method developed by Caspers *et al.* (2001).

The method uses the background corrected bands of protein (P: 2910-2966 cm^{-1}) and water (W: 3350-3550 cm^{-1}), as illustrated in figure 2, to calculate the water concentration using the following equation:

$$water\ concentration\ (\%) = \frac{\frac{W}{P}}{\frac{W}{P} + R} * 100$$

In this equation W is the integrated Raman signal of water, P is the integrated Raman signal of protein and R is proportionality constant describing the ratio between the Raman bands of water and protein in a solution with a water concentration of 50 %[25].

Signal background was estimated by a linear fit to the first 20 points (2600-2638 cm^{-1}) and the last 20 points (3762-3800 cm^{-1}) of the signal. The CH-band intensity (P) and the OH-stretching band intensity (W) were calculated with respect to this linear signal background.

The error in determining the water concentration is mainly caused by the error in estimating the linear background, represented by the red line in figure 2. The error is estimated by calculating the two most extreme possible baselines, one with the highest possible slope, and one with the lowest possible slope. The error in the water calculation is defined as half of the difference between the maximum and minimum water concentration values, calculated using these two extreme baselines[25].

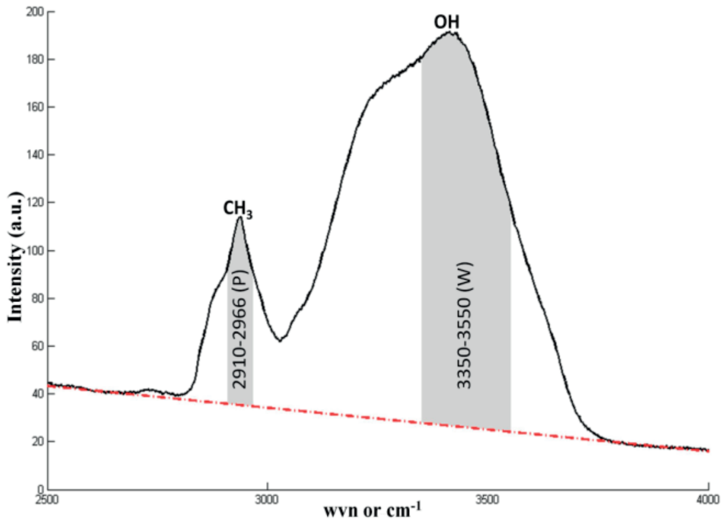

Figure 2. Example of a HWNR spectrum used to illustrate the factors W and P of equation 1. P is the Integrated Raman signal intensity of protein, represented by the gray bar located between 2910 and 2966 cm-1. W is the Integrated Raman signal intensity of water is represented the gray bar, located between 3350 and 3550 cm-1.

Statistical analysis

A Mann-Whitney U-test was used to determine if the water content of squamous cell carcinoma and the water content of healthy tissue showed a statistically significant difference.

The power to discriminate between healthy tissue and SCC was determined using a ROC (Receiver Operating Characteristic) curve. The ROC curve was obtained by determining the number of correct and incorrect classifications as a function of the threshold water concentration value to discriminate between the two groups. The Youden Index (highest combined specificity and sensitivity) was used to determine an optimal threshold value for using the water concentration as a diagnostic marker[27].

RESULTS AND DISCUSSION

One hundred and seventy Raman point measurements were performed on tongue resection specimens obtained from 14 patients. Each point measurement is represented by the average of acquisitions performed in the respective point.

Histology and Spectral Analysis

Seventy one of the 170 Raman point measurements were labeled SCC and 99 were labeled normal tissue after histopathological assessment of H&E stained tissue sections obtained from the measurement locations. Normal tissue comprised muscle, fat, connective tissue, blood vessels, salivary glands and/or nerve.

After normalization of the intensity of the spectra to the signal in the CH-stretching band ($2800cm^{-1}$ to $3040cm^{-1}$), the intensity of the OH-stretching vibration ($3450cm^{-1}$) is seen to be higher in spectra of SCC than in spectra of normal tissue, as shown in figure 3. The spectra in figure 3 were normalized with an extended multiplicative signal correction (emsc) to clearly illustrate the differences between normal and SCC measurements[28]. This normalization was not used for calculation of the water concentrations.

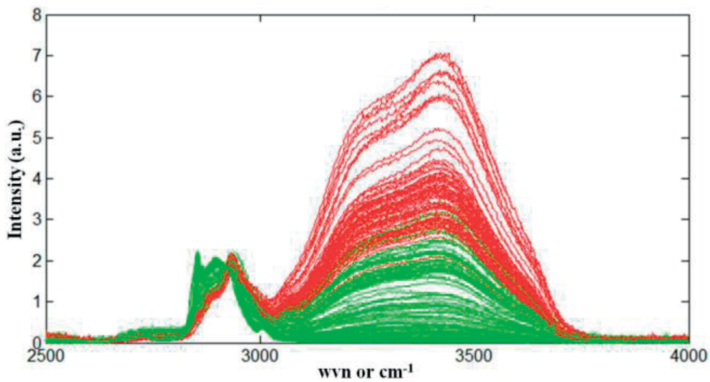

Figure 3. All 170 HWNR spectra normalized on the CH-stretching band ($2800cm^{-1}$ to $3040cm^{-1}$). Spectra are colored according to the histopathological evaluation. The green color represents spectra from healthy tissue and the red color represents spectra from SCC tissue.

The Raman experiments did not lead to tissue degeneration and did not interfere with routine histopathological evaluation of the resection specimen. The H&E sections performed as routine histopathological work-up showed, for all the measured locations, tissue without degeneration and without any damages.

Statistical significance of water concentration to differentiate normal tissue and SCC

For each spectrum the water concentration was calculated according to equation 1. Figure 4a shows a graphical representation of all water concentrations, arranged according to their histopathological annotation. For the majority of cases the water content of SCC was higher

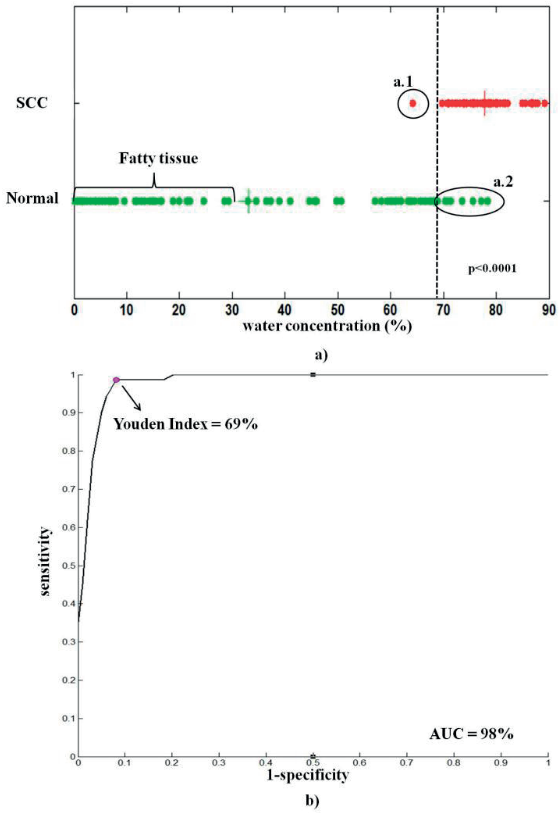

Figure 4. Scatter plot of the water concentration for all measurements (a) and the ROC-curve for discriminating SCC from normal tissue (b). (a) - the red points present the water concentration values for measurements in SCC and the green points show the water concentration values measured in healthy tissue. The p (<0.0001) value was calculated according with the Mann-Whitney u test. The vertical broken line represents the position of the cut off value calculated as Youden Index. (b) - at the Youden Index (water concentration of 69%) the sensitivity is 0.99 and the specificity is 0.92. The calculated area under the curve (AUC), representative for the discriminative power, is 0.98.

than that of normal tissue, as illustrated in figure 4a. The clear separation of data points indicates that the groups are significantly different, which was confirmed by a Mann-Whitney U-test confirming that the water concentration in SCC is significantly higher than in surrounding normal tissue ($p<0.0001$). This difference is illustrated in figure 5a, where a typical spectrum of SCC is shown together with the respective H&E stained tissue section.

Based on the distribution of the water concentration we identified two groups within the normal tissue, one with very low water concentration (0-30%) and one with moderate water concentration (>30%). The spectrum of the tissue with low water concentration is shown in figure 5b, characterized by a high intensity of the CH symmetric stretch of lipids (2724cm^{-1} and 2926cm^{-1}), CH_2 symmetric stretch of lipids (2860cm^{-1}), CH_2 asymmetric stretch of lipids and proteins (2900cm^{-1}) and unsaturated =CH stretch (3010cm^{-1})[29]. Histopathological correlation revealed adipose tissue at this measurement location, as shown in figure 5b1. This holds true in general. Whenever we encountered low water concentration this was in adipose tissue, which therefore is easy to distinguish from SCC (figure 4a). The second group of normal tissue is characterized by CH-stretching vibration typical for proteins, which corresponds to muscle and connective tissue based on histopathological evaluation, as shown in figure 5c1[29] and water concentration values higher than 30%. Figure 6c2 shows a representative spectrum of this group.

In order to verify whether adipose tissue was of major influence on the statistical significance of the differences in water concentration between SCC and all normal tissues measured, a new calculation was made after removing the adipose tissue measurements from our dataset. The difference between the water concentration in healthy tissue and SCC remained statistically significant ($p<0.0001$).

To investigate the use of the water concentration as a diagnostic marker during tumor resection surgery, a ROC curve was generated based on all measurements, figure 4b, where the true positive rate (sensitivity) was plotted against the false positive rate for different values of water concentration threshold[30]. The area under the curve proved to be 0.98. This further illustrates the high discriminatory power of water content as a diagnostic marker for SCC detection. The Youden Index was calculated for all points of the ROC curve and the highest value of the index was used as the optimum cut-off value, since it represents the best combination of specificity and sensitivity[27]. The Youden index is a water concentration of 69%, with a sensitivity of 99% and a specificity of 92%.

The mean error in the estimation of the water concentration for healthy non-adipose tissue and SCC is 0.95%. For a cut-off of 69±0.95% the sensitivity stays 99% but the specificity ranges between 86 and 92%. Therefore, errors in determination of the water content will only affect the specificity of the discrimination between healthy tissue and SCC.

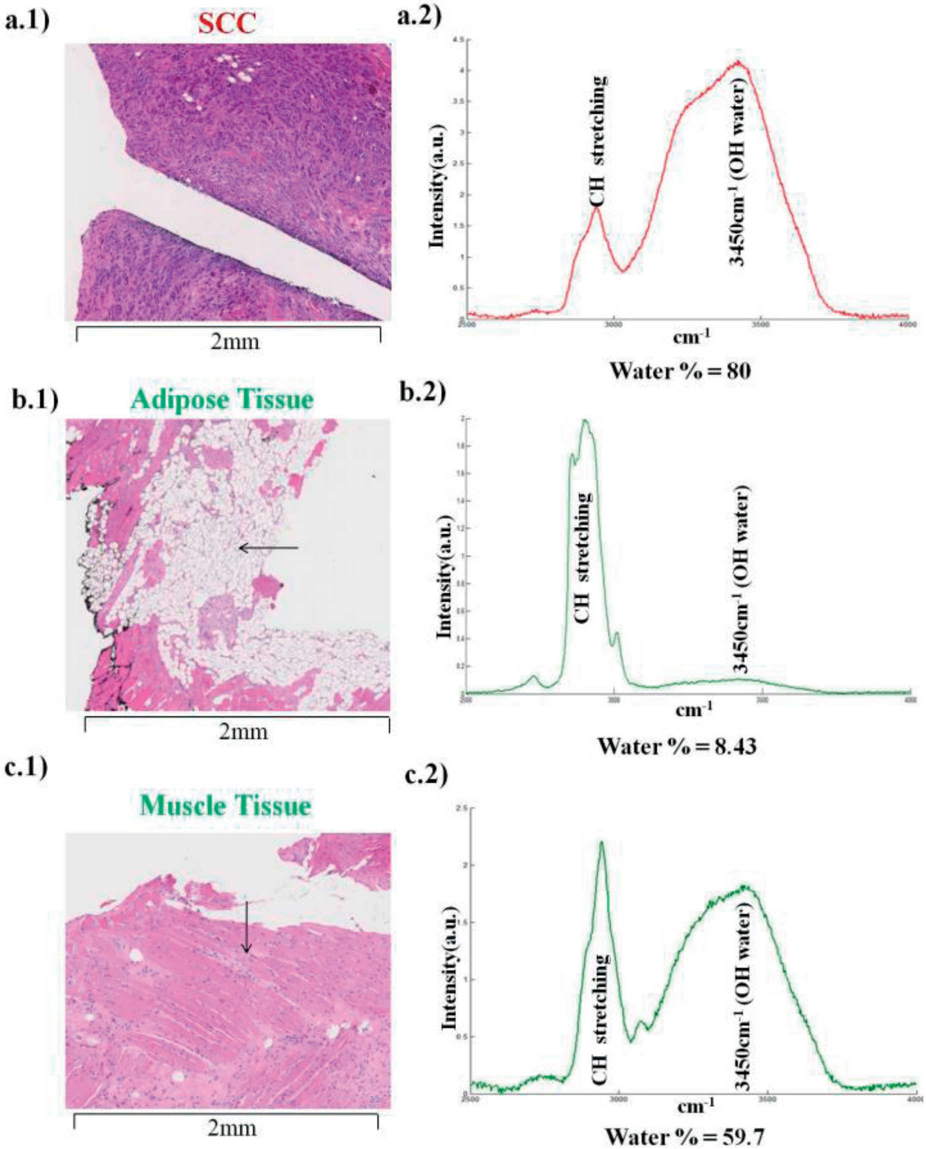

Figure 5. Examples of HWNR spectra measured in:
a.1) H&E stained thin tissue section of SCC a.2) typical Raman spectrum of SCC.
b.1) H&E stained thin tissue section showing adipose tissue (arrow) b.2) Raman spectrum of adipose tissue.
c.1) H&E stained thin tissue section showing muscle tissue (arrow) c.2) representative Raman spectrum of muscle.

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In figure 4a the highlighted points labeled a.1 and a.2 represent a measured water concentration in SCC below the cut-off concentration of 69% and a water concentration in healthy tissue above this cut-off value, respectively. The SCC-measurement showing a water concentration below the cut-off value of 69% was obtained in a tissue region that contained both SCC and normal tissue, as shown in figure 6. We cannot exclude that normal tissue was measured, however, histopathologically, this H&E stained section was identified as SCC.

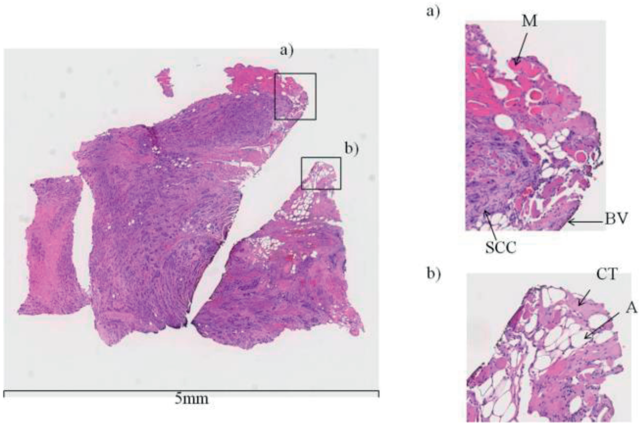

Figure 6. H&E stained thin tissue section from the only SCC-measurement that has a water concentration below the cut-off value 69%. The area where the measured point is probably located is represented by a) and b) and histologically this area contains muscle cells (M), a blood vessel (BV), some connective tissue (CT), adipose tissue (A) and tumor cells (SCC).

The eight measurements represented by label a.2 in figure 4a were of tissue locations with a mixture of connective tissue, muscle, blood vessels and nerves, showing water concentrations ranging between 71.4-78.4%. We do not have a clear explanation why the water concentration in these eight measurements is above the 69% cut off value.

The higher water concentration we found in SCC compared to healthy tissue was also found earlier in other tumors, such as melanoma, ductal carcinoma of the breast and basal cell carcinoma. However, these were qualitative observations[20-23].

Garcia-Flores *et al.* (2011) have reported a higher water concentration in chemically induced breast tumors in rats, than in normal breast tissue. In this case the normal tissue measurements appear to have been obtained from subcutaneous adipose tissue, which contains very little water, different from the tumor which is rich in water[20]. Philipsen *et al.* (2013) demonstrated by in vivo FT Raman spectroscopy a relatively stronger Raman signal contribution of water in skin lesions (including melanoma, basal cell carcinoma and benign pigmented lesions) than in normal skin. It should be noted that the spectral region assigned in this paper to water OH-stretching vibrations (3175 to 3265 cm^{-1}) strongly overlaps with NH-stretching vibrations from proteins and therefore is not the most suitable spectral region to assess water concentration in tissue[22]. Surmacki *et al.* (2013) also reported a higher intensity of the water Raman signal water in human breast tumor than in normal (adipose) breast tissue, which is low in water content by nature[23].

A first limitation of this study is related with the fact that the method to calculate the water concentration was devised for protein-water mixtures, and does not take the signals of lipids into account which will contribute to the 2910-2966 cm^{-1} band. The measurements with a high signal from lipids showed consistently low water contribution and were always associated with healthy tissue. For these reasons, we believe that these signal contributions from lipids do not influence our discrimination results. Limitations of this study also include the fact that we have a limited number of samples (fourteen) of only the tongue. Future investigation will also target other subsides within the oral cavity to see if we can extrapolate our results. Also, one can imagine that measurements of the transition zone between tumor and healthy surrounding tissue could be valuable in understanding and explaining the underlying mechanism. This is why we will keep on performing measurements and will focus on the peritumoral zone as this may contain inflammatory lesions which can influence the determining of the resection margin[21,31].

All specimens are rinsed with physiological salt solution prior measuring. We believe that this rinsing procedure does not influence the tissue water concentration measurements. Rinsing with physiological salt solution is standard procedure for both, the surgeon in the operation room and for the pathologist at the pathology department, and cannot be avoided. In the implementation of our technique, measuring on a rinsed surface will be part of the measurement protocol anyway.

Another limitation could be the fact that it is still difficult to correlate the measured points with pathology. Even though we used pins to mark the measurement spots, the pathologists found it hard to find the exact measurement spot in some cases. In some cases, the identified tissue contains both normal tissue and SCC, as shown in figure 6. Currently we are thinking of a different approach to mark the measurement spots after measuring, to overcome this problem.

The selection of the measurement points can be another limitation of the study, because the surgeons and pathologists select the points based on visual and tactile information, which could lead to a "selection bias".

Our ultimate goal would be the scanning of all resection margins, which is still far from the point measurements we describe in this study. Still, we believe that our results, promising as they are, could help to create a method to scan all resection margins. This technique can be integrated in a multi-fiber probe which can measure a bigger surface at once[32,33]. One could even imagine integrating multiple fibers in a sort of "flatbed scanner", thereby scanning a large surface at once. Also, the measurement time can be reduced drastically while maintaining discriminative power. We aim to be able to scan a resection margin in about one minute per cm^2. To select specific areas of the resection margin to scan, Raman spectroscopy could be combined with for instance auto-fluorescence to facilitate faster scanning, as described by Kong *et al.* (2013). By scanning all resection margins, we believe that the possible errors in correlating histopathology with the Raman measurements will decrease, as a 2D map is much easier to correlate than single point measurements.

Finally, this setup is not yet suitable for intra-operative use. Yet, these results can contribute to creating a method which can be used in an intra-operative system as well. Our ultimate goal would be the scanning of all resection margins, which is still far from the point measurements we describe in this study. Still, we believe that our results, promising as they are, could help to create a method to scan all resection margins.

CONCLUSIONS

In this study, high-wavenumber Raman measurements of fresh SCC tongue resection tissue specimens have demonstrated that SCC has significantly higher water content than surrounding healthy tissue. HWNRS can therefore be used to differentiate tumor and surrounding healthy tissue based on water concentration. The specific spectral information obtained in this study can be used for the development of an *in vivo* Raman spectroscopic method for border demarcation of SCC of head and neck. Raman spectroscopy is fast (measurements in the order of 1 second or less with real-time signal analysis) and can be applied through the use of hand-held fiber-optic probes at virtually any location of the head and neck area. This enables the development of both ex vivo and in vivo intra-operative applications for the purpose of assisting and guiding oncological surgical procedures towards adequate resection margins.

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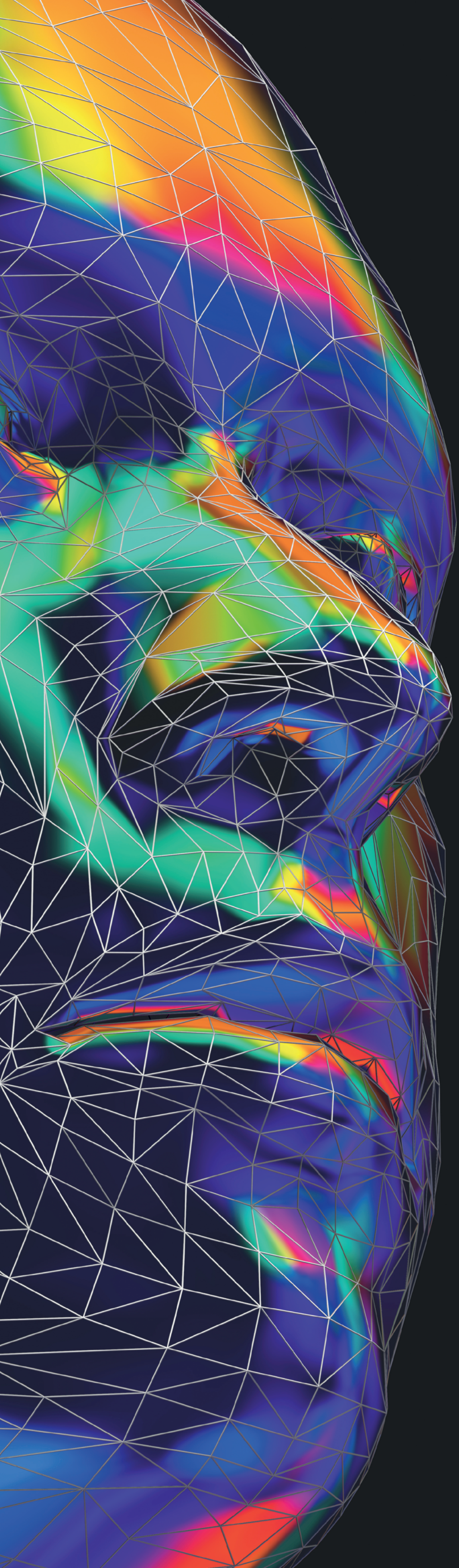

6

Water concentration analysis by Raman Spectroscopy to determine the location of the tumor border in oral cancer surgery

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ABSTRACT

Adequate resection of oral cavity squamous cell carcinoma (OCSCC) means complete tumor removal with a clear margin of more than 5 mm. For OCSCC 85% of the surgical resections appear inadequate. Raman spectroscopy is an objective and fast tool that can provide real-time information about the molecular composition of tissue and has the potential to provide an objective and fast intra-operative assessment of the entire resection surface. A previous study demonstrated that OCSCC can be discriminated from healthy surrounding tissue based on the higher water concentration in tumor.

In this study we investigated how the water concentration changes across the tumor border towards the healthy surrounding tissue on freshly excised specimens from the oral cavity. Experiments were performed on tissue sections from 20 patients undergoing surgery for OCSCC. A transition from a high to a lower water concentration, from tumor (76% ± 8% of water) towards healthy surrounding tissue (54% ± 24% of water), takes place over a distance of ≈ 4 to 6 mm across the tumor border. This was accompanied by an increase of the heterogeneity of the water concentration in the surrounding healthy tissue. The water concentration distributions between the regions were significantly different (p-values<0.0001). This new finding highlights the potential of Raman spectroscopy for objective intra-operative assessment of the resection margins.

INTRODUCTION

Oral cavity cancer is a major public health issue, with 300.000 new cases per year worldwide (1). Most oral cancers arise from the epithelium of the mucosal surface and are referred to as oral cavity squamous cell carcinoma (OCSCC). OCSCC mortality is high, with a 5-year survival of around 50% and 145,000 deaths per year worldwide (1,2). Despite advances in treatment modalities (surgery, radiotherapy and chemotherapy), these numbers have not shown significant improvement over the last decades (3,4). Important determinants of the clinical outcome of OCSCC patients are tumor subsite, TNM classification, age, comorbidity, and tumor histological characteristics (5-7). Surgery is the mainstay of treatment for OCSCC. Adequate tumor resection with acceptable remaining function and physical appearance is the main goal. At our institute, we follow the guidelines of the Royal College of Pathologists (United Kingdom). The distance between tumor and the nearest resection surface (DBTNRS) determines the adequacy of the surgical procedure. This distance is histologically measured in mm. A resection margin can be classified as clear (>5 mm of DBTNRS), close (1 to 5 mm of DBTNRS) and positive (<1 mm of DBTNRS) (8). Clear margins are regarded as adequate, close and positive margins as inadequate. Adequate resection margins are crucial for disease control and survival (8-14). Patients with inadequate resection margins often receive adjuvant therapy (chemotherapy and/or radiation), or re-resection. However, these can have a negative effect on patient morbidity.

6

Achieving adequate resection margins is challenging. The lack of reliable intra-operative guidance and the proximity of tumors to vital structures are the common causes of inadequate tumor resection. Despite comprehensive preoperative imaging of the tumor (by CT scan, MRI etc.), the surgeon decides where to cut, based on visual inspection and palpation of the tumor during the operation. Earlier, we have reported the surgical results obtained in two Dutch centers (Erasmus Medical Center Rotterdam and Leiden University Medical Center). For OCSCC surgery adequate resection margins were obtained in only 15% of the cases (9). A similar result was recently reported by the Harborview Medical Center and the University of Washington Medical Center in Seattle (USA) (11). Clearly, visual inspection and palpation of the tumor and surrounding tissue by the surgeon are insufficient to warrant adequate tumor resection.

Intra-operative assessment of resection margins by means of a frozen section procedure can be used (15). This procedure, in which the pathologist performs microscopic evaluation of a piece of suspicious tissue, is currently the gold standard of intra-operative diagnostics (15-17). The main limitation of the frozen section procedure is that only a fraction of the resection margins can be investigated. The method is prone to sampling error, which often leads to false negative results (9,18). As a result, the frozen section procedure is not very effective in improving surgical success rate. Ideally, the entire resection surface should be evaluated intra-operatively, which requires an objective and fast technology.

Intra-operative assessment of resection margins on the resection specimen (i.e. specimen driven approach) has been reported to be superior to assessment of the wound bed (i.e.

defect driven approach) by different groups. Specimen driven intra-operative assessment of resection margins leads to a higher surgical success rate and increase of patient survival than defect-driven or no intra-operative assessment at all (11,17-19).

Various techniques like ultrasonography, imprint cytology, and various optical techniques are being explored for intra-operative use in surgical oncology (20-28). Some of these techniques are being applied for OCSCC, which were recently reviewed by Ravi *et al* (2014). Optical techniques like high-resolution micro-endoscopy (HRME), optical coherence tomography (OCT), fluorescence spectroscopy, elastic light scattering spectroscopy, and Raman spectroscopy are promising because of their ease of use, relatively low cost and high speed in screening large tissue areas (20-28).

Raman spectroscopy is an optical technique that is being investigated for intra-operative evaluation of the surgical margins. Raman spectroscopy can be applied to assess the mucosa, as well as, the deep soft tissue layers (29-34). It is an objective technique based on inelastic scattering of monochromatic light that provides detailed quantitative and qualitative information about the molecular composition of tissue. The technique is non-destructive and there is no need for reagents or labeling, which promotes easier translation to the clinics (35,36).

The goal of our research is to develop a Raman spectroscopic technique for objective intra-operative assessment of the entire resection surface, with the ultimate goal to improve the success rate of OCSCC surgery. In a first pilot study we have demonstrated that Raman spectra of resection specimen discriminated tumor from healthy surrounding tissue with a sensitivity of 99% and a specificity of 92% (37). The primary discriminating factor of the Raman spectra proved to be the water concentration in the tissue. Raman spectroscopy is very suitable for rapid quantitative determination of the water concentration in tissue, as has been demonstrated by our group (38-40). The objectives of the current study were to investigate how the change in water concentration correlates with the border between tumor and surrounding healthy tissue and, consequently, to verify if this information can be used to assess resection margins.

MATERIAL AND METHODS

Medical Ethical Approval

This study was approved by the Medical Ethics Committee (MEC-2013-345) of the Erasmus MC Cancer Institute, University Medical Center Rotterdam. Prior to the operation, informed consent was obtained from the patients. Measurements were conducted ex-vivo on resection specimen of patients undergoing surgery for OCSCC. The allowed time for the experiments was 60 minutes, after which the resection specimen was put in formalin for routine histopathological evaluation.

Tissue samples and handling

Immediately after resection, the surgeon brought the specimen to the cutting room of the pathology department, which is in close proximity to the operating room. A dedicated pathologist and surgeon inspected the specimen together. This process included labeling of the anatomic sites and documentation of the specimen with diagrams and digital images (Figure 1.1).

After orienting and defining the resection margins, the pathologist and the surgeon surveyed all resection planes by visual inspection and palpation. After this, the pathologist cut the specimen in 3 - 5 cross sections (with a thickness of about 5 mm – 10 mm), perpendicular to the resection margin plane (Figure 1.2). For specimens comprising bone (i.e. mandibular resection specimens in patients with OCSCC invading the bone) the soft tissue was cut till the bone. The pathologist measured the distance between tumor and resection surface. Often, this macroscopic assessment only was sufficient to decide on the further course of the operation without the need for frozen sections. In case of an unclear tumor border the pathologist may decide to further refine the information by microscopic examination of frozen sections.

Provided with this intra-operative information regarding inadequate margins the surgeon continues to harvest more tissue from the wound bed (e.g. immediate re-resection) to achieve an adequate surgical result.

After this intra-operative diagnostic procedure, one of the specimen cross sections was chosen for Raman experiments (further called "Raman tissue section"). The cross section was regarded suitable when containing tumor and >5 mm of healthy looking surrounding tissue (Figure 1.2). The remaining specimen cross sections were immersed in formalin.

Blood was rinsed from the Raman tissue section using physiological salt solution (0.9% NaCl) and gently patted dry with gauze. The area of interest (i.e. tumor and >5 mm of surrounding healthy tissue) was macroscopically chosen by the pathologist. The Raman tissue section was inserted in a closed cartridge to avoid drying of the tissue. The upper side of the cartridge consists of a fused silica window. This cartridge allows the scanning of a 3x3 cm tissue area. The Raman tissue section was placed in the cartridge with the surface to be measured in contact with the fused silica window. Digital images of all handling steps were made, including images for the macroscopic representation of the tissue area measured (Figure 1.3).

After the experiment, the Raman tissue section was removed from the cartridge and immersed in formalin, together with the rest of the specimen to follow the routine procedure for final pathological processing.

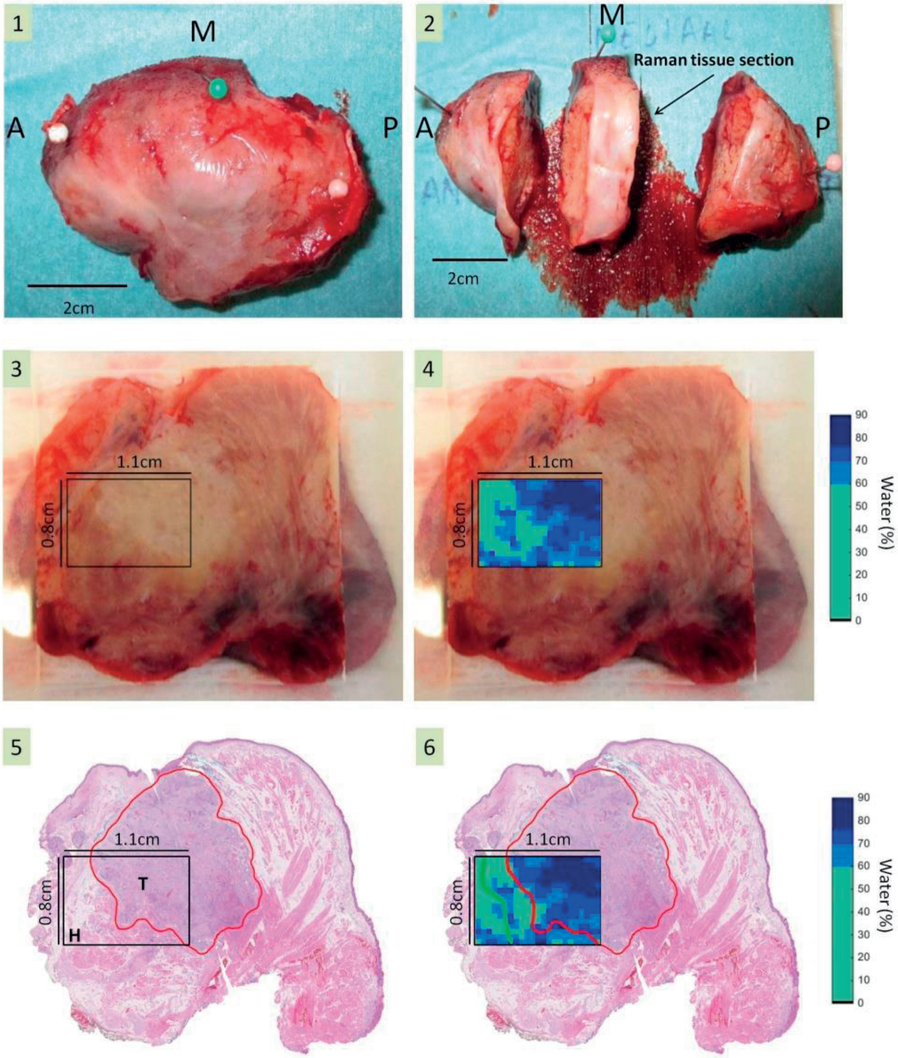

Figure 1. Overview of the experimental protocol. 1. Immediately after surgical resection, the specimen (excision of tongue SCC) was transferred to the pathology room and orientation was digitally recorded (anterior (A), posterior (P) and medial (M)). 2. The specimen was cut perpendicular to the resection surface in three sections for intra-operative assessment of the resection margins. Thereafter, a tissue section was chosen for the Raman experiment. 3. The Raman tissue section was inserted into a cartridge. The area to be measured was defined by the pathologist, containing tumor and >5 mm of surrounding healthy tissue, at least in one direction. 4. Raman mapping experiments were performed on a grid. The water concentration for each measured point was calculated. A two-dimensional image was obtained by using a nonlinear color scale to represent the water concentrations. 5. After Raman measurement, the specimen was routinely processed. H&E stained slide was made from the whole Raman tissue section within which pathologists identified the tissue area that was measured. The histopathological annotation of the tumor (T), healthy tissue (H) and of the tumor border (red line) was performed. 6. Based on the annotated tumor border in the H&E image (red line), the position of the adequate surgical margin (>5 mm of distance to the tumor border) was determined within the water map (green line).

Raman instrumentation and mapping experiments

Raman ex-vivo mapping experiments were performed using a confocal Raman microscope (CRM), built in-house. The equipment was placed in a laboratory close to the operating room. The setup, as explained in our previous work (37), comprised a multichannel Raman Module (HPRM 2500, RiverD International B.V., The Netherlands), a 671nm laser (CrystaLaser, CL671-150-SO) and a charge-coupled device (CCD) camera fitted with a back-illuminated deep depletion CDD-chip (Andor iDus 401, DU401A BR-DD, Andor Technology Ltd., UK). A microscope (Leica DM RXA2, Leica Microsystems Wetzlar GmbH, Germany) and a computer-controlled sample stage (Leica DM STC) were coupled with the Raman Module. Eighty mW of laser light was focused in the tissue by means of a microscope objective (0.4 numerical aperture) with a free working distance of 1.1 mm (N PLAN 11566026, Leica Microsystems B.V., The Netherlands). The depth resolution was 40 μm, experimentally determined. Spectral information was collected in the wavenumber range 2500 to 4000 cm^{-1} with a resolution <5 cm^{-1}.

For each measurement the cartridge with the tissue section was fixed on the microscope stage. The selected area was measured point–by-point using a grid. The grid cell size was between 300 μm per 300 μm to 1000 μm per 1000 μm, depending on the size of the tissue section and on the allowed time of 60 minutes to perform the experiment. In some cases, more than one map per specimen was measured depending on the size of the tissue section and on the allowed time. The acquisition time per spectrum was 1 second. Laser light was focused in the tissue at about 50 μm below the fused silica window surface.

Calibration and processing of spectra

All spectra were calibrated on the relative wavenumber axis and corrected for the wavelength dependent detection efficiency of the setup, according to instructions of the spectrometer supplier (RiverD International B.V., The Netherlands). Pre-processing of the spectral data was performed by removal of cosmic ray events and subtraction of the signal background generated in the optical path of the setup itself (39). MATLAB R2014b was used for data processing and data visualization.

The tissue Raman spectra showed varying levels of background signal originating from tissue autofluorescence. For the calculation of tissue water concentrations, the autofluorescent background signal was estimated by a 3rd order polynomial and subtracted from the measured spectra.

Spectra with a relative intensity lower than 5% of the average intensity of all spectra measured from the sample were discarded. Intensity of the spectra was determined for the range 2700 to 3100 cm^{-1} in which almost all spectral signatures from lipids and proteins are localized. Low signal intensities were encountered in cases where the tissue was locally not fully in contact with the measurement window.

The ratio of the Raman bands at 3390cm^{-1} and 2935cm^{-1} was used to determine the concentration of water per spectrum according to the method developed by Caspers *et al.* (2001) and described in detail in our previous study (38, 40).

Raman water maps

Raman water maps were created by plotting the water concentration as a 2D map using pseudo colors to represent the water concentration range. A convolution of the water map with a 3x3 averaging filter was applied, as shown in figure 1.4, to obtain values that are more representative of the local water concentration (reducing noise in the image), and for better visualization of the difference in water concentration between tumor and the surgical margins (41).

Histopathology

Histopathological evaluation of the measured areas was performed by two dedicated pathologists on routine hematoxylin and eosin (H&E) stained thin tissue sections. Subsequently, the H&E stained section was digitized, and the pathologists delineated healthy tissue, tumor and tumor border (Figure 1.5).

Data analysis

Based on the projection of the tumor border in the H&E image (red line) onto the Raman water map, each pixel was labeled as either tumor border, tumor or healthy (Figure 1.6). The precision with which the individual pixels could be annotated in this way is limited by the much lower resolution of the Raman map compared to the microscopic image. The error was estimated to be half of the Raman map pixel-size. Thereafter, the minimal Euclidean distance between each Raman map pixel and the tumor border was calculated. Based on these distances, the position of the adequate surgical margin (all pixels with distance >5 mm to the tumor border) was obtained (Figure 1.6).

For each map, the average and standard deviation of the water concentration were separately calculated for tumor, for the inadequate margin (i.e. distance from tumor border ≤ 5 mm), and for the adequate margin.

The Mann-Whitney U-test was used to determine if the distribution of the water concentrations in tumor, in inadequate margins and in adequate margins are significantly different from each other.

Next, we calculated the average water concentration of the tissue as a function of the distance to the tumor border. This was done by calculating the mean water concentration of pixels falling within a 0.5 mm distance interval and moving this interval from -15 mm (inside the tumor) to + 10 mm (in the healthy tissue). Likewise, the standard deviation in the water concentration was calculated as function of distance to the tumor border.

RESULTS

Twenty-five ex-vivo Raman mapping experiments were performed on fresh resection specimens from 20 patients treated by surgery for OCSCC. Table 1, shows patient and tumor characteristics.

Table 2. Patient and tumor characteristics. Number of maps measured per patient (Maps). Primary tumor location and pathological TNM classification (pTNM) of malignant tumors (42). Tumor size varied from less than 1cm (T1) to more than 4cm. In some patients, tumor had extended into the mandible (T4a). N-stage varied from no regional metastasis in lymph nodes to multiple lymph nodes with metastasis of 6cm or less in greatest dimension (N0-N2b). Distant metastasis was not encountered (M0).

Patient	Age	Gender	Maps	Primary Tumor location	pTNM
1	71	F	1	Lateral side of tongue	T2N2bM0
2	72	M	1	Floor of mouth	T2N2bM0
3	52	F	1	Floor of mouth	T3N2bM0
4	52	F	1	Lateral side of tongue	T1N0M0
5	54	M	1	Lateral side of tongue	T1N0M0
6	42	M	1	Lateral side of tongue	T1N0M0
7	59	F	1	Lateral side of tongue	T2N0M0
8	91	M	2	Lateral side of tongue	T1N1M0
9	52	F	1	Lateral side of tongue	T1N0M0
10	42	F	1	Lateral side of tongue	T4aN2bM0
11	67	M	2	Inferior alveolar process	T4aN0M0
12	60	F	1	Lateral side of tongue	T1N0M0
13	69	M	2	Lateral side of tongue	T1N0M0
14	61	M	1	Lateral side of tongue	T1N0M0
15	68	M	1	Lateral side of tongue	T1N0M0
16	79	M	2	Lateral side of tongue	T1N0M0
17	68	M	2	Retromolar trigone	T4aN2bM0
18	72	F	1	Tongue and floor of the mouth	T3N1M0
19	58	M	1	Lateral side of tongue	T2N0M0
20	61	F	1	Lateral side of tongue	T2N0M0

Each map had an average of 406 spectra (range comprehended between 97 to1250 spectra), and an average area of 240 mm^2 (from 18.9 to 624 mm^2). The average tumor area per map was 84mm^2 (range was between 13 mm^2 to 390 mm^2), the average inadequate margin area per map was 85 mm^2 (minimum value was 27.9 mm^2 and maximum value was 237 mm^2), and the average adequate margin area per map was 71 mm^2 (minimum and maximum values were respectively 4 mm^2 and 379.2 mm^2).

In total, 3526 Raman spectra from tumor were obtained. From the surrounding healthy tissue, 3620 spectra were obtained at a distance of less than 5mm from the tumor border (i.e. within the area of inadequate margin) and 3001 spectra were obtained at a distance greater than 5mm from the tumor border (i.e. from the area of adequate margins).

As an example, the results for three experiments performed on fresh resection specimens from three patients are shown in figure 2. The macroscopic images of the measured areas are shown in column A. Column B shows the water concentration maps.

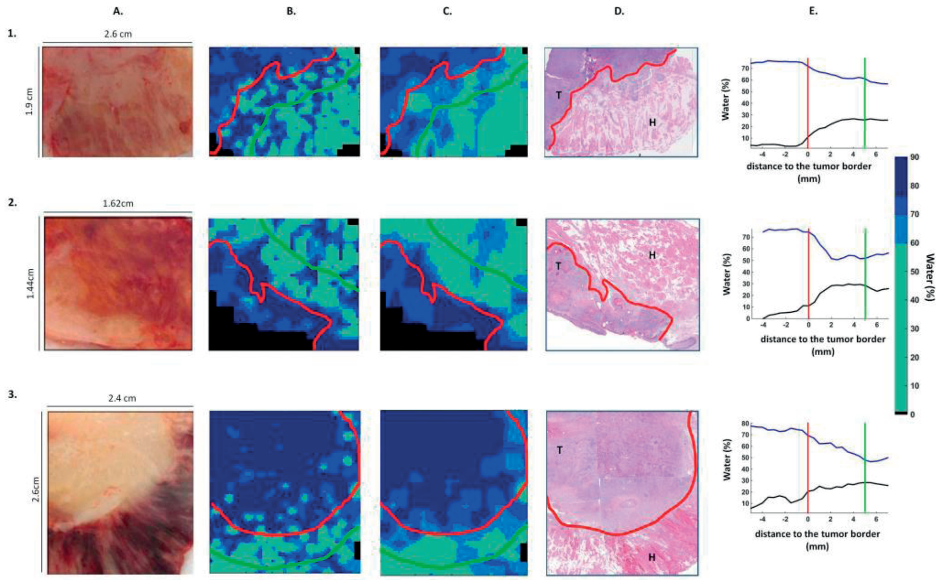

Figure 2. 1 – 3: Examples of the data obtained by means of mapping experiments on 3 Raman tissue sections from 3 patients. Panels column A: Photograph of the measured fresh tissue surface. Panels column B: Raman water map with indication of tumor border (red; based on final histopathology shown in panels of column D) and adequate surgical margin (green). Panels column C: Averaged Raman water map with indication of tumor border (red; based on final histopathology shown in panels of column D) and adequate surgical margin (green). Panels column D: H&E stained section obtained from the measured tissue surface, with tumor border (red), tumor (T), healthy surrounding tissue (H) indicated by pathologist. Panels column E: Graphs showing water concentration as function of the distance to the tumor border. Blue line: Average water concentration calculated per 0.5 mm distance interval. Black line: Standard deviation of the water concentration, per 0.5 mm distance interval. The red line at 0 mm represents the tumor border and the green line represents a distance of 5 mm from tumor border.

These maps were interpolated to a pixel size of 300 μm, which was the smallest step size used for mapping. In column C, the averaged water maps after interpolation to the same pixel size are presented. Column D shows the annotated H&E stained sections. Column E shows the average water concentration (blue line) and standard deviation (black line).

For each map the mean and standard deviation of the water concentration for tumor, in-adequate and adequate margins were calculated (table 2). The average water concentration in tumor is 76 ± 8%, in the inadequate margin it is 59 ± 24%, and in the adequate margin it is 54 ± 24%.

Table 3 – Average water concentration and respective standard deviation for each map. The water concentration was calculated specifically for the tumor, inadequate margin and adequate margin. Maps were ordered according to the TNM classification of tumors (42).

pTNM	Map	Patient	Concentration of water (%)					
			Tumor		Inadequate Margins		Adequate Margins	
			mean	std	Mean	std	Mean	std
T1N0M0	1	4	71	5	66	12	55	14
T1N0M0	2	5	71	5	65	20	62	19
T1N0M0	3	6	76	8	62	24	61	25
T1N0M0	4	12	76	6	54	28	58	24
T1N0M0	5	13	75	14	53	30	61	24
T1N0M0	6	13	76	11	49	30	57	31
T1N0M0	7	14	81	5	62	25	69	16
T1N0M0	8	15	77	4	66	21	61	26
T1N0M0	9	16	81	5	59	26	44	30
T1N0M0	10	16	77	12	57	26	43	32
T1N0M0	11	9	79	6	69	21	61	24
T1N1M0	12	8	73	10	55	26	46	33
T1N1M0	13	8	75	10	46	31	37	30
T2N0M0	14	7	78	5	60	24	55	24
T2N0M0	15	19	69	18	63	21	62	25
T2N0M0	16	20	81	3	70	23	62	24
T2N2bM0	17	1	80	9	65	25	55	30
T2N2bM0	18	2	76	6	54	20	60	22
T3N1M0	19	18	77	9	56	27	49	26
T3N2bM0	20	3	74	9	53	26	58	28
T4aN0M0	21	11	77	5	58	27	61	27
T4aN0M0	22	11	75	4	62	25	50	28
T4aN2bM0	23	10	76	8	64	18	42	21
T4aN2bM0	24	17	74	14	58	25	44	28
T4aN2bM0	25	17	75	13	52	27	43	27

Mann-Whitney U-tests show that these difference in water concentration between tumor, inadequate margin, and adequate margin are all significantly different with p-values < 0.0001.

In figure 3 the water concentration (blue line) is shown, calculated as the mean and standard deviation over all experiments, as a function of distance to the tumor border, using 0.5mm distance intervals. From the figure it is clear that, the water concentration in tumor is much higher than in the surrounding healthy tissue. The figure also shows that the drop-in water concentration coincides with the tumor border. The water concentration starts to decrease inside the tumor mass, close to the tumor border and continues to drop steeply until about 4 mm into the surrounding healthy tissue. From there the decline in water concentration continues with a smaller gradient. Interestingly, the standard deviation in water concentration values also differs between tumor and surrounding healthy tissue; from less than 10% inside the tumor to more than 15% just outside the tumor.

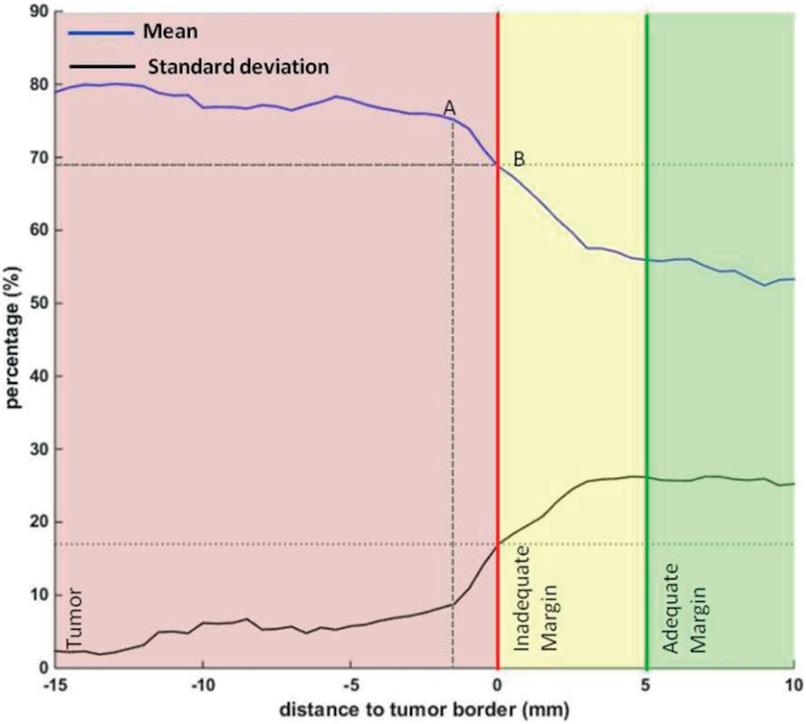

Figure 3. Water concentration profile from inside the tumor towards adequate margin. All individual water concentration percentages of the 25 maps were averaged per interval to calculate the mean (blue) and standard deviation (black) of the water concentration as a function of the distance to the tumor border. The red line at 0 mm indicates the tumor border. The green line at 5 mm indicates the beginning of the adequate surgical margin.

DISCUSSION

The aim of our research is the development of a clinical tool for intra-operative guidance of surgical-oncological procedures motivated by the main goal of surgery: adequate tumor resection and preservation of function and physical appearance. Of the many factors that affect the clinical outcome of patients with OCSCC, only the resection margins, can be influenced by the surgeon and pathologist. The objective intra-operative assessment of resection margins is the key to increase the number of adequate resections in surgical oncology, therefore, an objective tool for assessment and guidance is needed.

Multiple techniques are being explored for intra-operative use in surgical oncology (20-28). Until now, fluorescence spectroscopy (20), diffuse reflectance spectroscopy (21), elastic light spectroscopy (22), HRME (23) and OCT (24) have explored in-vivo delineation of the tumor at the mucosal surface, prior to surgery. However, eighty-seven percent of inadequate margins are found in the deeper (submucosal) soft tissue layers (43). Therefore, the design of these studies is not perfect to be applied at the submucosal layers of soft tissue, which is where the majority of inadequate margins are found.

OCT is a promising technique that has been used to investigate OCSCC resection margins. A recent study published by Hamdoon *et al* (2015) concluded that OCT is a valuable tool in the assessment of surgical margins. This study reported that the diagnostic accuracy was about 85%. However, they mentioned that the use of OCT-technology is limited, because the created image can be affected by the lack of normal tissue perfusion. Therefore, the resolution and contrast of the OCT images are influenced by the "ex-vivo nature" of the approach (44,45). Moreover, not only OCT but also HRME has as disadvantage that it requires complicated subjective image-interpretation (23,24,44,45).

Raman spectroscopy has proved to be a reliable technique that can be applied to assess mucosa as well as the deep soft tissue layers (31,36-38). This objective and non-destructive technique was used in our first study, where it showed to be accurate in discriminating OCSCC from the surrounding healthy tissue. In this previous study, we showed, by means of high-wavenumber Raman spectroscopy, that water concentration within the tumor (OCSCC) is significantly higher than in the surrounding healthy tissue enabling discrimination between tumor and healthy tissue with 98% accuracy (37). The notion that certain tumors contain more water than surrounding healthy tissue was not new; already in 1971 water content was described as one of the discriminators between tumor and healthy tissue. Diagnostic instruments like MRI use the differences in water between the relaxation times of normal and malignant tissues to generate contrast between the two (46).

In the current study, we investigated how the water concentration changes from inside the tumor towards the adequate surgical margin. The results show a clear correlation between the tumor border and the change in water concentration. The transition from a high-water concentration inside the tumor to a lower water concentration in the surrounding tissue takes place as a negative gradient over a distance of about 4-6 mm across the border of the tumor. By analyzing this negative water concentration gradient (Figure 3) we observed that the decrease in water concentration from tumor towards the adequate margin is accompanied by an increase in the standard deviation of the water concentration, i.e. the heterogeneity increases. Inside the tumor, the water concentration was higher than 69%, with a relatively low standard deviation of less than 15%. This low standard deviation indicates that OCSCC is homogeneous concerning water concentration, regardless of pTNM classification (Table 2). Inside the tumor, at about 1.5 mm distance to the tumor border, the water concentration of the tumor starts to decrease, and the standard deviation starts to increase (Figure 3.A). The average precision with which the Raman image could be annotated with the image of the H&E-stained section was ±0.38 mm (from ±0.15 mm to ±0.5 mm) and was determined by the resolution of the Raman measurements as explained in the materials and methods section. The increase in the standard deviation can indicate that close to the tumor border, the water concentration heterogeneity increases, possibly explained by the presence of stroma, blood vessels and lymphatic vessels (47). Another interesting finding is that at approximately 4 mm beyond the tumor border the standard deviation of the water concentration levels off at about 26%. This high variance of the water concentration in the surrounding healthy tissue is due to the heterogeneity in these areas comprising fat tissue, muscle (M) and vessels (Figure 4).

In this study we show the water concentration distribution across the tumor border. The shape of the water profile from inside the tumor towards the adequate margin for OCSCC is a new finding, as well as, the increase in water concentration heterogeneity at the tumor border.

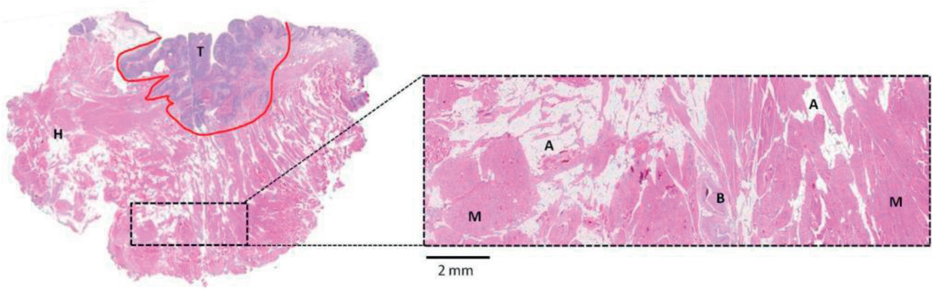

Figure 4. H&E stained section obtained from a measured tissue surface, with tumor border (red line), tumor (T) and healthy surrounding tissue (H) indicated by pathologist. A representative region of the adequate margin was enlarged and the tissue structures annotated. Tissue structures present are muscle (M), adipose tissue (A) and blood vessels (B).

We are currently devising fiber optic probe configurations and fiber optic probe measurement strategies to capture this information in a way that can be implemented for rapid intraoperative assessment of resection specimens.

We believe that Raman spectroscopy is a promising candidate for comprehensive intraoperative inspection of the surgical margins for OCSCC resection specimens, which will fit in the surgical workflow and can help to significantly improve the percentage of adequate resections.

We expect that water concentration analysis will be will be proven equally useful in localizing the tumor border in other locations of the body and plan to expand this line of investigation accordingly.

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7

General discussion

GENERAL DISCUSSION

Until the late 19th century, surgery was more or less the only available treatment modality for oral cancer. Only after the discovery of the X-ray in 1895 by the German physicist Wilhelm Conrad Röntgen radiotherapy became the favored treatment for Head and Neck cancer in the early 20th century. This was due to the fact that the morbidity was much lower compared to surgery and treatment could be more easily provided by community hospitals at that time. (85)

In the early 1940s surgery regained its popularity because of growing evidence of poor survival rates obtained with radiotherapy (5 year survival rates of around 5%) and important improvements in general anesthesia techniques. Also, the commercial production of penicillin made it possible to counter postoperative infections. (86)

Chemotherapy as treatment for cancer was also introduced around the 1940s and initially showed promising results. Currently however there is no convincing evidence that conventional chemotherapy significantly improves survival in oral cavity squamous cell carcinoma (OCSCC) patients. Nonetheless drugs such as cetuximab, an epidermal growth factor antibody, have been shown to be able to improve survival when combined with postoperative radiotherapy (PORT) for selected patients with tumor-positive resection margins and extracapsular lymph node spread. At present, radiotherapy or chemoradiation can be used for the treatment of OCSCC in a number of settings. (87, 88) These settings include PORT as an adjuvant treatment to primary surgery, for locoregional tumorcontrol for patients in whom surgery is not recommended, and as salvage treatment for patients with recurrent disease or in a more advanced stage of disease. According to the Dutch guidelines the indications for PORT may include close or tumor-positive resection margins, perineural tumor invasion, spidery growth pattern, large tumors (T3-T4), and multiple lymph node metastases, especially when extranodal extension is seen upon histological evaluation. (89) Brachytherapy can be used to treat small primary tumors and as an adjuvant to external beam radiotherapy.

Currently, surgery is the mainstay of treatment for OCSCC.

Importance of resection margins

The primary goal of OCSCC surgery is complete resection of the tumor with a surrounding margin of healthy tissue of more than 5 mm (adequate margin) in case of soft tissue resection. In case of bone involvement a tumor-free bone resection surface should be obtained. (17) Adequate resection margins lead to higher survival and a marked reduction of local recurrence. (23, 67)

At the same time, especially with the complex Head and Neck anatomy comprising numerous important (cranial) nerves and major blood vessels, it is vital to spare healthy tissue as much as possible. Therefore the surgeon is always in a delicate balance between above mentioned aims.

There is much debate in literature about the definition of clear, close and tumor-positive margins, and this debate seems to increase in recent years.

Because there is much evidence that resection margins of >5 mm improve patient outcome (e.g., local control, disease-free survival, overall survival), accordingly there should be agreement on 5 mm as definition of a clear margin. (19, 24, 67) Yet, other authors found that resection margins of <5 mm could be sufficient, especially for early-stage OCSCC. (14, 80) Nason *et al.* stated that survival improves with each additional millimeter of clear surgical margin and proposes a minimum margin of 3 mm to be considered an adequate resection.(80) Zanoni *et al.* showed that for OCSCC of the tongue, resection margins between 2.2 and 5 mm cause no greater risk of local recurrence, than margins >5 mm. (81) Jang *et al.* reported hardly any effect of resection margin status on local recurrence, but did this only for small (<3 mm maximum diameter) T1 tumors, as did Barry *et al.* for T1/T2 tumors. Dik *et al.* concluded that a margin of 3 mm with ≤2 selected adverse histological features is as safe as a margin of 5 mm in chance of developing a local recurrent lesion. (66, 82, 90) Remarkably, some studies even showed that only a margin of less than 1 mm was associated with an increased risk of local recurrence. (91, 92)

However, the evidence these authors put forward to decide what is an adequate margin is still very fragmented. This was also encountered by a review of studies on resection margins for OCSCC surgery; a notable variation in definition of adequate resection margins was encountered. (67) Close resection margins (1-5 mm of radial clearance) are associated with poor patient outcome, with a three-fold increased risk of tumor recurrence and a negative impact on patient survival. (28, 93, 94)

In 2016 Buchakjian *et al.* showed that the most important predictor of local recurrence and overall patient survival was the tumor margin status upon final histological evaluation. (70) Comparable results were found earlier by Varvares et al. showing a disease-free survival (DFS) benefit of margins >5 mm compared to margins <5 mm. (24)

Tumor-positive bone resection margins are also associated with a worse 5-year survival. (95, 96)

Until sufficient unambiguous evidence proving otherwise is found, a margin >5 mm should be pursued.

Unfortunately, for OCSCC adequate resection margins (of >5 mm) are hard to achieve. Some authors report 15-17% adequate resection margins and 43% tumor-positive resection margins. (24, 67) In case of bone involvement the affected bone should be removed with a macroscopical clear bone margin. Yet, tumor-positive bone resection margins are encountered in up to 21.2% of cases after segmental mandibulectomy. A more sparing marginal resection has many advantages in terms of function preservation, but the percentage of tumor-positive bone resection margins can be as high as 35,7%. (14, 95-98)

In case an inadequate margins is encountered based on final pathology, a second operation is mostly not an attractive option, for instance because reconstruction has taken place during primary surgery. As a result, adjuvant therapy in the form of PORT is usually indicated. This is an extra burden for the patient, which in the vast majority of cases results in additional morbidity and reduced quality of life. (65)

Therefore, the importance of "first-time-right" oral cancer surgery is clear. The results to date show that the information regarding tumor location and tumor size, obtained from pre-operative imaging, and the hands and eyes of the surgeon do not suffice to warrant successful tumor resections.

Importance of intraoperative assessment of resection margins

There is a clear need for additional information and feedback during the operation. Intraoperative assessment of resection margins (IOARM) can provide such information.

Defect-driven assessment

For defect-driven intraoperative assessment, the surgeon takes (multiple) tissue samples from the wound bed for frozen section (FS) histopathologic analysis. Traditionally, intraoperative assessment of the resection margins based on the frozen section procedure is the most chosen technique for head and neck cancer. (42)

Although frozen section analysis is a well-known procedure, available in many centers, studies have reported that it has no impact on regional control or survival in OCSCC patients. (24, 70, 75, 99-101)

Frozen section analysis shows high accuracy in tissue classification, but has shown to be poor in predicting final margin status. One of the explanations for this is that the method is time-consuming and laborious, leading to relatively few tissue samples that can be analyzed intraoperatively. Therefore the method is prone for sampling error. Large cohort studies showed no benefit with respect to local recurrence or survival, when a re-resection was performed because of a positive frozen section margin based on defect-driven intraoperative assessment. (70, 100) This may be partially caused by difficulty of relocating the exact location of the frozen section tissue sample in the wound bed after frozen section analysis. Because of the large variation of tissue histology in the Head and Neck region relocation may be particularly difficult, and therefore, a correct additional resection is not always achieved. (41, 44, 68, 102, 103) In 2001 Kerawala et al. performed an interesting experiment to explain this difficulty of relocation. They asked surgeons to place sutures in the wound bed and then pointed out exact coordinates of each suture based on fixed reference points. The surgeon was then blinded from the wound bed for 5 minutes, during which the sutures were removed. The surgeon then attempted to replace the sutures in their original locations. After that, new measurements were taken and compared to the earlier demarcated reference points. The mean peripheral margin error was 9 mm, whereas deeper areas in the wound bed were 12 mm discrepant. Additionally, there was no preponderant direction for error; thus, they argue that wide re-excisions would need to be taken in excess of 2 cm in all directions to correct the magnitude of these error. (102) This is not feasible in most OCSCC cases.

Thus, defect-driven intraoperative assessment seems insufficient to achieve "first time right" surgery.

Specimen-driven assessment

Instead of sampling suspicious tissue from the wound bed for frozen section analysis, in case of specimen-driven IOARM the resection specimen is analyzed.

A survey in 2005 showed that over 90% of Head and Neck surgeons performed defect-driven IOARM and only 14%–24% performed specimen-driven IOARM during OCSCC surgery. (19) However, since then there is growing evidence that specimen-driven IOARM is superior to defect-driven IOARM. (18, 24, 68, 73, 74, 104, 105) In 2017 Mair *et al.* showed that specimen-driven IOARM by ways of macroscopic examination and measurement of margins is as accurate as specimen-driven IOARM with frozen section analysis. (75) The American Joint Committee on Cancer (AJCC) has suggested specimen-driven IOARM to be considered standard of care. (71) At our institute, we have implemented a comprehensive specimen-driven IOARM since 2013, which has been updated after critical reviews in the following years. Figure 1 shows an example of the specimen-driven IOARM procedure as is performed at our institute in recent years. Before resection of the tumor, the surgeon places numbered tags in a pair-wise manner on both sides of the resection line, both superficially and deep in the wound bed (Figure 1.A). After resection of the tumor with a macroscopical adequate margin, one tag of each pair remains attached to the specimen and the other tag stays in the wound bed. These tags are later used to relocate a possible inadequate margin in the wound bed. This relocation method was described in more detail by van Lanschot *et al.* (76).

Next, the specimen is taken to the pathology department for assessment of the resection specimen. The surgeon and the pathologist select an anatomical template that best illustrates the anatomical orientation of the resection specimen and wound bed (Figure 1.B). The specimen is inspected and palpated to locate suspicious areas (i.e., areas close to the resection surface that might be an inadequate margin). Next, the pathologist makes one or more parallel (partial or complete) incisions, perpendicular to resection surface with a mutual distance of approximately 5mm (Figure 1.C).

In most cases, this enables clear macroscopical visualization and facilitates measurement of the margin of healthy tissue on the cross-sectional side with a ruler (Figure 1.D).

Frozen section samples can be taken from the specimen in case of doubt. For instance, in cases when tumor border is not clearly visible and margin can therefore not be determined with certainty (e.g. in case of previous surgery resulting in scar tissue, previous radiotherapy resulting in fibrosis). Recent data (2018-2019) show that in 22% of cases frozen section samples were taken during specimen driven intraoperative assessment.

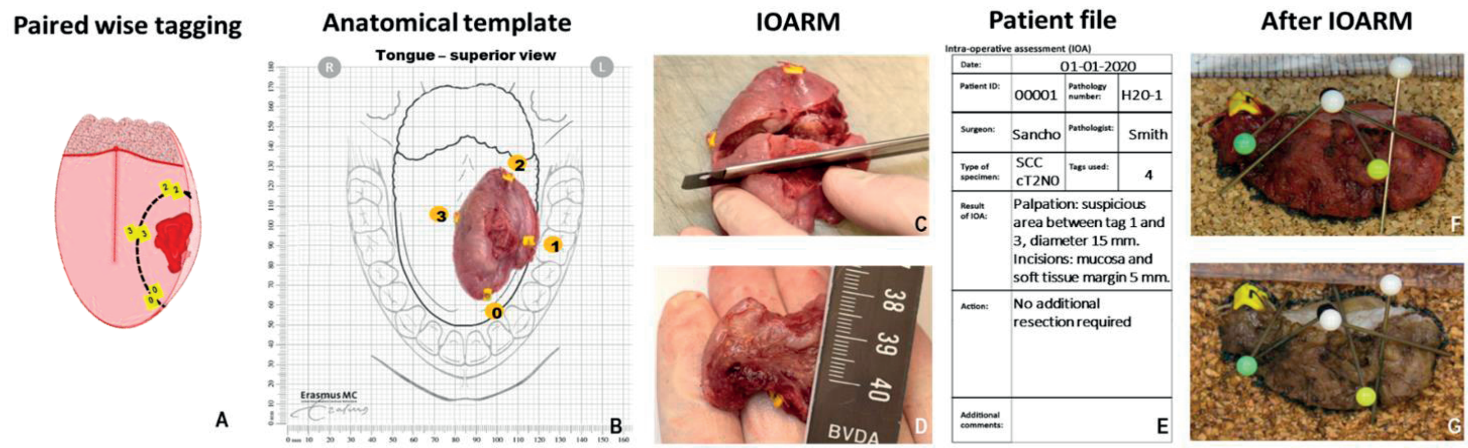

Figure 1. Specimen-driven intraoperative assessment (adapted from Smits et al. Frontiers)

H. Paired wise tagging on both sides of the resection line, performed during surgery (76).

I. Anatomical template, used to maintain orientation, tags are noted on the template.

J. Grossing of the tissue, perpendicular incisions must be approximately 5 mm from each other.

K. Measuring the margin with a ruler.

L. Patient file, used for patient information, reporting results and recommendations.

M. Cross section of fresh tissue placed against cork to maintain shape and orientation during fixation.

N. Cross section after fixation shows no shrinkage of tissue or change in shape.

If no inadequate margins are suspected, the surgeon can return to the operating room and close the wound. In contrast, if an inadequate margin is detected on the specimen, the numbered tags are used by the surgeon to relocate this area in the wound bed. It can then be determined if an additional resection is possible or not. In some cases close relation of the resection to structures such as cranial nerves or the mandible might lead to the surgeon accepting the inadequate margin. However, in case of close relation to the mandible a marginal mandibulectomy or at least periosteal stripping should be considered. The recommended thickness of the additional resection is precisely indicated by the pathologist (in millimeters). For example, if the initial margin is 2 mm upon specimen-driven IOARM, the pathologist recommends an additional resection of tissue with at least 4 mm thickness to achieve a margin of more than 5 mm.

The entire process of specimen-driven IOARM, including the conclusion and the recommendation for additional resection, is recorded and stored in the patient file (Figure 1.E).

Next, to maintain the anatomical orientation and shape of the specimen, the tissue sections are placed between pieces of cork at the original location in the specimen, and held in place by needles (Figure 1.F, 1.G) prior to formalin fixation.

After the intraoperative assessment, the resection specimen enters the routine procedure for pathological examination.

Preferably, as shown above, the entire IOARM process including photographs is recorded and stored in the patient file. This information can then be used during final pathologic assessment and multi-disciplinary consultations.

The complete workflow is shown in a recent video publication from our institute in ? Journal of Visualized Experiments. (106)

Results of the specimen-driven IOARM as showed in figure 1 were published in 2020. (105) An overall accuracy comparing margin status assessment during specimen-driven IOARM and final histopathology of 63.1% was calculated. In 53% of cases an extra resection was taken after specimen-driven IOARM. This lead to improvement of resection margin upon final histopathology in 70% of cases.

According to final pathology 58% of patients had adequate resection margins, 26% close resection margins and 16% tumor-positive resection margins after specimen-driven IOARM between 2013-2017. This compared to only 23% adequate and 29% tumor-positive resection margins after defect-driven IOARM in this period of time. Furthermore, a significant reduction in tumor recurrence and significant improvement of disease-specific survival was found for the specimen-driven IOARM group.

Yet, although specimen-driven IOARM has led to a significant increase of adequate resection margins in OCSCC, we believe that there may be hurdles for wide implementation of this method. One of the main concerns of implementing specimen-driven IOARM as described in detail above include the fact that grossing fresh tissue may feel counter-intuitive to pathologists potentially deteriorating the anatomical orientation and the shape or size of the specimen when protocols are not followed. These obstacles may potentially affect the final, postoperative pathologic assessment. (77, 78) Another concern is that specimen-driven IOARM may be more time-consuming than defect-driven IOARM, because of the distance between the operating room and department of pathology. Furthermore, it is not realistic to expect that specimen-driven IOARM can be easily adopted by every medical center because a dedicated team of head and neck surgeons and pathologists that are trained on this matter is required. Finally, because personnel involved can change between surgeries or in time, results of specimen-driven IOARM can be prone to subjectivity. Therefore other techniques should be investigated to further approach 100% adequate resection margins.

Objective intraoperative assessment of resection margins

Apart from striving to 100% adequate resection margins, ideally an IOARM method is objective and fast. Dozens of techniques were investigated in the last decades, varying from chromosomal analysis to high-resolution microendoscope (HRME) and imaging techniques such as MR and PET-CT. (31, 35, 52, 107) Also, the potential benefits of various optical techniques like optical coherence tomography (OCT) and elastic scattering spectroscopy (ESS) have been investigated. (49, 50) Some of the most relevant techniques are listed below:

Ultrasound

Ultrasound has been introduced as a technique that could delineate the deep margin in oral tongue squamous cell carcinoma. (108) Moreover, ultrasound has been shown to be accurate in determining the depth of invasion of squamous cell carcinoma of the tongue with a depth of invasion up till 10 mm. (109) Unfortunately, correlation between depth of invasion exceeding 10 mm estimated with ultrasound and found after histological evaluation is difficult. Therefore ultrasound seems unsuitable for intraoperative assessment of resection margins in locally advanced stage (T3-T4) OCSCC. Furthermore, prolonged operative times are described and the technique is highly operator dependent as compression of the tissue with the ultrasound probe leads to discrepancies in reporting the tumor thickness. (110) Finally, with the current available ultrasound probes it seems virtually impossible to deploy this technique for other subsites than the oral tongue. Some authors even showed that intraoperative ultrasound can only be used for examining the anterior part of the tongue. (109)

Fluorescent imaging

Fluorescent optical imaging is based on the interaction between tissue and penetrating light energy to expose unique tissue characteristics. This can be achieved by utilizing a light source or laser to reach targeted fluorophores within the tissue that are capable of producing a distinct emission which can then be captured either by human sight or a camera. (74) These fluorophores can either be endogenous (i.e., hemoglobin in autofluorescence imaging) or exogenous (i.e., fluorescein administered intravenously). Fluorescence imaging could be suitable to be used to delineate specific tissues within the surgical wound bed, and more specifically to clarify tumor margins. (111, 112) Preclinical trials have demonstrated the potential of tumor detection using near-infrared (NIR) fluorescence. (113) The absorption coefficient of tissue is optimal when using light in the NIR region. This results in a minimal light absorption and nonspecific autofluorescence, resulting in an increase in tissue penetration. (114) However, approval of the use of organic fluorophores by the US Food and Drug Administration (FDA) is needed. Indocyanine Green (ICG) showed great potential as a fluorescent dye injected the day prior to surgery. (115, 116) Another possibility is the use of topical agents such as γ-glutamyl hydroxymethyl rhodamine green (HMRG), which yields a specific fluorescent product in the presence of enzymes that is upregulated in OSCC tumors. (117, 118)

Narrow bang imaging

Narrow band imaging (NBI) targets hemoglobin as endogenous chemophore utilizing video imaging with specific light source bands of 415 and 540 nm. Hemoglobin has a specific peak wavelength absorption that corresponds exactly to the light source bands used in NBI, resulting in a minimum of reflectance at those wavelengths. The result is a low signal in areas with higher hemoglobin levels due to rich vascularity, such as in neoplastic lesions. (119) Comparison between conventional white light examination and NBI guided resection of OCSCC showed an improvement in final margin status with the use of NBI. (120) Another study showed that the use of NBI in OSCC surgery led to improved local recurrence rates and improved disease-specific survival. (121)

Raman spectroscopy

Raman spectroscopy is among the most promising optical techniques for intraoperative assessment of resection margins. Raman spectroscopy is an optical technique that does not require sample preparation, like the injection of dyes in NIR. (122, 123) Raman spectroscopy provides real-time information about the molecular composition of the tissue. (60, 124) Multiple authors have investigated the potential of Raman spectroscopy in OCSCC diagnosis and OCSCC surgery guidance. (60, 64, 125, 126) Earlier studies have shown that Raman spectroscopy can discriminate between OCSCC and surrounding healthy tissue with a sensitivity of 99% and a specificity of 92%. (83, 127) At the Erasmus Medical Center in Rotterdam, Raman spectroscopy is implemented in an instrument employing a fiber-optic needle probe (Figure 2). This fiber-optic needle is driven into the specimen. Based on the Raman spectra collected through the needle along the insertion path, it is determined whether the needle tip is in healthy or tumor tissue. This principle is used to measure the resection margin in millimeters. This method takes a few seconds per measurement and enables objective measurement of resection margins without the need for cutting the specimen. This prototype instrument is now validated in a research setting by collecting Raman measurements in the majority of the current OCSCC resection specimens (figure 3). (128)

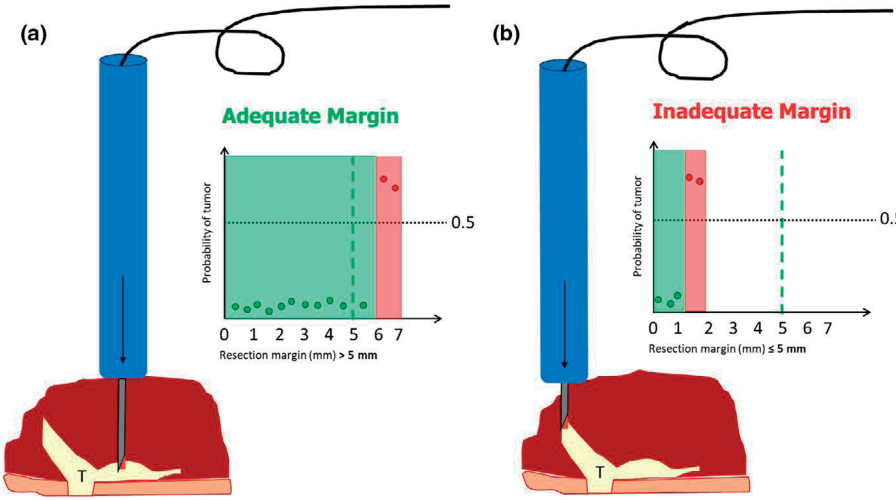

Figure 2. Illustration of specimen-driven IOARM based on Raman spectroscopy.

The fiber-optic needle is driven into the specimen, from the resection surface towards the tumor border. Raman spectra are collected along the insertion path at each 0.5 mm of depth. In the graphs each measurement is presented as a dot; the x-axis shows the measured resection margin in millimeters, the y-axis shows the probability of individual measurements to be classified as tumor or not.

2a: Example of adequate margin (6 mm), no additional resection is recommended.

2b: Example of inadequate margin (1.5 mm), an additional resection is recommended.

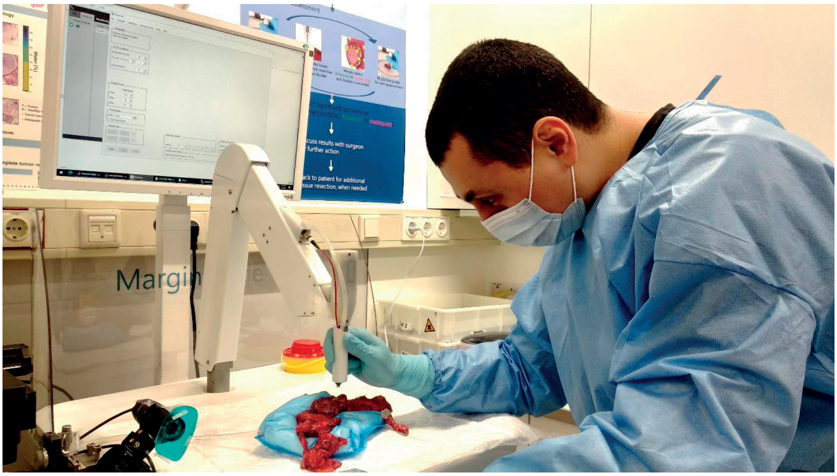

Figure 3. Prototype Raman instrument employing a fiber optic-needle probe. The fiber-optic needle is driven into the OCSCC specimen, from the resection surface towards the tumor. Based on the Raman spectra collected along the insertion path, the location of the tumor border can be determined. (130)

In addition to soft tissues, Raman spectroscopy can also be used to assess osseous resection margins. (129) When shown to be feasible and reliable, this Raman spectroscopic approach could solve the persisting problem of the lack of any reliable and feasible intraoperative assessment of bone resection margins. (95, 96)

Findings of this thesis

The main goal of this thesis was to elaborate on different ways to improve resection margins in OCSCC surgery. But the starting point in terms of resection margins was investigated first in two Dutch academic centers.

At the Erasmus University Medical Center (Erasmus MC) and Leiden University Medical Center (LUMC) data of 291 patients who were surgically treated for OCSCC were extracted from the patient history files. Histopathological reports were obtained from all patients. Resection margins (soft tissue) were defined (according to the Royal College of Pathologists) as clear: smallest distance between tumor and resection border >5mm, close: smallest distance between tumor and resection border 1-5mm and positive: distance from tumor to resection border <1 mm. (17) Clear margins are regarded adequate, close and positive margins inadequate. Furthermore a literature review was carried out describing all publications reporting on OCSCC surgery and resection margins. Main findings include a total of striking 85% inadequate margins for both centers. Furthermore, for Erasmus MC, significant positive impact of adequate resection margin compared to the inadequate resection margin was seen in case of, local recurrence, regional recurrence, metastasis and death. At the LUMC similar results were seen for chance of developing local recurrent lesions. Furthermore, Erasmus MC data showed that survival decreased in the group of patients with inadequate resection margins ($p=0.016$). LUMC data also showed a decrease of survival in the group with inadequate resection margins, not statistically significant ($p=0.07$). The eight studies found in the literature review totaled 2557 patients. Definition of clear resection margins varied between ">2 mm" and ">7 mm", and some studies did not even give the definition of a clear resection margin. (16, 20, 29, 131-135) Statistical analysis was not carried out on the literature review data because of lack of required information.

Most research focuses on soft tissue resection margins, were as only few investigate bone resection margins. In case of resection and subsequent reconstruction of bone, more specifically the mandible, it is of great importance that resection margins are clear at primary surgery. After segmental mandibulectomy, free flap reconstruction often combined with transposition of the fibular bone is conducted. This means that second stage surgery in case of a tumor-positive bone resection margin is not desirable. A retrospective review was carried out in which medical records of patients undergoing segmental mandibulectomy for OCSCC with bone involvement between 2000-2012 were analyzed. A total of 127 patients were included. Tumor-positive bone resection margins were found in 21% of patients. Soft tissue resection margin status did not contribute independently to the bone margin status. Overall 5-year survival was significantly lower in the group with tumor-positive resection margins ($p<0.005$).

Both for soft tissue and bone resection margins there are different methods to improve resection margin status. Intraoperative assessment of resection margins (IOARM) seems to have an important potential in lowering the number of inadequate resection margins. A cohort of patients surgically treated for OCSCC at the Erasmus MC, between 2010-2012 was studied retrospectively and compared to results of a prospectively collected cohort between 2013-2017. The frequency, type and results of intraoperative assessment of resection margins were compared. 174 patients were included from 2010-2012, 241 patients were included from 2013-2017. An almost 7-fold increase in the frequency of specimen-driven assessment was seen between the two periods, from 5% in 2010-2012 to 34% in 2013-2017. When performing specimen-driven assessment, 16% tumor-positive resection margins were found in 2013-2017, compared to 43% tumor-positive resection margins overall in 2010-2012. We found a significant reduction of inadequate resection margins for specimen-driven intraoperative assessment ($p <0.001$). Also, tumor recurrence significantly decreased, and disease-specific survival significantly improved when performing specimen-driven intraoperative assessment. Yet even when specimen-driven intraoperative assessment of resection margins is performed, 42% inadequate margins (16% tumor-positive, 26% close) are found. Therefore it seems crucial to elaborate on techniques to strive towards 100% adequate resection margins. Optical techniques such as Raman spectroscopy may have great potential in examining the resection specimen for inadequate margins.

High-wavenumber Raman spectroscopy is a reliable technique to determine the water content of tissues which may contribute to differentiate between tumor and healthy tissue. From fourteen patients undergoing OCSCC surgery, the water content was determined at 170 locations on freshly excised tongue specimens using the Raman-bands of the OH-stretching vibrations (3350-3550cm^{-1}) and of the CH-stretching vibrations (2910-2965cm^{-1}). The results were correlated with histopathology. The water content values from squamous cell carcinoma measurements were significantly higher than from surrounding healthy tissue ($p <0.0001$). Squamous cell carcinoma could be detected with a sensitivity of 99% and a specificity of 92% using a cut-off water content value of 69%. The findings of this study can potentially be used to develop a method based on Raman spectroscopy to scan all resection margins of the resection specimen. When combining multiple Raman point measurements a Raman map can be created in order to delineate the tumor border.

Using high-wavenumber Raman spectroscopy the water concentration gradient from the tumor border towards the healthy surrounding tissue was measured. Raman tissue sections were cut from the resection specimen and subsequently a 2D map was created containing hundreds of point measurements (average 406 spectra). These 2D maps could be correlated with final histopathology. A transition from high to low water concentration was seen from the tumor (76 ±8% of water) toward the healthy surrounding tissue (54% ± 24% of water). The water concentration distributions between the separate regions were significantly different ($P<0.0001$).

CURRENT STATUS AND FUTURE DIRECTIONS

Currently specimen-driven IOARM is performed in more than 90% of patients at our institute. However, we strive for 100% especially because there is no good reason to not perform specimen-driven IOARM.

Unfortunately we learned that not many centers are performing specimen-driven IOARM and there is scarce literature on this topic. So in order to make this method as easy to use as possible, we are continuously taking measures to further optimize it. Already since 2013 this method has evolved from basic evaluation of the specimen with non-mandatory incisions to evaluate the submucosal tumor extension, to a well-structured and well-documented IOARM as off today. It is crucial that all personnel involved in the specimen-driven IOARM is well aware of its importance and its workflow. The steps as carefully described in the protocols of the Pathology department should be followed during all OCSCC surgery.

Furthermore, we believe it is of great importance to learn from our mistakes. That is one of the reasons that we have implemented a bi-weekly multidisciplinary meeting since 2013. All OCSCC resections are discussed in detail by Head and Neck pathologists and surgeons. In this way the most accurate communication is ensured. We have seen that surgeons might be unaware of the extension and the shape of the tumor under the mucosa. Judged from the mucosa surgeon may determine tumor diameter as 1 cm while the tumor extends submucosally and reaches the diameter of for instance 3 cm. This means that in many cases the initial resection margin will be inadequate, despite of preoperative imaging and staging. Inadequate margins can be caused by delay between imaging and surgery and imaging specificity.

The bi-weekly multidisciplinary meetings have given us more knowledge about how to overcome inadequate resections. Also, postoperative treatment plans are discussed, while histological findings such as differentiation grade and perineural growth contribute to a possible indication of postoperative radiotherapy. Finally the bi-weekly meetings are an ideal way to gather information about resection margins in a prospective manner. We believe that with gathering data on all resection margins for almost ten years already, we will soon be able to match certain resection margin outcome with specific patient outcome. For instance, maybe a resection margin of 3 mm proves to be sufficient in early stage oral tongue squamous cell carcinoma.

Either way, the multidisciplinary bi-weekly meetings should be easily adapted by other hospitals.

Although we believe that specimen-driven IOARM and multidisciplinary meetings will improve the current OCSCC patient care, we will probably not be able to reach 100% adequate margins and 100% implementation of the protocol by all hospitals. To approach this goal development of objective techniques such as Raman spectroscopy is the key.

Currently, we are developing a system for Raman spectroscopic IOARM as shown in figure 3, together with RiverD International (Rotterdam, The Netherlands) and art photonics (Berlin, Germany). The Raman-system is being developed to provide an objective IOARM, which can be performed by all medical personnel, instead of only by trained surgeons and pathologists, as is the case for current method of IOARM.

The system makes use of a fiber-optic needle probe to determine the resection margin. This takes a few seconds per measurement location, enabling IOARM of a specimen with multiple measurements per resection surface within a limited amount of time. (130)

A project that aims to combine the advantages of objective IOARM by Raman spectroscopy, with the advantages of fluorescence-guided surgery, using tumor specific fluorescent labeling (e.g. by cRGD-ZW800-1) has recently started at the Erasmus University Medical Center. (136) By combining these optical techniques it might be possible to further improve the rate of adequate oral cavity cancer operations.

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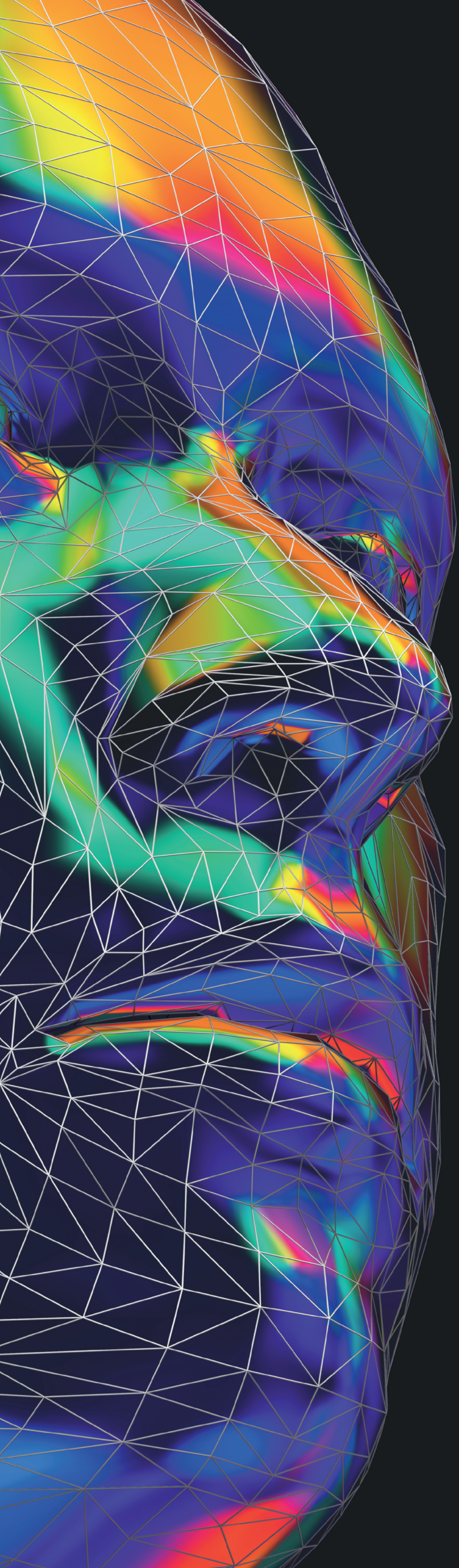

8

Summary

SUMMARY

With a global incidence of around 350.000 oral squamous cell carcinoma (OCSCC) is a major health concern. Remarkably, over the past decade the incidence has risen with approximately 33% in some Western countries. With that, oral cancer has become a top 10 cancer in more and more countries over the last couple of years. The primary choice of treatment is surgery, with postoperative radiotherapy on indication. The most important goal of surgery is to excise the tumor with adequate resection margins (>5 mm distance between tumor border and resection surface), because patient outcome is negatively affected when adequate resection margins are not achieved.

In **Chapter 2** we performed a "zero measurement" of resection margins. The definition of resection margins as described by the Royal College of Pathologists was used. Margins with the smallest distance between tumor and resection border >5mm are considered clear, margins with the smallest distance between tumor and resection border 1-5mm are considered close and margins with a distance from tumor to resection border <1 mm are considered tumor-positive. Clear margins are regarded adequate, close and positive margins inadequate. A percentage of 85% inadequate margins was found in Erasmus MC and Leiden UMC. This percentage was than compared to a literature review of other centers reporting resection margins in OCSCC surgery. Multiple studies were found reporting 30-65% inadequate margins. Remarkably, results in terms of overall survival and disease recurrence were comparable to our findings. Differences in definitions of resection margins could be a major factor as literature reports inadequate resection margins comprising margins varying between 2-10 mm. We also showed a significant difference in recurrence of disease, metastasis and survival in favor of the patients with adequate resection margins. We hypothesize that the main reason for the high number of inadequate margins is the lack of a method to evaluate the entire resection surface intraoperatively.

In **Chapter 3** we evaluated the bone resection margins in patients who underwent segmental mandibulectomy at Erasmus MC. In a similar way as for soft tissue resection margins in Chapter 2 we assessed the number of tumor-positive bone resection margins and compared our findings to the literature. Furthermore, we evaluated the effect of tumor-positive bone resection margins on patient outcome. We found 21% tumor-positive bone resection margins compared to 2-20% reported in literature. It was demonstrated that patients with tumor-positive bone resection margins had worse overall survival upon Cox regression analysis.

Just as for soft tissue margins, the main reason for the high number of tumor-positive bone resection margins seems to be the lack of a method to evaluate the bone resection margins intraoperatively.

After discussing potential methods and techniques to lower the number of inadequate resection margins we decided to evaluate the role of intraoperative assessment of resection margins in **Chapter 4.** The 15% adequate margins found between 2010-2012 and described in chapter

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2 were compared to the period between 2013-2017. Intraoperative assessment of resection margins was used in 61% of cases between 2013-2017 compared to 14% between 2010-2012. Furthermore, the number of adequate resection margins increased from 15% to 32% and the number of tumor-positive resection margins decreased from 43% to 26%. Sub analysis showed best results with specimen-driven intraoperative assessment; 58% adequate margins and only 16% tumor-positive margins. Upon statistical analysis adequate resection margins resulted in lower recurrence rates and improved disease-specific survival. Therefore, we recommend specimen-driven intraoperative assessment as standard of care for OCSCC surgery.

Even though an increase from 15% adequate margins to 58% adequate margins when implementing specimen-driven assessment is remarkably, we strive for further improvement. Other methods and techniques to evaluate the resection margin should be investigated. In **Chapter 5** we describe the great potential of Raman spectroscopy to differentiate between tumor and surrounding healthy tissue. Measurements were obtained from freshly excised tongue specimens and the results were correlated with final histopathology of the corresponding locations. When investigating the OH-stretching vibrations (3350-3550cm^{-1}) it became clear that squamous cell carcinoma showed significant higher water content than surrounding healthy tissue. A sensitivity of 99% at a specificity of 92% could be established when using a cut-off water content value of 69%.

These results clearly show the difference in water concentration between squamous cell carcinoma and healthy tissue. Yet one could imagine that in order to be able to establish a clear margin around the tumor more information regarding the water concentration of that margin should be gathered. Therefore in **Chapter 6** we describe Raman measurements on freshly excised tissue, focusing on the water concentration of the peritumoral margin. A significant gradient transition from higher water concentration (76%± 8%) towards lower water concentration (54%± 24%) was found when measuring from the tumor towards the margin of 5 mm. This highlights the potential of Raman spectroscopy to be used for measuring the resection surface in order to establish adequate resection margins.

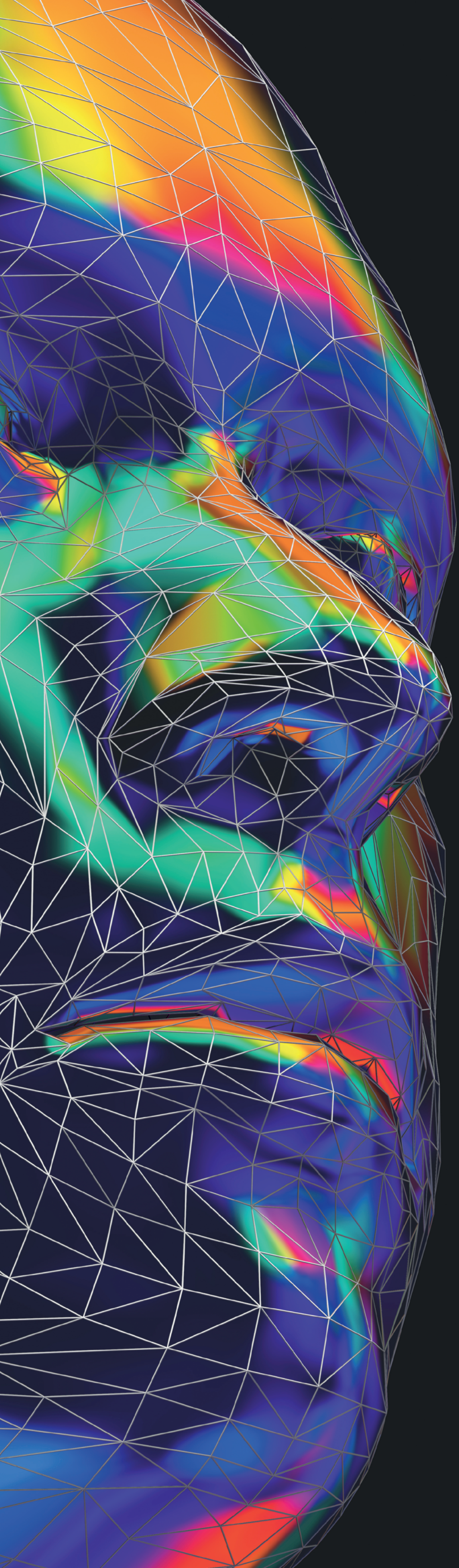
SAMENVATTING

Met een wereldwijde incidentie van rond de 350.000 is mondholte plaveiselcelcarcinoom een groot gezondheidsprobleem. Opvallend genoeg is deze incidentie het afgelopen decennium met ongeveer 33% gestegen in een aantal Westerse landen. Daarmee komt mondholte kanker in steeds meer landen in de top 10 van meest voorkomende kankersoorten voor. De eerste keuze van behandeling is chirurgie, met postoperatieve radiotherapie op indicatie. Het belangrijkste doel van chirurgie voor mondholte kanker is om de tumor met adequate resectie marges (>5 mm afstand tussen tumor en resectie oppervlak) te verwijderen, omdat de prognose van de patiënt negatief beïnvloed wordt als adequate marges niet bereikt worden.

In **Hoofdstuk 2** hebben we een nul meting verricht voor wat betreft resectie marges voor mondholte plaveiselcelcarcinoom. Hiervoor werd de definitie voor resectie marges gebruikt zoals deze door de Royal College of Pathologists is opgesteld. Marges met een minimale afstand van meer dan 5 mm tussen tumor en resectievlak worden beschouwd als ruim, marges met een afstand van 1-5 mm worden beschouwd als krap en marges van <1 mm worden beschouwd als tumor-positief. Ruime marges worden adequate marges genoemd, krappe en tumor-positieve marges worden inadequate marges genoemd. Een percentage van 85% inadequate marges werd gevonden in het Erasmus MC en Leiden UMC. Dit percentage werd vergeleken met een review van de literatuur van andere ziekenhuizen die resectie marges van mondholte plaveiselcel-carcinoom rapporteerden. De gevonden studies rapporteerden 30-65% inadequate marges. Opvallend genoeg waren de uitkomsten voor wat betreft overleving en recidiverende ziekte vergelijkbaar met onze studie. Verschillen in de definitie van resectie marges kunnen hierbij een grote rol hebben gespeeld, aangezien in de literatuur een definitie van 2-10 mm voor adequate marges wordt beschreven. We toonden in deze studie ook aan dat patiënten met een adequate resectie marge een significant betere overleving hadden en significant minder recidiverende ziekte of afstand metastasering. De belangrijkste oorzaak voor het hoge aantal inadequate marges lijkt het gebrek aan een betrouwbare methode om het resectievlak intra-operatief te beoordelen.

In **Hoofdstuk 2** hebben we de bot resectie randen onderzocht van patiënten die een segmentresectie van de mandibula ondergingen in het Erasmus MC. Op een vergelijkbare manier als in Hoofdstuk 2 werden de tumor-positieve bot resectie randen gemeten en vergeleken met de literatuur. Daarnaast werd het effect van een tumor-positieve bot resectie rand op de overleving onderzocht. Een percentage van 21% tumor-positieve bot resectie randen werd gevonden, vergeleken met 2-20% in de literatuur. Patiënten met een tumor-positieve bot resectie rand hadden een significant slechtere overleving. Ook voor bot resectie randen lijkt het ontbreken van een betrouwbare methode om intra-operatief het resectievlak te kunnen beoordelen de voornaamste reden voor het hoge aantal tumor-positieve marges. In **Hoofdstuk 4** werd de rol van intra-operatieve beoordeling van de resectie randen onderzocht. De 15% adequate marges die tussen 2010-2012 werden gevonden en beschreven zijn in Hoofdstuk 2 werden vergeleken met de periode 2013-2017. Intra-operatieve beoordeling van resectie randen werd in 61% van de gevallen gebruikt tussen 2013-2017 vergeleken met in 14% van de gevallen tussen 2010-2012. Daarbij steeg het aantal adequate resectie marges van 15% naar 32% en daalde het aantal tumor-positieve marges van 43% naar 26%. Sub analyse van de verschillende methoden liet zien dat de beste resultaten werden behaald met op het resectie preparaat gebaseerde intra-operatieve beoordeling; 58% adequate marges en 16% tumor-positieve marges. Statistische analyse liet zien dat patiënten met een adequate resectie marge minder recidiverende ziekte hadden en een betere ziekte-specifieke overleving. Daarom adviseren wij de preparaat-gebaseerde intra-operatieve beoordeling van het resectievlak als standaard keuze bij chirurgie voor mondholte plaveiselcelcarcinoom.

Ook al is een stijging van 15% naar 58% adequate marges een opvallend mooi resultaat, we streven naar verdere verbetering. Andere technieken om de resectie marges te beoordelen moeten worden onderzocht. In **Hoofdstuk 5** beschrijven we de potentie van Raman spectroscopie om onderscheid te kunnen maken tussen tumor en gezond weefsel. Er werden metingen verricht van verse resectie preparaten van de tong en deze metingen werden gecorreleerd aan de corresponderende histopathologische coupes. Opvallend genoeg bleek dat op het moment dat de OH-stretching vibraties (3350-3550cm^{-1}) werden geanalyseerd dat plaveiselcelcarcinoom significant meer water bevatte dan omliggend gezond weefsel. Een sensitiviteit van 99% bij een specificiteit van 92% werd bereikt bij een water afkapwaarde van 69%. Deze resultaten laten het duidelijke verschil in water concentratie zien tussen plaveiselcelcarcinoom en gezond weefsel. Toch kan men zich voorstellen dat om een adequate marge rondom de tumor vast te stellen meer metingen van deze marge nodig zullen zijn. Daarom richten wij ons in **Hoofdstuk 6** op Raman metingen van de peritumorale marge. Een statistisch significante transitie van een hoge water concentratie (76%± 8%) naar een lage water concentratie (54%± 24%) werd gevonden vanaf de tumor tot aan de marge van 5 mm. Dit laat zien dat Raman spectroscopie geschikt is om te worden gebruikt om het resectievlak te meten en een adequate resectie marge te bewerkstelligen.



9

Addendum

ABBREVIATIONS

ACE: Adult Comorbidity Evaluation
AHNS: American Head and Neck Society
AJCC: American Joint Committee on Cancer
ANOVA: analysis of variance
AUC: area under the curve
CCD: Charge-Coupled Device
CI: confidence interval
CRM: confocal Raman microscope
CT: computed tomography
DBTNRS: distance between tumor and the nearest resection surface
DSS: disease-specific survival
EMSC: extended multiplicative signal correction
ESS: elastic scattering spectroscopy
FDA: Food and Drug Administration
FOM: floor of the mouth
FS: frozen section
FT: Fourier-Transform
H&E: hematoxylin and eosin
HMRG: hydroxymethyl rhodamine green
HNSCC: head and neck squamous cell carcinoma
HRME: high-resolution micro-endoscopy
HWVN: high wavenumber region
HWNRS: high-wavenumber Raman spectroscopy
ICG: Indocyanine Green
IOARM: intraoperative assessment of resection margins
IR: infrared
LUMC: Leiden University Medical Center
MC: medical center
MEC: medical ethical committee
MRI: magnetic resonance imaging
NA: not applicable
NBI: Narrow band imaging
NIR-FT: near infrared Fourier Transform
NS: not specified
OCSCC: oral cavity squamous cell carcinoma
OCT: optical coherence tomography
OPT: Panoramic radiography

OR:	odds ratio
OSCC:	oral squamous cell carcinoma
PET:	positron emission tomography
PORT:	postoperative radiotherapy
ROC:	Receiver Operating Characteristic
RR:	relative risk
SCC:	squamous cell carcinoma
SPECT:	single-photon emission computed tomography
STD:	standard deviation
THz:	terahertz
WHO:	World Health Organization

PhD PORTFOLIO

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PhD-period: 2012 – 2021
Promotor: Prof. dr. R.J. Baatenburg de Jong

	Year	Workload (Hours/ECTS)
1. PhD training		
General academic skills		
- DOO Samenwerken	2017	1.0 ECTS
- DOO ziekenhuis management	2017	1.0 ECTS
- DOO Communicatie	2016	1.0 ECTS
- Teach the teacher	2016	1.0 ECTS
- DOO Ethiek	2017	1.0 ECTS
- DOO EBM	2017	1.0 ECTS
- DOO gezondheidsrecht	2017	1.0 ECTS
Courses		
- BROK (Basis Registratie Onderzoek Klinische Trials)	2012, 2016	2.0 ECTS
- Systematisch literatuuronderzoek in Pubmed	2012	5 hours
- Systematisch literatuuronderzoek andere databases	2012	3.5 hours
- Molmed: basic introduction course on SPSS	2015	1.0 ECTS
- Research integrity	2014	0.3 ECTS
- Omgaan met groepen (tutoraat)	2015	1.0 ECTS
- Firefighting and evacuation	2015	0.7 ECTS
- Basic Life Support	2017	3 hours
- Course on oncology, NVV Oncology	2017	2.0 ECTS
Oral presentations		
- NWHHT young researcher's day (oral)	2013	1.0 ECTS
- Daniel den Hoed scientific day (poster)	2013	1.0 ECTS
- Daniel den Hoed scientific day (oral)	2015	1.0 ECTS
- Scientific meeting of Dutch society (oral)	2014	1.0 ECTS
- Scientific meeting of Dutch society (oral)	2015	1.0 ECTS
- Scientific meeting of Dutch society (oral)	2017	1.0 ECTS
- Scientific meeting of Dutch society (oral)	2018	1.0 ECTS
- NWHHT, Groningen (oral)	2014	1.0 ECTS
- IFHNOS, New York (poster)	2014	1.0 ECTS
- NWHHT, Rotterdam (poster)	2013	1.0 ECTS
- SPEC, Krakow (oral and poster)	2014	1.0 ECTS
- ENT Scientific day, Rotterdam (oral)	2014	1.0 ECTS
- ENT Scientific day, Rotterdam (oral)	2015	1.0 ECTS
- 9th AHNS, Seattle (oral)	2016	1.0 ECTS
- IFHNOS, Buenos Aires (oral)	2018	1.0 ECTS

Other		
- Quaterly progress meetings Raman group	2014-2020	2.0 ECTS
- ENT residency program courses (endoscopy, nasal surgery, mouth pathology, functional endoscopic sinus surgery, radiology)	2016-2020	1.0 ECTS
- Multiple oral presentations ENT-department, Erasmus MC, Rotterdam		
- Basical surgical exam	2012-2021	20 hours
	2016	10 hours
Scientific awards		
- Best Poster award – SPEC 2014 Shedding New Light on Disease – 17-22 August 2014, Krakow, Poland. Towards Raman-guided tumor surgery: discriminating squamous cell carcinoma from healthy tissue based on water content	2014	
- Beter Horen Wetenschapsprijs 2021		
	2021	
2. Teaching		
- ICK and PKV education Otorhinolaryngology medical students, Erasmus MC, Rotterdam	2012-2021	30 hours
- Oncology and airway in ENT for OR assistants, Erasmus MC, Rotterdam	2014, 2016	8 hours
- Traumatic ENT for ER residents, Erasmus MC, Rotterdam	2012, 2013	8 hours
- "Tutorschap" medical students, Erasmus MC, Rotterdam		
	2015-2016	30 hours

LIST OF PUBLICATIONS

M.C. van Opstal, L. Claes, **R.W.H. Smits**, F. de Jong. Type-D personality, psychosomatic symptoms and voice handicap in female voice patients: A perspective on vocal communication. Audiological Medicine, 2010;8: 179-183.

R.W.H. Smits, H. Marres, F. de Jong. The relation of vocal fold lesions and voice quality to voice handicap and psychosomatic well-being. Journal of Voice. Volume 26, issue 4. July 2012, Pages 466-470.

R.W.H. Smits, P.D. Gobardhan, M.P. Poortmans, E. Tetteroo, G.P. vd Schelling. Primair plaveiselcelcarcinoom van de mamma, een zwarte zwaan onder de borstmaligniteiten. Nederlands Tijdschrift voor Heelkunde. Jaargang 21, nummer 3, mei 2012.

E.M. Barroso, **R.W.H. Smits**, T.C. Bakker Schut, I. ten Hove, J.A. Hardillo, E.B. Wolvius, R.J. Baatenburg de Jong, S. Koljenovic, G.J. Puppels. Discrimination between oral cancer and healthy tissue based on water content determined by Raman Spectroscopy. Anal Chem. 2015, 87, 2419-2426.

R.W.H. Smits, S. Koljenovic, J.A. Hardillo, I. ten Hove, C.A. Meeuwis, A. Sewnaik, E.A.C. Dronkers, T.C. Bakker Schut, T. P.M. Langeveld, J. Molenaar, V. Noordhoek Hegt, G.J. Puppels, R.J. Baatenburg de Jong. Resection margins in oral cancer surgery: Room for improvement. Head & Neck, April 2016; E2197-E2203.

E.M. Barroso, **R.W.H. Smits**, C.G.F. van Lanschot, P.J. Caspers, I. ten Hove, H. Mast, A. Sewnaik, J.A. Hardillo, C.A. Meeuwis, R. Verdijk, V. Noordhoek Hegt, R.J. Baatenburg de Jong, E.B. Wolvius, T.C. Bakker Schut, S. Koljenovic, G.J. Puppels. Water concentration analysis by Raman Spectroscopy to determine the location of the tumor border in oral cancer surgery. Cancer Res; 76(20) October 15, 2016.

R.W.H. Smits, R.M.L Poublon, A.P. Nagtegaal. Een bijzondere presentatie van een patiënt met een hangend ooglid. Nederlands Tijdschrift voor Keel- Neus- en Oorheelkunde, Jan 2017.

I. dos Santos, E.M. Barroso, T. Bakker Schut, P. Caspers, C.G.F. van Lanschot, **R.W.H. Smits**, J.A. Hardillo, A. Sewnaik, I. ten Hove, H. Mast, C. Meeuwis, T. Nijsten, E.B. Wolvius, R.J. Baatenburg de Jong, G.J. Puppels, S. Koljenovic. Raman Spectroscopy for cancer detection and cancer surgery guidance: translation to the clinics. Analyst. Jul 2017.

E.M. Barroso, I. ten Hove, T.C. Bakker Schut, H. Mast, C.G.F. van Lanschot, **R.W.H. Smits**, P.J. Caspers, R. Verdijk, V. Noordhoek Hegt, R.J. Baatenburg de Jong, E.B. Wolvius, G.J. Puppels, S. Koljenovic. Raman spectroscopy for assessment of bone resection margins in mandibulectomy for oral cavity squamous cell carcinoma. European Journal of Cancer 92 (2018) 77-87.

R.W.H. Smits, I. ten Hove, E.A.C. Dronkers, T.C. Bakker Schut, H. Mast, R.J. Baatenburg de Jong, E.B. Wolvius, G.J Puppels, S. Koljenovic. Evaluation of bone resection margins of segmental mandibulectomy for oral squamous cell carcinoma. Int. J. Oral Maxillofac. Surg. 2018; 47:959-964.

C.G.F. van Lanschot, H. Mast, J.A. Hardillo, D. Monserez, I. ten Hove, E.M. Barroso, F.L.J. Cals, **R.W.H. Smits**, M.F. van der Kamp, C.A. Meeuwis, A. Sewnaik, R. Verdijk, G.J van Leenders, V. Noordhoek Hegt, T.C. Bakker Schut, R.J. Baatenburg de Jong, G.J. Puppels, S. Koljenovic. Relocation of inadequate resection margins in the wound bed during oral cavity oncological surgery: A feasibility study. Head & Neck. 2019;1-8.

Y. Aaboubout, I. ten Hove, **R.W.H. Smits**, J.A. Hardillo, G.J. Puppels, S. Koljenovic. Specimen-driven intraoperative assessment of resection margins should be standard of care for oral cancer patients. Oral Diseases 2020;00:1-6.

R.W.H. Smits, C.G.F. van Lanschot, Y. Aaboubout, M. de Ridder, V. Noordhoek Hegt, E.M. Barroso, C.A. Meeuwis, A. Sewnaik, J.A. Hardillo, D. Monserez, S. Keereweer, H. Mast, I. ten Hove, T.C. Bakker Schut, R.J. Baatenburg de Jong, G.J. Puppels, S. Koljenovic. Intraoperative assessment of the resection

specimen facilitates achievement of adequate margins in oral carcinoma. Front. Oncol. 10:614593. Dec 2020.

Y. Aaboubout, Q.M. van der Toom, M. de Ridder, M. de Herdt, B. van der Steen, C.G.F. van Lanschot, E.M. Barroso, M. Nunes Soares, I. ten Hove, H. Mast, **R.W.H. Smits**, A. Sewnaik, D. Monserez, S. Keereweer, P.J. Caspers, R.J. Baatenburg de Jong, T.C. Bakker Schut, G.J. Puppels, J.A. Hardillo, S. Koljenovic. Is the depth of invasion a marker for elective neck dissection in early oral squamous cell carcinoma? Front. Oncol. 11:628320. Mar 2021.

R.W.H. Smits, V. van Dis, F. Datema. Large chondroid syringoma in the reconstructed nasal tip: diagnostic dilemmas and surgical management. JAAD case reports. Available online 24 July 2021.

CURRICULUM VITAE

Roeland Smits werd op 27 oktober 1985 geboren in Amsterdam, waarna hij op 4-jarige leeftijd naar Deventer verhuisde. Op 11-jarige leeftijd verhuisde hij vervolgens naar het Brabantse Veghel om daar in 2003 zijn diploma te behalen aan Gymnasium Bernrode te Heeswijk Dinther. Na niet geselecteerd te worden voor de pilotenopleiding, begon hij met de studie Geneeskunde aan de Universiteit van Utrecht. Tijdens het co-schap KNO-heelkunde in het Diakonessenhuis te Utrecht werd de interesse voor dit vak gewekt. Nadat hij in 2010 zijn artsexamen behaalde, werkte hij eerst als arts niet in opleiding tot specialist (ANIOS) bij de afdeling Heelkunde in het Amphia Ziekenhuis te Breda. In 2012 werd hij tot zijn genoegen aangenomen als ANIOS bij de afdeling KNO-heelkunde van het Erasmus MC Rotterdam, waarnaast hij startte met zijn promotieonderzoek onder begeleiding van prof. Baatenburg de Jong, dr. Koljenović en dr. Puppels. In 2016 startte hij met de opleiding tot KNO-arts in het Erasmus MC. In augustus 2021 rondde hij de specialisatie af waarop hij sinds september 2021 als KNO-arts werkzaam is in de Vijfmeren Kliniek te Hoofddorp. Vanaf 1 januari 2022 zal hij als KNO-arts werkzaam zijn in het Ijsselland ziekenhuis te Capelle aan de Ijssel.
In augustus 2016 trouwde Roeland met Wendy Malskat. Samen hebben ze drie kinderen, Florian (18 september 2015), Hannah (2 januari 2017) en Olivier (14 oktober 2020).