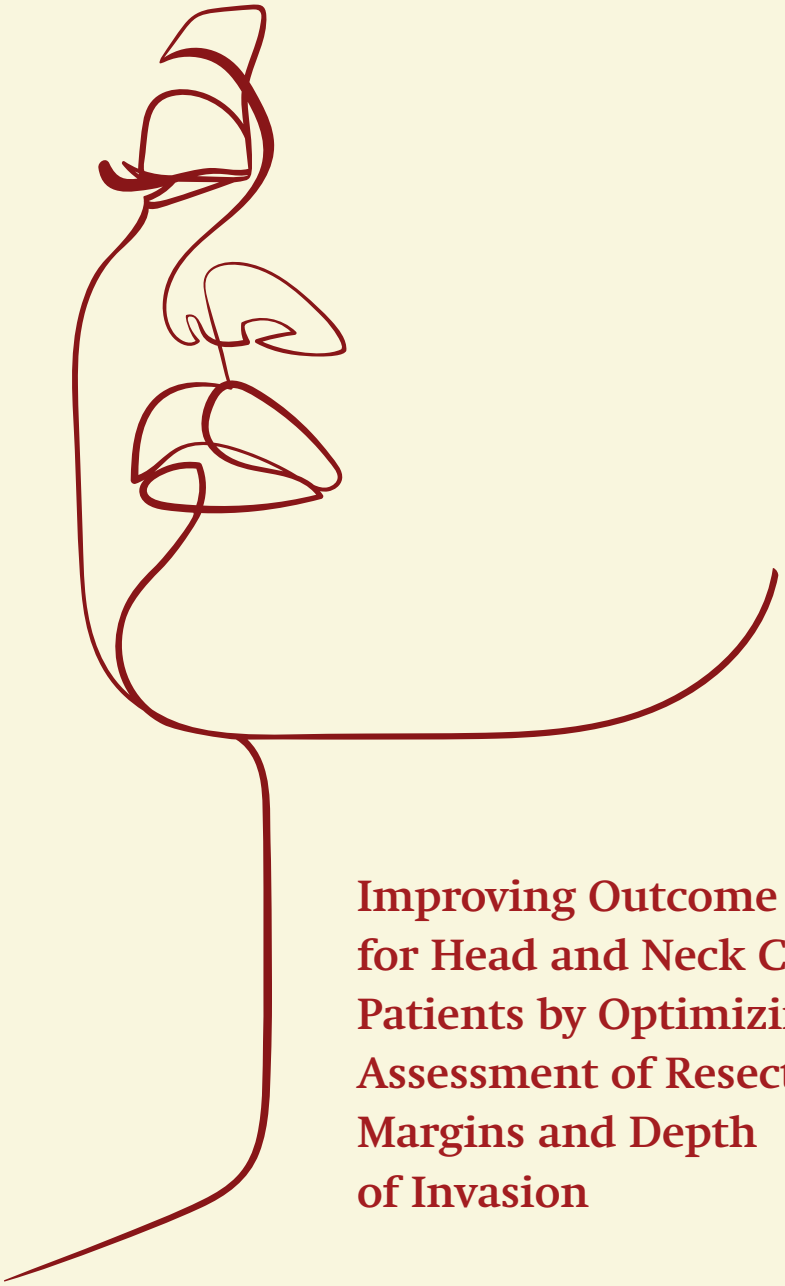

Improving Outcome for Head and Neck Cancer Patients by Optimizing Assessment of Resection Margins and Depth of Invasion

C.G.F. VAN LANSCHOT

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Het verbeteren van de uitkomst voor patiënten met hoofd-hals kanker door optimalisatie van de beoordeling van resectiemarges en invasiediepte.

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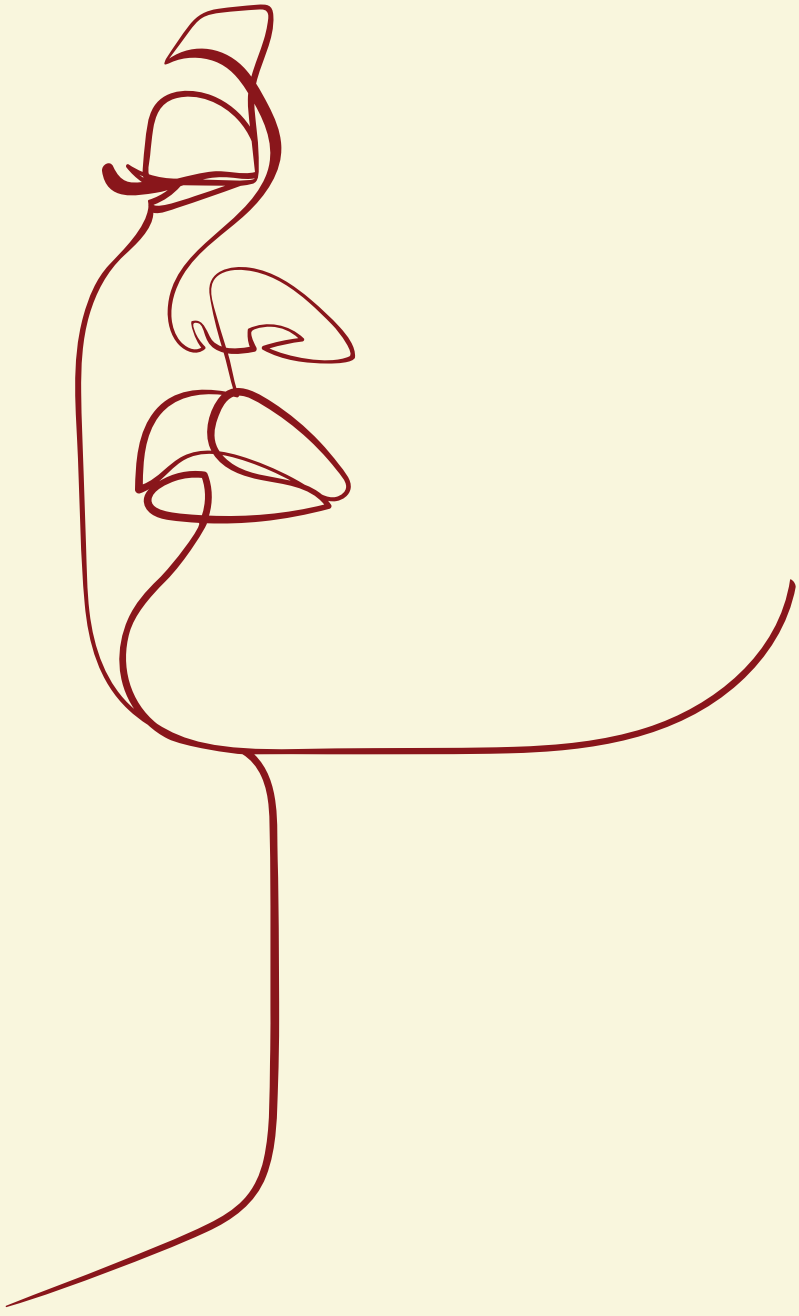

Chapter 1

General introduction

1.1 SURGICAL ONCOLOGY

Cancer has been a major health problem for a long time, affecting all populations in the world. Extensive research has been done and is ongoing in all surgical oncological disciplines, leading to new and various treatment modalities and improving survival rates.

Early and accurate diagnosis of cancer allows for timely intervention and provides the best chance of cure. The current gold standard for diagnosis is histopathological examination. In case of biopsies, only small portions of a lesion are taken for histopathological examination, which poses a risk of sampling error. Moreover, the pathology report relies on subjective assessment, leading to inter and intra operator variability. This diagnostic method is also invasive and time-consuming (1). Clearly, a technique that facilitates representative biopsies, along with accurate and *in vivo* diagnosis, is required. Currently, extensive research is being conducted on non-invasive, rapid, and accurate techniques to address this clinical need.

Cancer can be treated through various modalities (i.e., surgery, radiotherapy, chemotherapy, or a combination of these). When deciding on the appropriate treatment modality, prognostic parameters play a crucial role. Prognostic parameters rely on patient and tumor characteristics. Ongoing research aims to identify the most significant prognostic factors that impact survival. However, an even more critical question is how to influence these prognostic factors?

Surgery is the oldest and most common mainstay of cancer treatment. The primary goal of oncological surgery is to achieve complete tumor removal with adequate resection margins, ensuring a sufficient distance from the tumor border to the surrounding healthy tissue. Different guidelines exist for resection margins in surgical oncology, tailored to each organ. It is essential to strike a balance between tumor removal with adequate margins and preserving organ function as much as possible. Over time, surgical techniques have become more refined, allowing for organ-preserving treatments. However, these preserving techniques may sometimes result in residual disease, making close collaboration between surgeons and pathologists crucial.

Various intra-operative methods are available to guide surgeons in achieving adequate tumor resection while preserving the safety of surrounding structures and function. The initial assessment is performed by the surgeon's eyes and hands, but this approach is often limited to superficial evaluation, potentially

missing tumor extension into tissue depth. Currently, intra-operative assessment of resection margins with frozen section analysis is available. In this process, the surgeon takes samples from suspicious areas in the wound bed and sends them to the pathologist for histopathological analysis. However, frozen section analysis is time-consuming, laborious, and can be prone to sampling errors (2). Thus, there is a clear clinical need for an objective, rapid, and non-invasive intra-operative technique for assessing resection margins.

Evidently, there is room for improvement in surgical oncology, especially in Head and Neck oncology. This thesis will focus on optimizing surgical treatment in this field, aiming to enhance patient outcomes.

1.2 HEAD AND NECK ONCOLOGY

1.2.1 Epidemiology

The head and neck area comprises the lip, oral cavity, pharynx, larynx, and paranasal sinuses, as well as the major and minor salivary glands (3, 4). Head and neck cancer is the 6th most common malignancy in the world, significantly impacting the quality of life (5, 6). This is due to the complex nature of the region, which is vital for basic physiological functions (e.g., eating and breathing), senses (such as smell, taste, and hearing), and speaking (5, 6).

The majority of head and neck cancers (>90%) originate from the squamous lining of the mucosa and are referred to as squamous cell carcinomas (3, 5, 7, 8). Incidence rates and anatomic distributions vary globally, but all regions report high mortality rates (8, 9). Tobacco and alcohol (ab)use, the areca nut (betel quid), and poor oral hygiene are the most common risk factors for squamous cell carcinoma in head and neck cancer (3, 5, 10 - 13). A more recently discovered risk factor is the human papillomavirus (HPV), especially the high-risk HPV 16 subtype, which is associated with tonsil and tongue base cancer (3, 7, 10).

1.2.1.1 The oral cavity

The oral cavity comprises the anterior 2/3 of the tongue, the inner mucosal surface of the lips, upper and lower alveolar ridge, retromolar trigone, floor of the mouth, the hard palate, and the buccal mucosa (Figure 1) (3). The incidence of oral cavity cancer is 11 per 100,000 individuals. Men are more susceptible to this type of cancer, and the mortality rate is higher among men as well. The 5-year overall survival rate ranges from 50% to 65% (10, 14). The most common presentation of oral cavity cancer is that of an ulcerated lesion in the oral cavity.

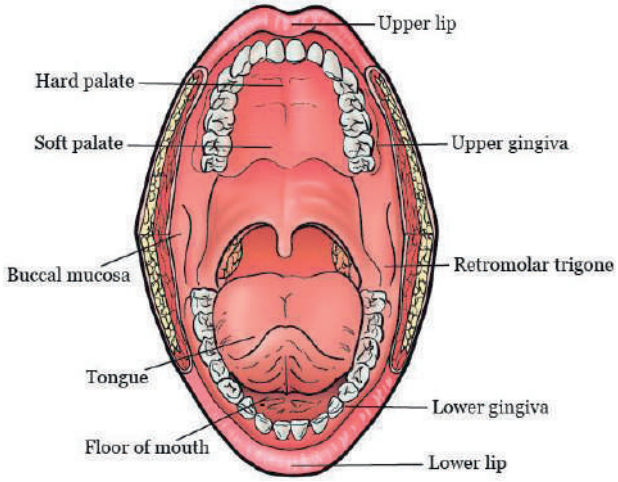

Figure 1. Schematic representation of the oral cavity.
https://www.mskcc.org/cancer-care/patient-education/mouth-cancer

1.2.1.2 The larynx

The larynx connects the oropharynx airway to the tracheal airway and lies anteriorly in the neck (Figure 2). It is divided into three subsites; 1. Supraglottic larynx, which comprises the epiglottis, aryepiglottic folds, and the false cords. 2. The glottic region, housing the true vocal cords with their muscular structures. 3. The subglottis, containing the mucosal lining of the cricoid cartilage ring (7). The subsite distribution for laryngeal cancer is approximately 30% supraglottic, 65% glottis, and 5% subglottic. The incidence of laryngeal cancer is 3.1/100,000, with men being more susceptible to it. The 5-year overall survival rate is approximately 60-63% (10, 15). Hoarseness is an early symptom of glottic cancer, leading to early diagnosis and a cure rate of 80-90%. In contrast, supraglottic cancer is often diagnosed at an advanced stage (3, 15).

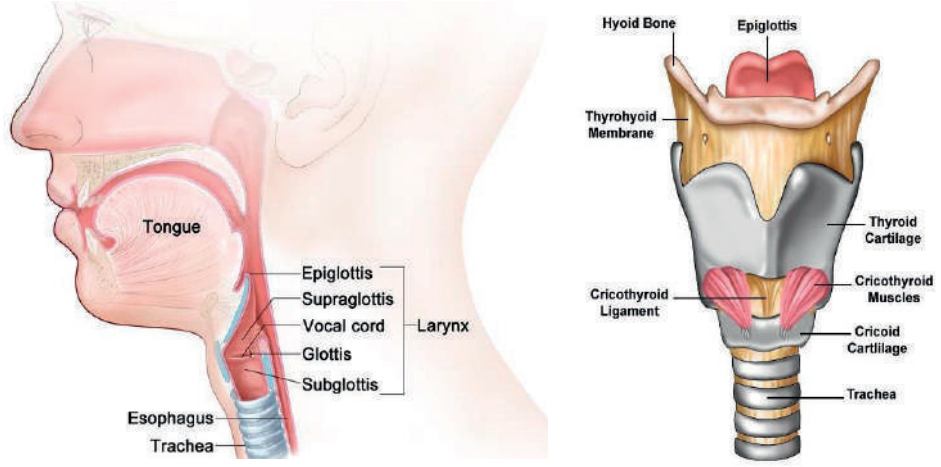

Figure 2. Schematic representation of the anatomy of the larynx.
https://www.aacr.org/patients-caregivers/cancer/childhood-laryngeal-tumors/childhood-laryngeal-tumors-treatment-pdq/
https://www.studocu.com/row/document/tanta-university/human-anatomy-and-physiology-course/anatomy-of-larynx/46413322

1.2.1.3 The hypopharynx

The hypopharynx lies posteriorly in the neck and contributes to digestion (Figure 3). The posterior wall of the oropharynx continues as the posterior wall of the hypopharynx, while the anterior wall of the hypopharynx is known as the post-cricoid region. The lateral "pockets" of the hypopharynx are referred to as the pyriform sinuses. Separating the hypopharynx from the larynx are the aryepiglottic folds. The inferior aspect of the hypopharynx marks the beginning of the cervical esophagus (7). The pyriform sinus is the most common site for hypopharyngeal cancer, with an incidence of 0.63/100,000 (16). In the Western world, 70% of the patients with this condition are Caucasian males, and the 5-year overall survival rate is 30–35% (17). The poor prognosis is a result of diagnosis at an advanced stage (3). Patients affected by hypopharyngeal cancer often remain asymptomatic until laryngeal invasion or nodal metastasis occurs. Symptoms in early-stage disease are nonspecific and may mimic benign conditions such as laryngopharyngeal reflux or globus sensation (17).

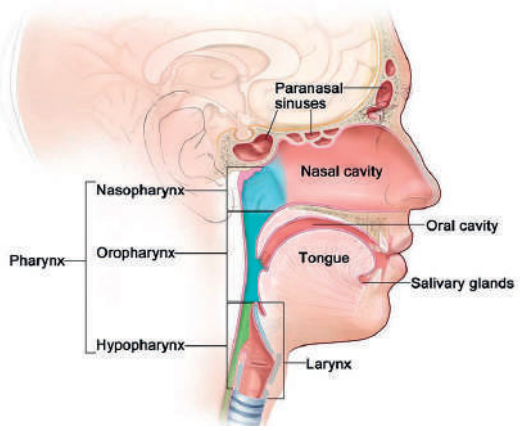

Figure 3. Schematic representation of the anatomy of the hypopharynx.
https://www.ncbi.nlm.nih.gov/books/NBK65972/figure/CDR0000062966__219/

1.2.2 Diagnosis

In the first place, diagnosis is made through clinical examination and confirmed by histopathology. Radiology plays a key role in tumor staging and treatment planning. Using ultrasound (with fine needle biopsy if indicated), CT, or MRI, the full local extent of the primary tumor is evaluated, along with the assessment of regional nodal and distant spread of the cancer (3, 7).

The TNM classification, developed by The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control, is used to stage the tumor, and serves as a major determinant for treatment and prognosis. This internationally accepted classification defines the size and extent of the (primary) tumor (T1-T4), the presence of nodal metastasis (N0-N3), and the presence of distant metastasis (M0-M1) (18, 19). Staging before treatment is referred to as clinical staging or the clinical TNM (cTNM).

Pre-operative pathological diagnosis is performed on biopsies, while intra-operative diagnosis is accomplished through frozen section analysis or diagnostic excision. The final diagnosis is determined postoperatively by the histopathological assessment of the resection specimen. Pathological measurements, such as tumor size, lymph node involvement, perineural growth, cartilage involvement for laryngeal tumors, and depth of invasion for oral cavity tumors, are defined (3, 7). This information is then used to determine the pathological TNM (pTNM).

Sometimes, the pathological stage differs from the clinical stage, especially if the surgery reveals that the tumor has spread more than was observed on imaging modalities. The pathological stage provides more precise information, which is valuable for deciding if post-operative treatment is necessary, as well as predicting treatment response and prognosis. However, it's important to note that pTNM is only available for patients who have been treated with surgery.

1.2.3 Prognostic factors derived from surgery

Prognostic factors play a crucial role in predicting locoregional control and patient survival. Treatment modalities are selected based on these factors, including the TNM classification (4). Prognostic factors also heavily influence decisions regarding post-operative management (20 - 22). Adjuvant treatment is determined by tumor characteristics, such as extra nodal extension, perineural invasion, vascular invasion, and pT3-T4 status, identified in the final histopathology report (3).

Two important prognostic factors are resection margins and depth of invasion (DOI). Among all prognostic factors, resection margins are influenced by the surgeon and pathologist. Determination of DOI could alter the course of the operation if available pre- or intra-operatively.

1.2.3.1 Resection margins

In general, for head and neck oncological surgery, resection margins play a vital role as prognostic factors (23 - 27). The Royal College of Pathologists defines resection margins as follows: clear >5 mm, close 1-5 mm, and positive <1 mm. Carcinoma in situ and high-grade dysplasia are also considered positive margins (28). Achieving clear margins is crucial for reducing the risk of local recurrence (3). Additionally, the National Comprehensive Cancer Network guidelines consider positive margins as an indication for re-resection or adjuvant therapy (3).

Intra-operative assessment of resection margins is a must. To guide the surgeon towards achieving an adequate resection (i.e., clear margins), intra-operative assessment with frozen section analysis is often employed. During this procedure, the surgeon samples tissue from the most suspicious areas in the wound bed, a method known as defect-driven assessment. However, this defect-driven method has shown low sensitivity in detecting inadequate margins (29). Moreover, it is time-consuming, and only a limited number of tissue samples can be examined, leading to sampling errors and underestimation of inadequate margins (29). Furthermore, the defect-driven method cannot provide the margin size in millimeters; it can only indicate the presence or absence of tumor-positive margins. To overcome these limitations, specimen-driven intra-operative assessment has been advocated. In this approach, the pathologist and surgeon together assess the resection specimen visually, by palpation, and by making incisions perpendicular to the resection plane. If the tumor border is not clearly identified by inspection, the assessment is refined with frozen section analysis (29, 30). This approach provides immediate feedback on whether additional resection is necessary. Recent studies show that this type of intra-operative assessment is superior to defect-driven assessment due to better visualization and less sampling error (31 – 34). The AJCC 8th edition has adopted the specimen-driven intra-operative assessment as the standard of care in the current guidelines for oral cavity cancer. A schematic representation of intra-operative assessment of resection margins is provided in Figure 4.

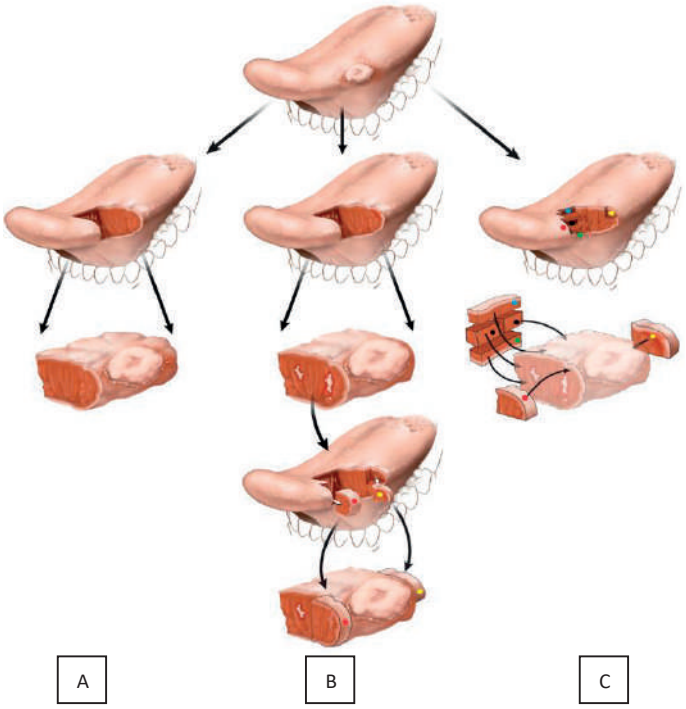

Figure 4. Schematic representation of a tumor resection of the tongue.
A: no intra-operative assessment was performed. The complete resection specimen is sent for routine histopathology. B: The resection margins were examined from the resection specimen and found to be inadequate (i.e., specimen-driven assessment). The surgeon revised the margins by performing an additional resection (red and yellow dots) from the tumor bed. Below, the additional resections are projected onto the resection specimen. C: Five tissue samples are taken 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 assessment).
Reproduced with permission from S. Chiosea MD, University of Pittsburgh Medical Center and Laura Pliego, Medical Illustrator.

1.2.3.2 Depth of Invasion

Depth of invasion is a known predictor for nodal involvement, and nodal spread is a crucial prognostic factor, particularly when extra nodal extension is present. In patients with advanced oral squamous cell carcinoma (OCSCC) or OCSCC with clinical involvement of the neck lymph nodes (i.e., lymph node metastasis (LNM)), a therapeutic neck dissection (ND) is indicated. For patients with early-stage OCSCC or OCSCC with clinically uninvolved neck lymph nodes but DOI >4 mm, an elective ND (ND level 1-3) is performed. DOI >4 mm is a recognized risk factor for occult nodal metastases. However, patients undergoing a ND face the risks and morbidity associated with the procedure, such as damage to the accessory nerve, marginal branch of the facial nerve, dysfunction of the trapezius muscle, and shoulder impairment (35, 36).

There is a need for reliable predictors to identify patients with clinically negative (N0) necks and to reduce the number of unnecessary elective NDs. In early-stage oral cavity cancer, DOI is considered an independent predictor for LNM. Pathologically, DOI is measured from the level of the basement membrane of the adjacent normal mucosa. A "plumb line" is dropped to the deepest point of tumor invasion (4), as shown in Figure 5. An elective ND is recommended for DOI >4 mm and should be considered in selective cases with DOI between 2-4 mm, based on clinical judgement (3). Unfortunately, DOI is usually determined only days after initial surgery based on the final pathological evaluation. As a result, in some cases, a second surgery is required for an elective ND. Accurately measuring DOI pre- or intra-operatively could save time and enable an elective ND during the initial surgery.

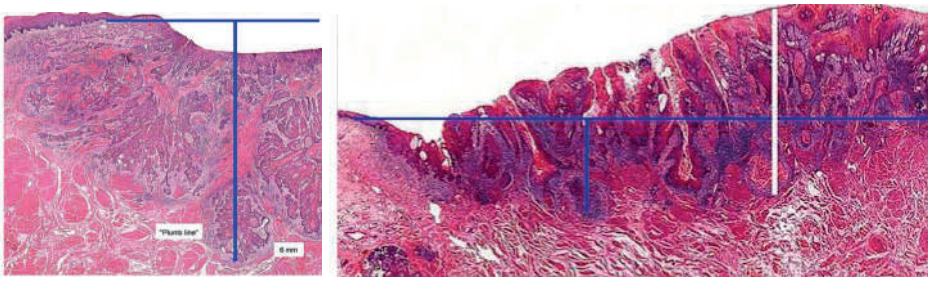

Figure 5. Depth of invasion measured pathologically from the level of the basement membrane of the adjacent normal mucosa. A "plumb line" is dropped to the deepest point of tumor invasion. *https://med.libretexts.org/Bookshelves/Anatomy_and_Physiology/Atlas_of_Otolaryngology_Head_and_Neck_Operative_Surgery/06%3A_Clinical_Practice_Guidelines_for_Head_and_Neck_Cancers/6.08%3A_Cancer_of_Oral_Cavity/6.8.09%3A_9._Oral_Cancer_-_Depth_of_Invasion*

1.2.4 Treatment

Curative treatment with function preservation in the complex head and neck area is of utmost importance. Single-modality treatment with surgery is recommended in 30-40% of patients with early-stage disease (e.g., small primary tumors with no nodal involvement) (3). The choice of treatment modality depends on institutional expertise and the associated morbidity of the treatment. Emerging minimally invasive surgeries, such as robotic or laser surgery, may prove useful in reducing morbidity (3). For advanced stage disease (e.g., larger primary tumors with invasion of underlying structures and/or nodal involvement), combined-modality treatments are recommended in 60% of head and neck cancer patients (3).

1.2.4.1 The oral cavity

For oral cavity cancer, surgery is the primary treatment modality. The specific treatment is dictated by the TN-stage. In cases where the DOI is >4 mm, an elective ND is performed due to the risk of occult metastasis. However, surgery can have an impact on critical functions such as chewing, swallowing, and speech, making it crucial to strike a balance between resection and function. There is ongoing debate regarding whether patients with N0 necks should receive an elective ND. Literature suggests that elective ND results in reduced regional recurrence and improved overall survival rates (37, 38). On the other hand, the watchful waiting protocol helps in reducing morbidity (39).

1.2.4.2 The larynx

For laryngeal cancer, it is crucial to take into account the potential loss of speech and swallowing dysfunction when selecting the best treatment option. In cases of early-stage cancers, endoscopic (laser) surgery or radiotherapy are recommended. Both treatment modalities have similar local control rates (40 – 42). However, transoral laser microsurgery offers better laryngeal preservation and overall survival (43). For advanced stage cancer, a total laryngectomy is the standard treatment. The need for adjuvant treatment (i.e., systemic and/or radiotherapy) depends on the presence of adverse prognostic factors (3).

1.2.4.3 The hypopharynx

For hypopharyngeal cancer, early-stage disease is treated with larynx-preserving surgery. However, hypopharyngeal tumors are often diagnosed in advanced stages, requiring aggressive combined treatment. Treatment options may include chemotherapy (followed by surgery in case of partial or no response), surgery followed by (chemo) radiation, or concurrent chemo radiation. Surgical intervention typically involves total laryngectomy with partial pharyngectomy (3).

1.3 ROOM FOR IMPROVEMENT

1.3.1 Current practice

1.3.1.1 Limitations of intra-operative assessment of resection margins

Surgery remains the primary treatment for OCSCC. The impact of resection margins on clinical outcomes has been extensively discussed due to divergent surgicopathological approaches and varying definitions of resection margins.

Additionally, outcome evaluations are often based on different criteria, making comparisons across studies unreliable (44).

Earlier results from our group support the general conclusion that inadequate margins (i.e., <5 mm) adversely affect patient outcomes. Our study found that 85% of OCSCC cases had inadequate resection margins based on final histopathology (45). Among all prognostic factors, physicians can only improve resection margins. To achieve better control over resection margins, intra-operative assessment with frozen section analysis is available, but there is still room for improvement.

The first step towards enhancement would be adopting a universal definition of resection margins. We propose following the guidelines set by the Royal College of Pathologists, which we currently utilize at our institute (28). This approach will facilitate clear comparison of resection margin status between different institutes. At our institute, we advocate using specimen-driven intra-operative assessment of resection margins. Our institutional protocol for specimen dissection and sampling of resection margins for microscopic evaluation during surgery is likely more comprehensive compared to other institutes.

We strongly support the adoption of specimen-driven assessment as the standard of care for all OCSCC surgeries, in accordance with the latest guidelines from the AJCC (4). However, both defect-driven and specimen-driven methods, as described earlier, have limitations since they are subjective, time-consuming, and prone to sampling errors. Consequently, various objective optical techniques are currently under extensive investigation for intra-operative assessment of resection margins.

One critical issue is the accurate relocation of inadequate margins. The technique is only meaningful if it enables a correct additional resection. The head and neck region is known to present challenges in relocating inadequate margins, making it difficult to achieve an optimal additional resection (3, 9, 30, 47). Therefore, there is a need to develop methods for precise relocation to optimize the use of intra-operative assessment of resection margins.

Furthermore, the demand for retaining satisfactory function and acceptable physical appearance adds complexity. The Royal College of Pathologists' guidelines define a clear margin as >5 mm (28), and achieving an adequate margin may require resection of adjacent functional structures (3). However, in some subsites of the head and neck region, the anatomy is complex, and resection margins are

constrained by surrounding critical structures and limited tissue thickness. This raises the question of whether a clear margin of >5 mm is always feasible.

1.3.1.2 Limitations of method of measurement of depth of invasion

Although DOI is considered the best predictor for lymph node metastasis and, consequently, elective ND, its prognostic role is not widely recognized (3, 4). The incorrect use of its definition and measurement methods poses known issues. Moreover, there is no universally accepted cut-off value for DOI to determine the necessity of performing an elective ND. Reported cut-off values range from 1.5 mm to 10 mm. Therefore, guidelines backed by well-executed research on large cohort studies are of great importance.

1.3.2 Future: optical techniques

In recent decades, there have been significant advancements in optical techniques, providing unique opportunities for objective, real-time, and rapid imaging. These techniques utilize specific properties of light to capture anatomical or chemical characteristics of tissue (9). The interaction of light with tissue is influenced by its composition, which is made up of cells, molecules, and atoms. When light interacts with tissue, it can be absorbed or scattered (48). At room temperature, most molecules are in their lowest energy state, also known as the ground state. If light hits a molecule in the ground state, energy can be absorbed, causing the molecule to reach a higher energy level. This is called an electronic excited state. The molecule will eventually release its energy and return to the ground state. The emitted energy can be observed as light, a phenomenon referred to as fluorescence. If the molecule returns to the ground state at a later time through an excited triplet state, it is referred to as phosphorescence (48, 49).

Light can also interact with matter through scattering, as depicted in Figure 6. When the energy of incoming light matches that of the scattered light, it is called elastic scattering or Rayleigh scattering (48 - 50). On the other hand, inelastic scattering, known as Raman scattering, occurs when energy is transferred between the light and the molecule. Due to constant vibrations of the molecules, the frequency of the scattered light can shift. If the energy is transferred from the light to a vibration mode of a molecule, the frequency decreases, leading to an increase in the wavelength of the scattered light, termed Stokes scattering. Conversely, if the molecule transfers energy from a vibration mode to the light, the frequency increases, causing the wavelength to decrease, known as Anti-Stokes scattering. The specific combination and conformation of atoms in a molecule determine its possible vibration modes, making the inelastic scattering frequencies of the molecule

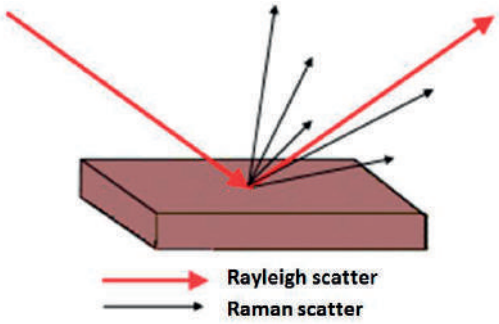

Figure 6. Schematic representation of interaction between light and a sample.
The red arrow represents the fraction of light which is elastically scattered (Rayleigh scattering). The black arrows represent a smaller fraction of light which is scattered due to inelastic light scattering (Raman scattering).

highly specific. Anti-Stokes scattering is unlikely to occur at room temperature as it requires the molecule to be in an excited state. Thus, Stokes scattering is currently the more commonly used method for analysis (49, 50).
Various optical techniques based on absorption or scattering, such as fluorescence imaging, high-resolution micro endoscopy, narrow-band imaging, optical coherence tomography, elastic scattering spectroscopy, confocal microscopy, and Raman spectroscopy, are being investigated for their potential application in head and neck cancer diagnosis and intra-operative assessment.

For an optical technique to be integrated into clinical practice, it must provide added value compared to current methods. As mentioned earlier, there is a demand for improved diagnosis and intra-operative assessment. The technique must be objective, rapid, user-friendly, operator-independent, and offer high resolution. Ideally, it should be applicable *in vivo*, preferably without the need for reagents and labels to seamlessly integrate into the oncological workflow. Raman spectroscopy fulfills all these requirements.

1.3.2.1 Raman spectroscopy

Raman spectroscopy is based on inelastic scattering and was discovered by Sir C.V. Raman and K.S. Krishna in 1928 (Figure 7). Sir C.V. Raman was awarded the Nobel Prize in Physics in 1930 for this significant discovery. However, Raman scattering was almost forgotten until it was re-discovered following the invention of the laser in 1960. The laser's invention enabled the acquisition of high-quality Raman spectra. Nevertheless, it was only in the years after 1969, with the invention of the charged-coupled device (CCD), that Raman Spectroscopy took a substantial step towards practical use. The CCD facilitated the accelerated collection of Raman spectra as the entire spectrum could be measured simultaneously. Subsequently,

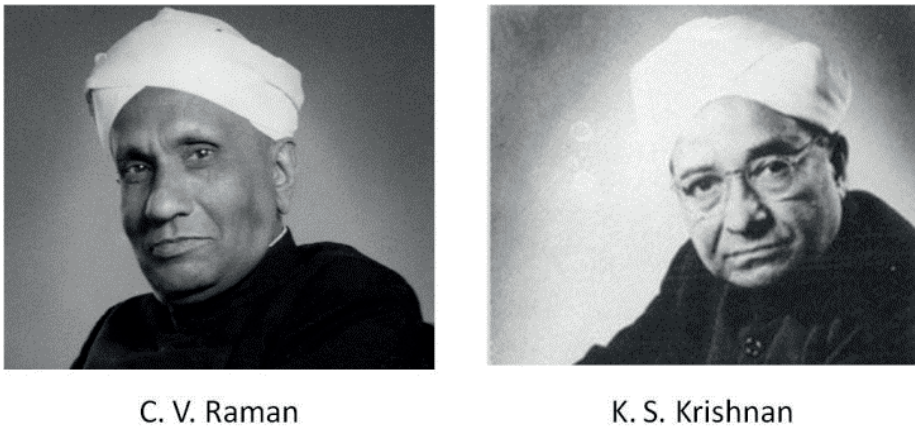

Figure 7. Portraits of C. V. Raman and K. S. Krishnan.
https://www.researchgate.net/figure/Sir-C-V-Raman-and-Sir-K-S-Krishnan_fig3_283136005

in 1990, a confocal Raman microscope was introduced, enabling the collection of Raman spectra from cells and cell organelles (51).

A Raman spectrum represents a plot of all the molecular vibration modes present in the sample. The intensity of the scattered light is on the y-axis, while the wavelength, typically expressed in relative wavenumbers (cm^{-1}), is on the x-axis. Each molecule exhibits a highly specific Raman spectrum. The position and intensity of the peaks in the spectrum provide valuable information about the composition and conformation of the molecule. As a tissue sample comprises a complex combination of numerous molecules, its Raman spectrum is a linear combination of the Raman spectra of these molecules and their interactions. Thus, it becomes highly specific to the tissue sample, resembling an optical fingerprint. Any changes occurring in the tissue will be reflected in alterations in the Raman spectrum, allowing Raman spectroscopy to distinguish cancer from healthy tissue.

The Raman spectrum can be divided into two regions: the fingerprint region, with wavenumbers ranging from 200 cm^{-1} to 2000 cm^{-1}, and the high wavenumber region, spanning from 2500 cm^{-1} to 4000 cm^{-1} (Figure 8). The fingerprint region contains detailed information on molecule-specific vibrations, whereas the high wavenumber region predominantly conveys information about the CH-, OH-, and NH-stretching vibrations of molecules. In this thesis, we employed high wavenumber Raman spectroscopy due to its advantages over the fingerprint region. The high wavenumber region offers a stronger Raman signal and reduced fluorescence background (52). When considering the implementation of the technique in a clinical setting, for example, using an optical fiber, fingerprint Raman spectroscopy

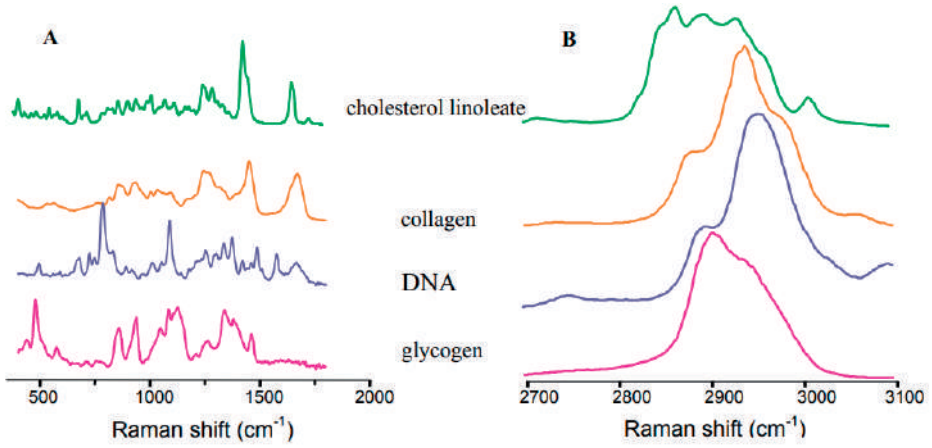

Figure 8. Raman spectra of pure chemical compounds, obtained in fingerprint region (A) and in high wave number region (B).
https://www.researchgate.net/figure/Raman-spectra-of-commercially-available-pure-chemical-compounds-obtained-in-fingerprint_fig1_7536065

faces challenges due to the strong fused silica background signal generated by the fiber (52 - 54), which is absent in the high wavenumber region. Furthermore, the signal intensity of fingerprint Raman spectroscopy is relatively low, potentially leading to long signal integration times that render it impractical for clinical use (52 - 54).

Earlier work by our group demonstrated the potential of Raman spectroscopy in distinguishing OCSCC from healthy tissue. Initially, a database was established by *Cals et al.*, comprising Raman spectra of various structures encountered in tongue tissue, including squamous cell carcinoma (55). In a subsequent study, the authors successfully differentiated OCSCC from different surrounding healthy structures, such as adipose tissue, nerve, muscle, gland, connective tissue, and squamous epithelium, achieving an accuracy of 93%-100% (56). Continuing their research, the authors developed a model for discriminating between OCSCC and healthy tissue in the tongue with an accuracy of 91%, sensitivity of 100%, and specificity of 78% (57). These studies exclusively utilized the fingerprint region. *Barroso et al.* continued the research by focusing on the high wavenumber region and managed to discriminate between oral cancer and healthy tissue based on water concentration, attaining a sensitivity of 99% and specificity of 92% (58) (Figure 9).

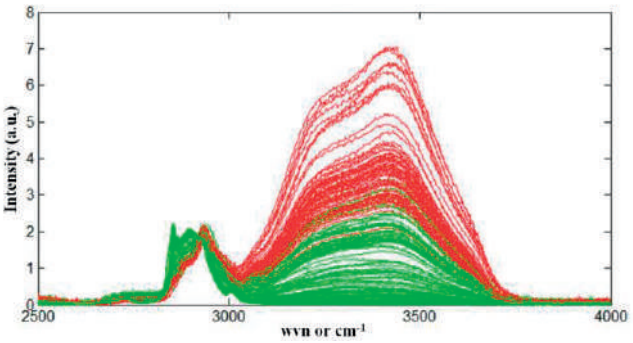

Figure 9. High wavenumber spectra normalized on the CH-stretching band (2800 cm^{-1} to 3040 cm^{-1}). Spectra are colored according to the histopathological evaluation. The green color represents spectra from healthy tissue and the red color represents spectra from oral cavity squamous cell carcinoma.

1.4 SCOPE OF THIS THESIS

The main objective of this thesis is to optimize surgical treatment in Head and Neck cancer by improving the assessment of prognostic factors that can be enhanced through intra-operative evaluation of resection margins and accurate measurement of depth of invasion. The focus of this thesis will be on cancer of the oral cavity, larynx, and hypopharynx.

For oral cavity cancer, we conducted a study (**Chapter 2**) to evaluate the value of specimen-driven intra-operative assessment. We compared the margin status in the period before and after introducing this assessment as the standard of care at our institute. In **Chapter 3**, we build on earlier results by our group, investigating the use of water concentration in tissue to discriminate between tumor and healthy tissue. We explore whether changes in water concentration correlate with the distance between tumor border and surrounding healthy tissue, aiming to verify if this information can aid in assessing resection margins.

As previously mentioned, accurate additional resection is crucial for meaningful intra-operative assessment of resection margins. Thus, **Chapter 4** reports on a reliable and objective method for relocating inadequate margins and evaluates its ease and accuracy in the surgicopathological workflow. Additionally, we describe the development of a semi-automatic instrument for rapid and easy placement and removal of tags, a collaboration between the Erasmus MC and the faculty of Industrial Design Engineering at Delft University of Technology, detailed in **Chapter 5**. We also address the surgeons' desire for biocompatible, soluble, and

radio-opaque tags, which can be utilized during PORT, enabling a more precise target volume. Chapter 5 further outlines the study design set up.

In addition to optimizing intra-operative assessment, we analyze the influence of occult nodal disease. **Chapter 6** evaluates whether a DOI of 4 mm is indeed the optimal cut-off value for an elective ND in oral cancer patients. We also analyze the effectiveness of elective ND in **Chapter 7** by studying the rate of regional recurrence and its impact on survival in T1-T2 buccal squamous cell carcinoma in a multicenter western population.

While a clear margin (>5 mm) is generally accepted, achieving it during laryngeal and hypopharyngeal surgery is not always possible. In **Chapter 8**, we identify the resection surfaces and measure the maximum feasible margins per subsite in the larynx and hypopharynx, proposing a new guideline for maximum feasible but adequate resection margins. Additionally, in **Chapter 9**, we investigate the clinical relevance of resection margins in laryngeal and hypopharyngeal surgery through statistical analysis for recurrences and survival rates. In **Chapter 10**, we explore whether Raman spectroscopy can be used to differentiate laryngeal squamous cell carcinoma from surrounding non-cancerous tissue.

Finally, **Chapter 11** presents a general discussion on the results and limitations of this thesis. We delve into the needs of surgical treatment and discuss future perspectives for optical techniques, aiming for objective, fast, and user-friendly intra-operative assessment of resection margins to guide surgeons towards achieving adequate resection margins.

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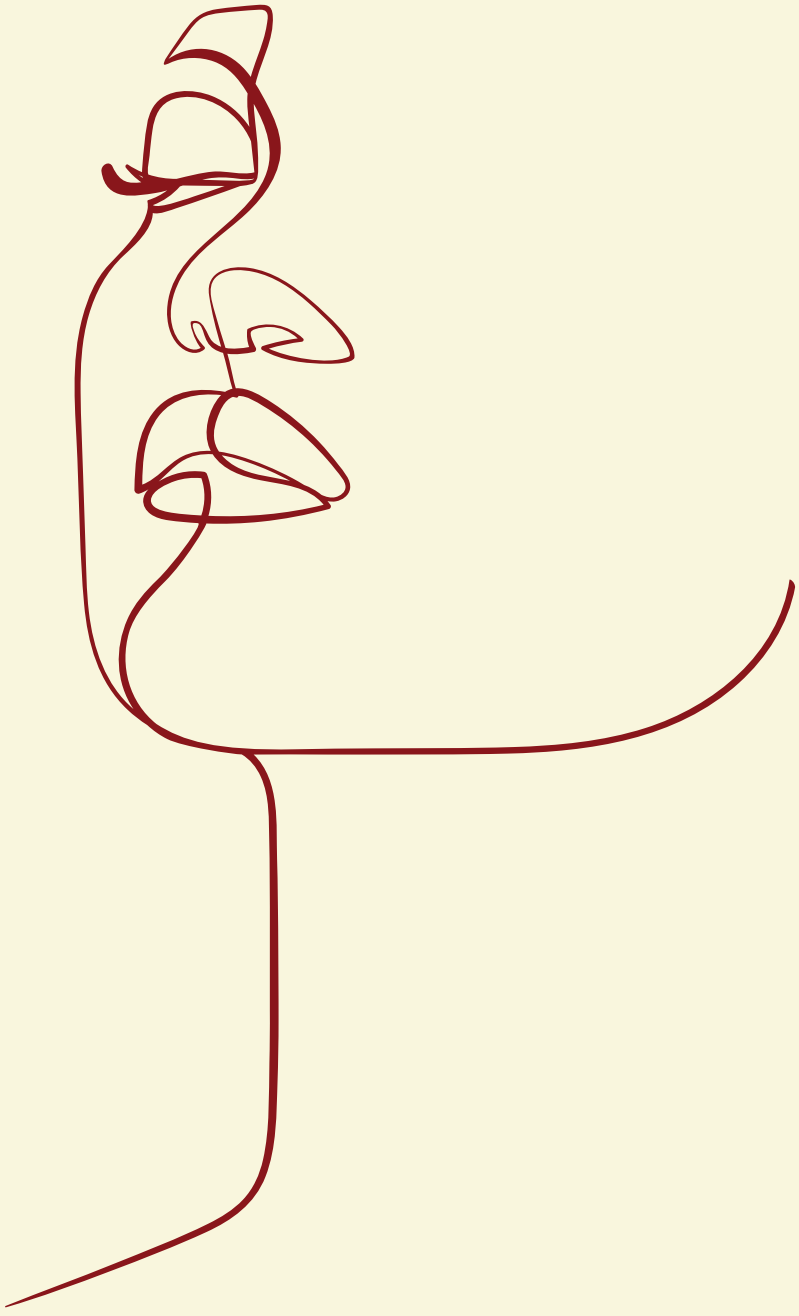
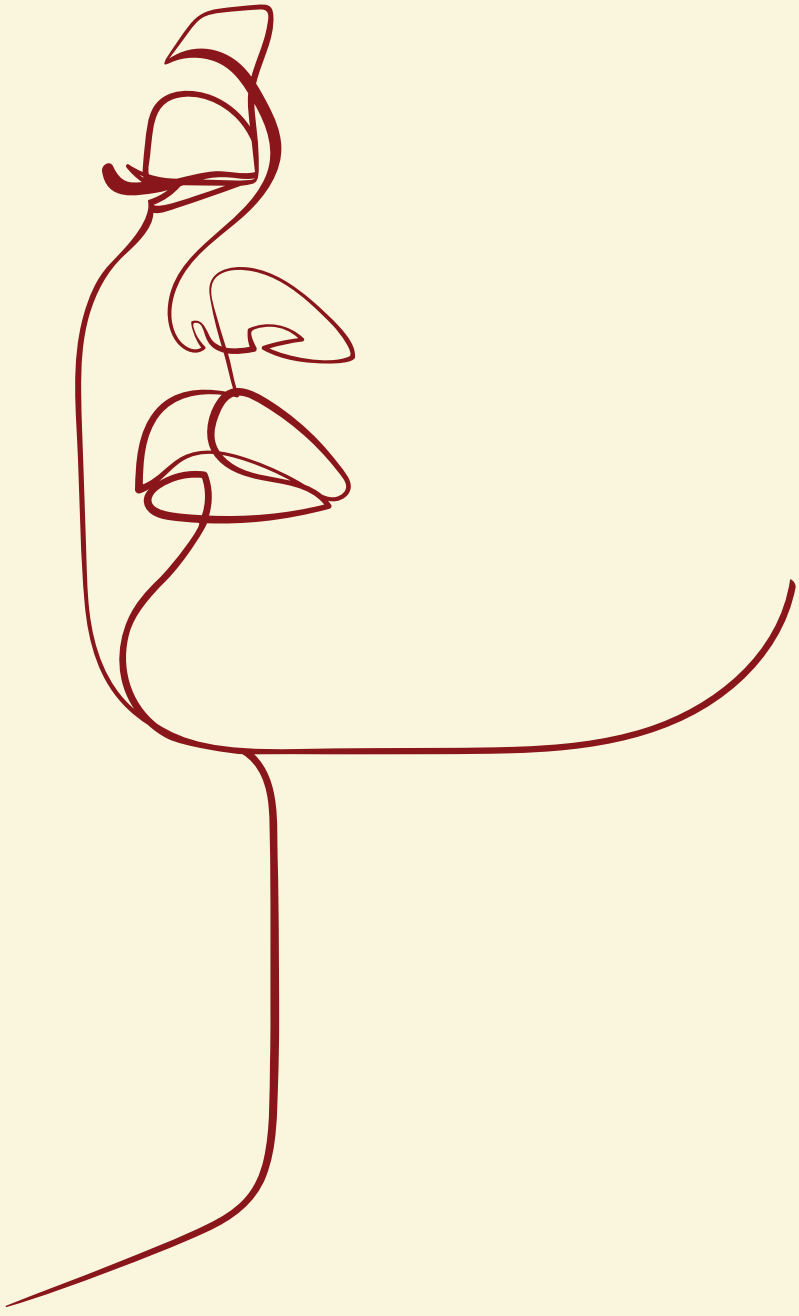


Oral cavity



Chapter 2

Intra-operative assessment of the resection specimen facilitates achievement of adequate margins in oral carcinoma

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2.1 ABSTRACT

Background

Inadequate resection margins in oral cavity squamous cell carcinoma have an adverse effect on patient outcome. Intra-operative 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 intra-operative 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 intra-operative 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 intra-operative assessment ($p < 0.001$). Also, tumor recurrence significantly decreased, and disease-specific survival improved when performing specimen-driven intra-operative assessment.

Conclusions

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

2.2 INTRODUCTION

Patients with inadequate tumor resection margins often receive adjuvant treatment (radiotherapy, chemo radiation and/or re-operation), which leads to higher morbidity (1).

Moreover, inadequate resection margins in oral cavity squamous cell carcinoma (OCSCC) lead to a significantly worse clinical outcome (2-4).

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 (3). Equally low numbers of adequate OCSCC resections were reported by other authors (2, 4).

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, intra-operative 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 intra-operative assessment). For the detection of inadequate margins during OCSCC surgery, this defect-driven frozen section procedure has been shown to have low sensitivity (5-9). 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 (10-15). 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 intra-operative 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 intra-operative assessment is superior to defect-driven assessment due to better visualization, less sampling error and it has been recommended in the latest AJCC guidelines (4, 6, 16-21).

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

This study aimed to evaluate the value of specimen-driven intra-operative 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).

2.3 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 intra-operative assessment was not standard of care, has been described earlier (3).

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, intra-operative assessment and final histopathology. The type of intra-operative 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 (22). 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 intra-operative assessment

Figure 1 shows an example of the specimen-driven 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.* (23).

Next, the specimen is taken to the pathology department for intra-operative 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 5 mm (Figure 1.C).

In most cases, this enables the 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 intra-operative 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 intra-operative assessment, the resection specimen enters the routine procedure for the final pathological examination.

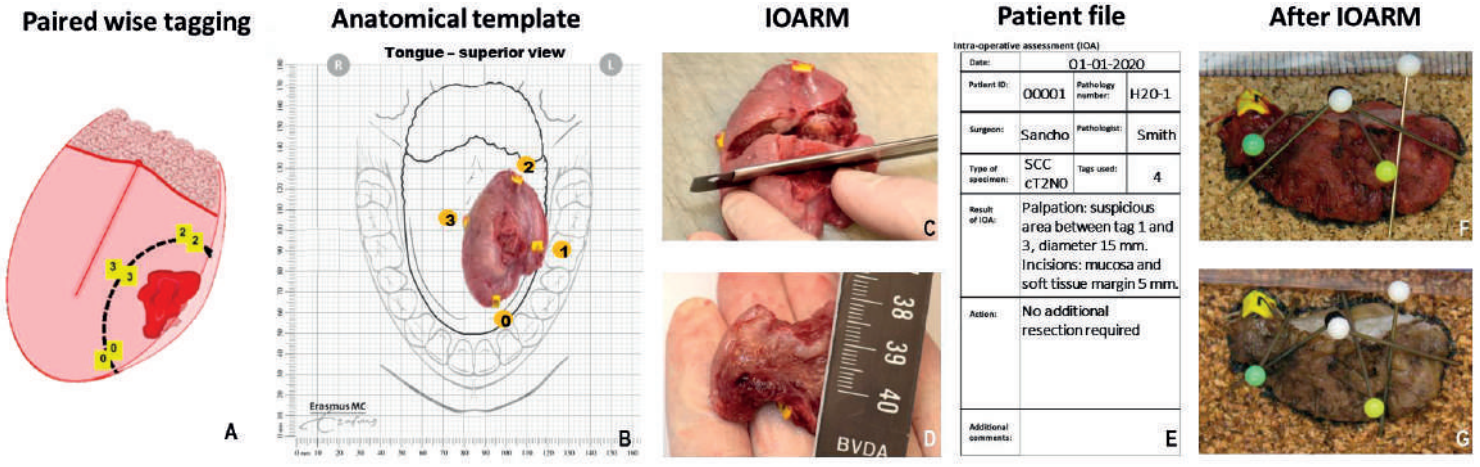

Figure 1. A. Paired wise tagging on both sides of the resection line, performed during surgery (23). B. Anatomical template, used to maintain orientation, tags are noted on the template. C. Grossing of the tissue, perpendicular incisions must be 5-6 mm from each other. D. Measuring the margin with a 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.

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 intra-operative assessment types (i.e., 'no intra-operative 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 intra-operative 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.

2.4 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.

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

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'.

Table 2. Frequency and type of intra-operative assessment of resection margins.

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

Defect-driven intra-operative 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 intra-operative assessment, 2) defect-driven assessment, and 3) specimen-driven assessment. The results are shown in Table 4.

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 intra-operative assessment 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 intra-operative assessment

The impact of intra-operative 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 intra-operative assessment was not significant (RR 0.93, 95% CI 0.79 to 1.09). Comparison between specimen-driven assessment and no intra-operative 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 intra-operative 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 intra-operative 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.

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

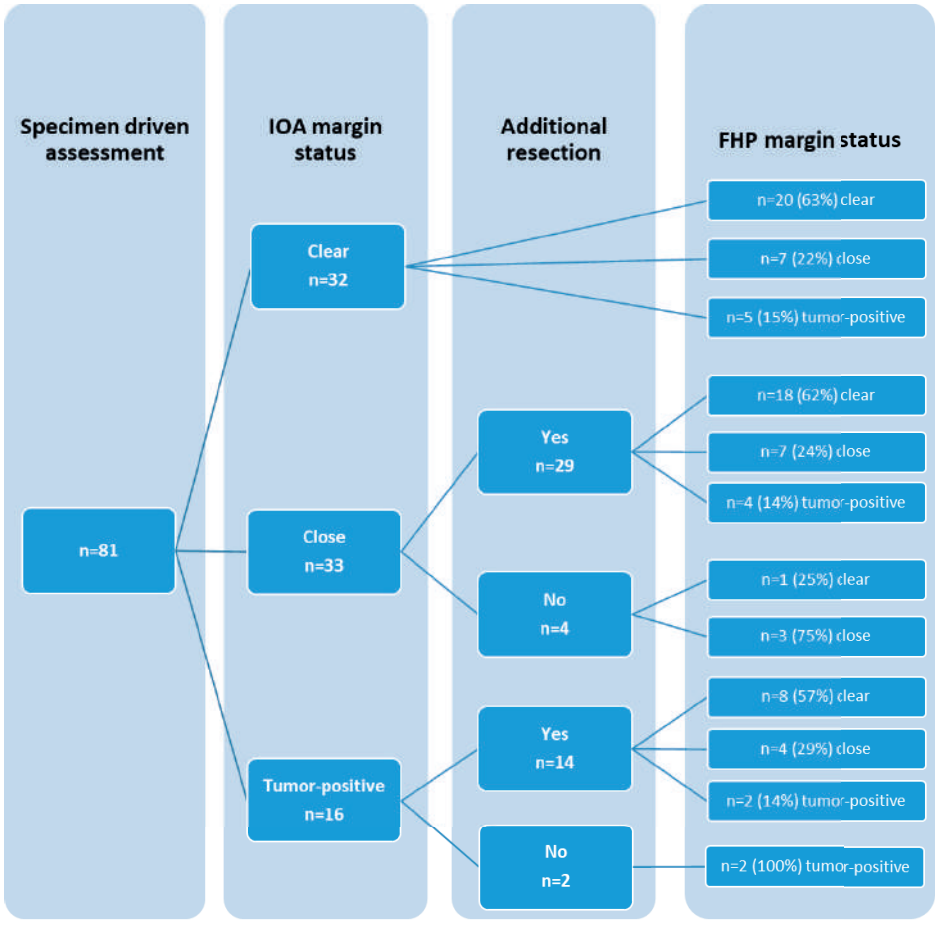

Figure 2. Comparison of margin status based on intra-operative assessment (IOA) and margin status based on final histopathology (FHP), including additional resection.

margins with tumor-positive resection margins, there was a significant difference (both $p < 0.001$). Kaplan Meier curves are shown in Figure 5.

Candidates contribution

The candidate contributed to gathering, analyzing, and interpreting data. Additionally, the candidate was actively involved in drafting the article, revising it critically for significant intellectual content, and contributing to the final revision of the version intended for publication.

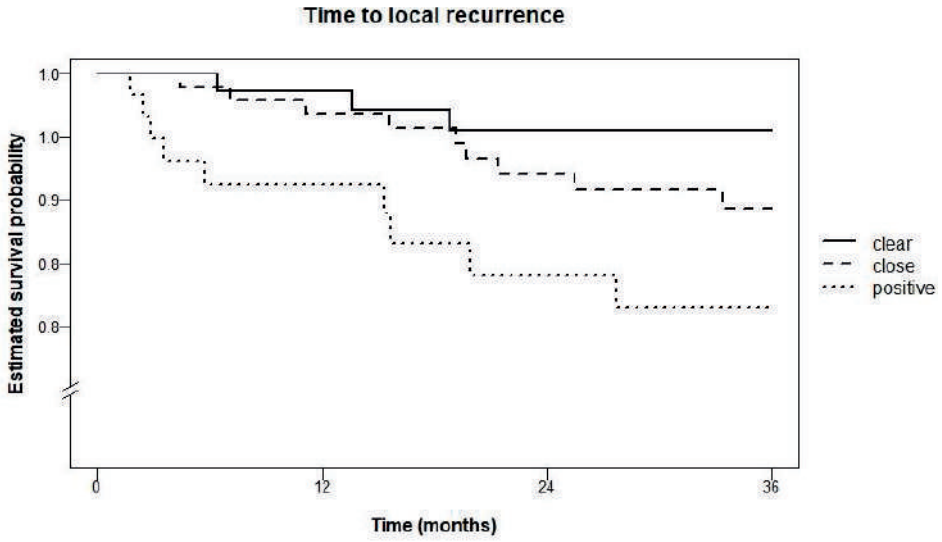

Figure 3. Kaplan Meier estimations of time to local recurrence in months.

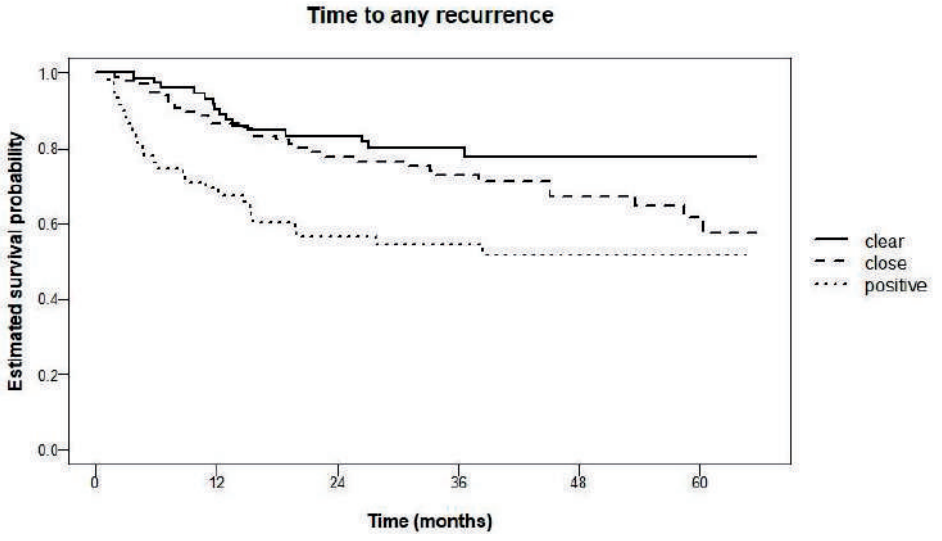

Figure 4. Kaplan Meier estimations of time to any recurrence (local, regional, distant metastasis) in months.

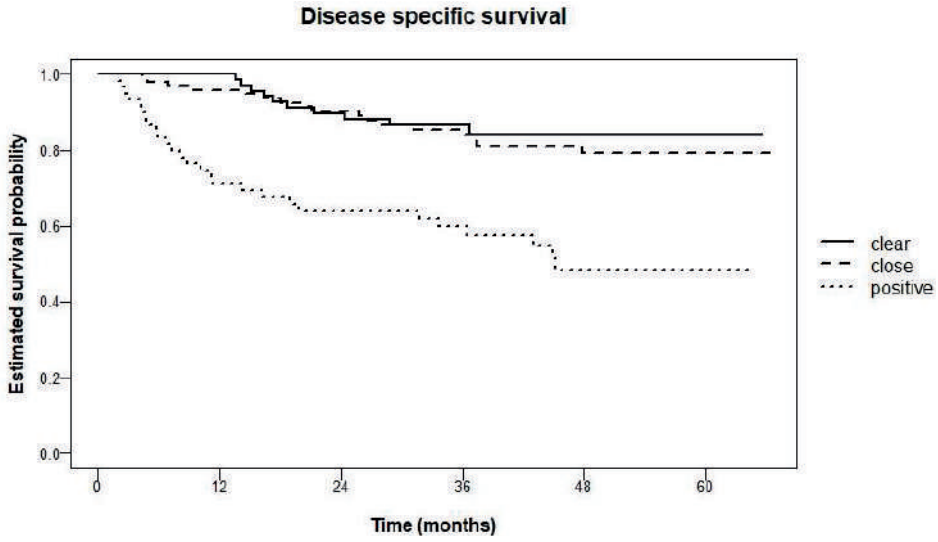

Figure 5. Kaplan Meier estimations of disease-specific survival in months.

2.5 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 intra-operative 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 (2, 4).

To improve the status of resection margin at our institute, a comprehensive specimen-driven intra-operative 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 intra-operative 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 intra-operative 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 intra-operative 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 intra-operative 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 intra-operative 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 intra-operative 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 intra-operative 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 intra-operative 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 (4, 6, 18-20). We therefore advocate specimen-driven assessment as standard of care during OCSCC surgery. This is in line with the latest guidelines of the AJCC (16).

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 peroperative planning is of essential importance, it unfortunately does not always reflect the intra-operative 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 (24, 25). 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 (26). Several authors suggest that resection margins between 2-3 mm could be sufficient while not hampering patient outcome (27-29). 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 refining 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 (30, 31). The combination of these techniques is being developed to allow for a rapid and accurate specimen-driven intra-operative assessment of all resection surfaces that will fit in the surgico-pathological workflow.

Only by intra-operative 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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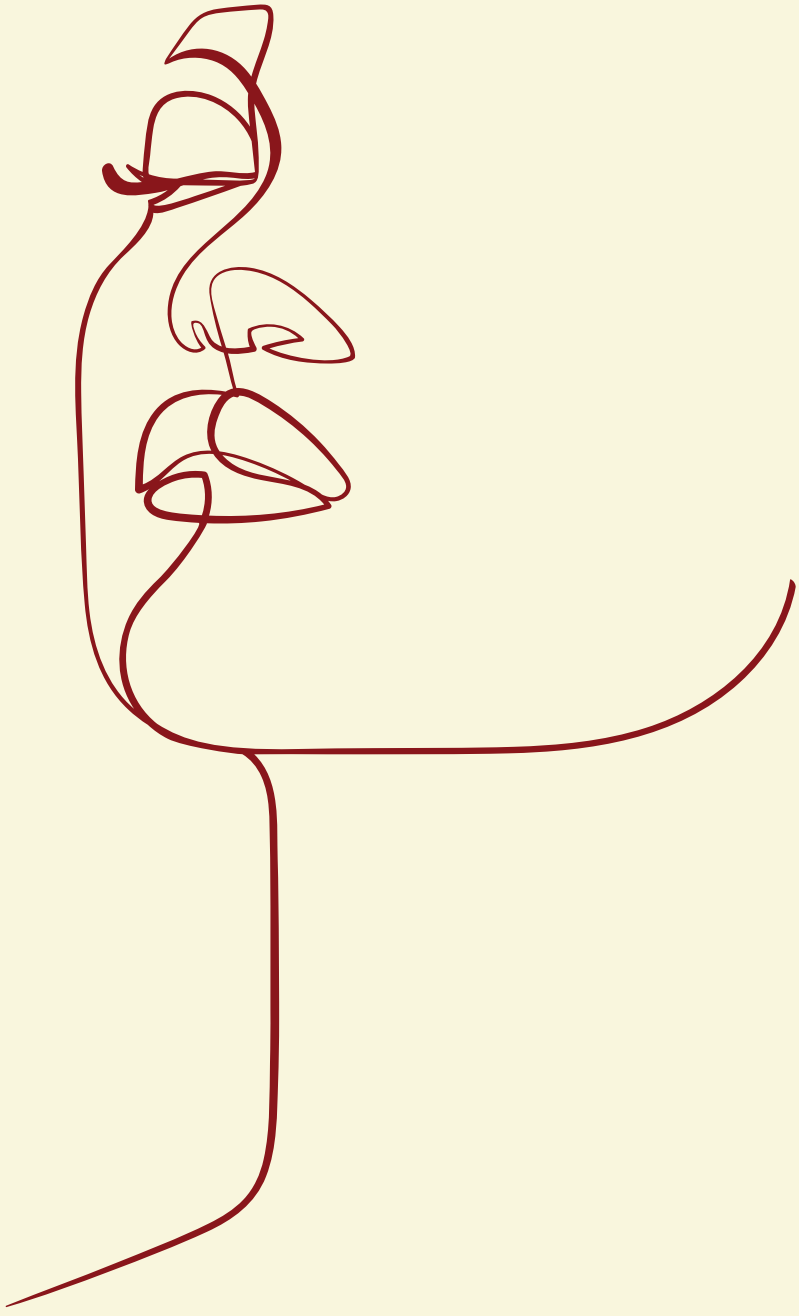

Chapter 3

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

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3.1 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.

3.2 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.

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.

3.3 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.

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

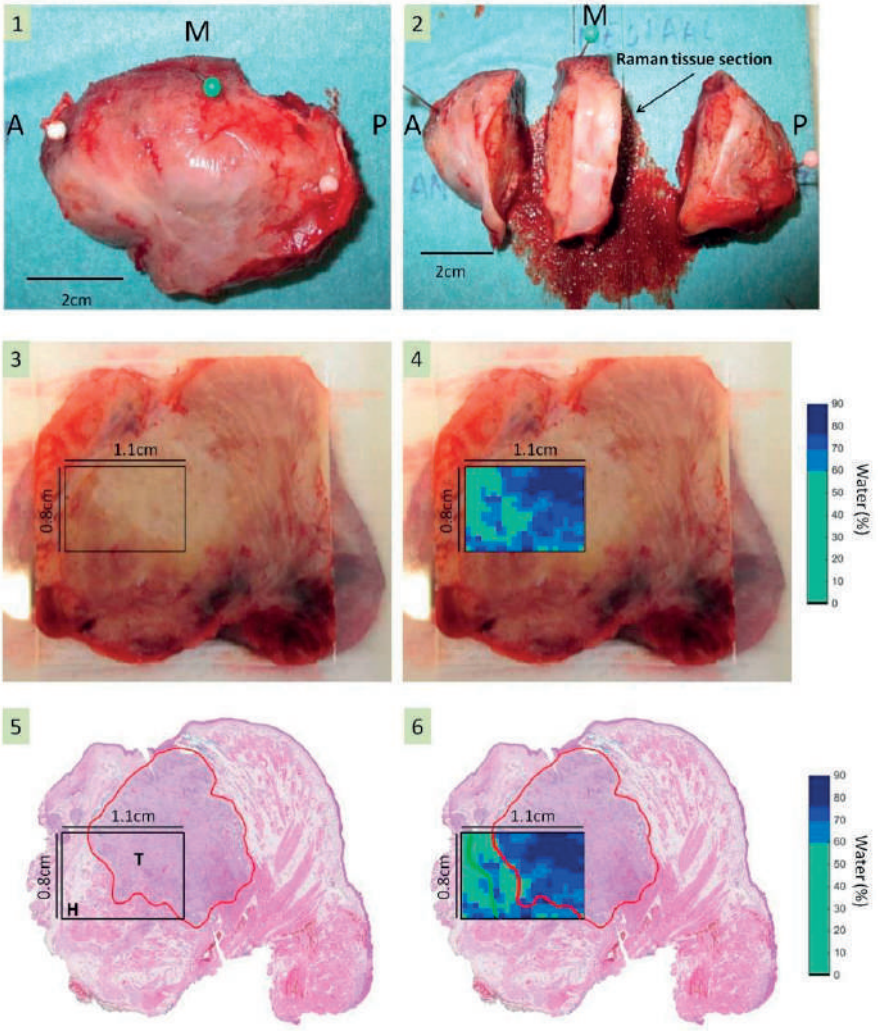

Figure 1. Overview of the experimental protocol.
1. Immediately after surgical resection, the specimen (excision of tongue SCC) was transferred to the pathology room and orientation was digitally recorded (anterior (A), posterior (P) and medial (M)). 2. The specimen was cut perpendicular to the resection 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).

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). Preprocessing 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 autofluorescence 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 3390 cm^{-1} and 2935 cm^{-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.

3.4 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 1. 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 1 cm (T1) to more than 4 cm. 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 6 cm 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 84 mm^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 5 mm from the tumor border (i.e., within the area of inadequate margin) and 3001 spectra were obtained at a distance greater than 5 mm 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.

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, inadequate 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%.

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.

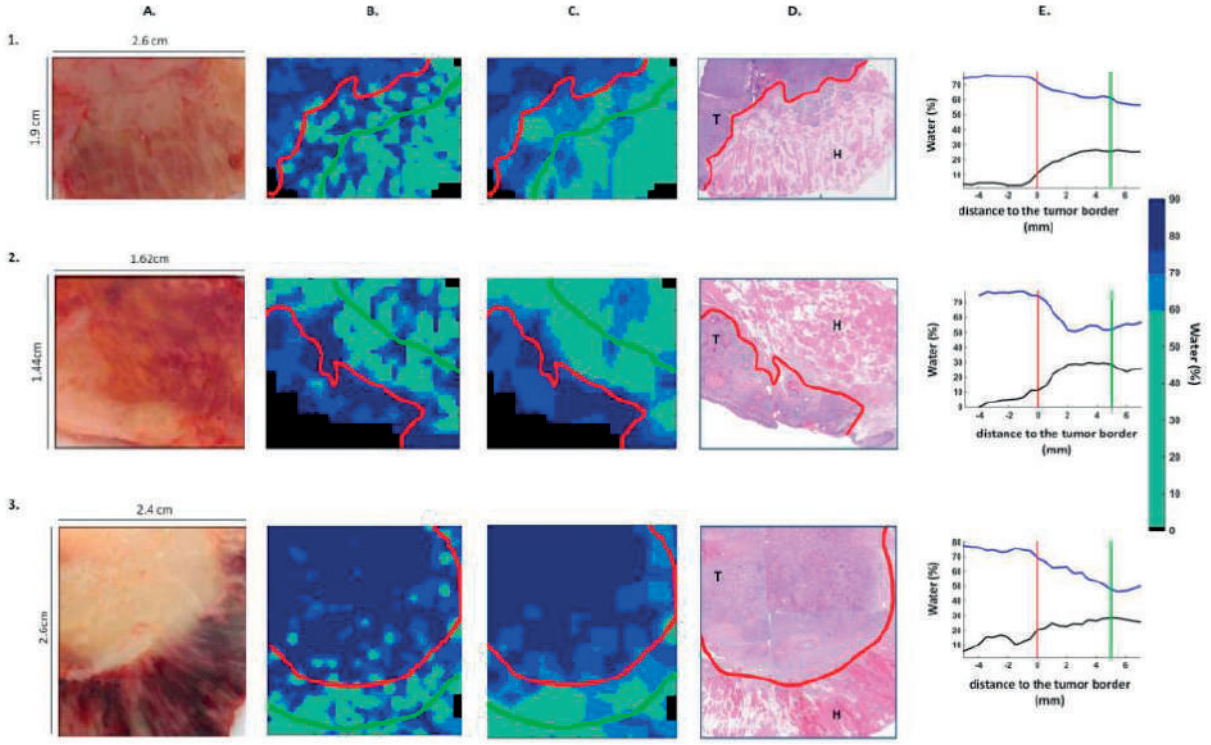

Figure 2. 1. 3: Examples of the data obtained by means of mapping experiments on 3 Raman tissue sections from 3 patients.
Panels column A: Photograph of the measured fresh tissue surface. Panels column B: Raman water map with indication of tumor border (red; based on final histopathology shown in panels 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.

Table 2. 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

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 0.5 mm 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 concentra-

tion values also differs between tumor and surrounding healthy tissue; from less than 10% inside the tumor to more than 15% just outside the tumor.

Candidates contribution

The candidate contributed to gathering, analyzing, and interpreting data. Additionally, the candidate was actively involved in drafting the article, revising it critically for significant intellectual content, and contributing to the final revision of the version intended for publication.

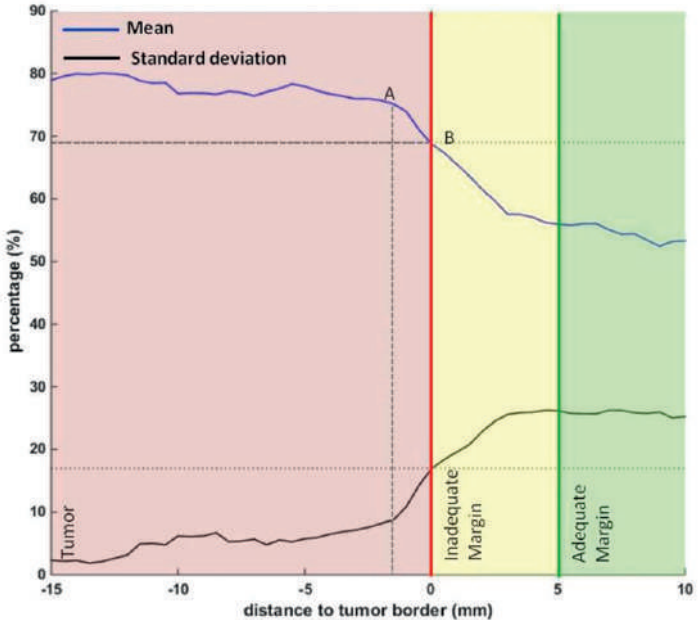

Figure 3. Water concentration profile from inside the tumor towards adequate margin.
All individual water concentration percentages of the 25 maps were averaged per interval to calculate the mean (blue) and standard deviation (black) of the water concentration as a function of the distance to the tumor 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.

3.5 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 increasing

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 the 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 was shown 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).

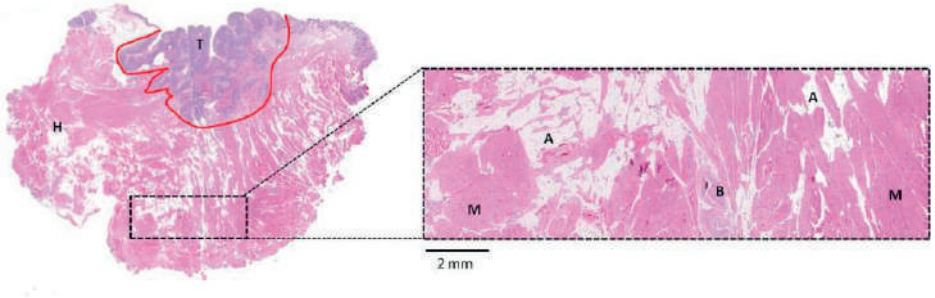

Figure 4. H&E stained section obtained from a measured tissue surface, with tumor border (red line), tumor (T) and healthy surrounding tissue (H) indicated by pathologist. A representative region of the adequate margin was enlarged, and the tissue structures annotated. Tissue structures present are muscle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

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.

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 intra-operative assessment of resection specimens.

We believe that Raman spectroscopy is a promising candidate for comprehensive intra-operative 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 prove 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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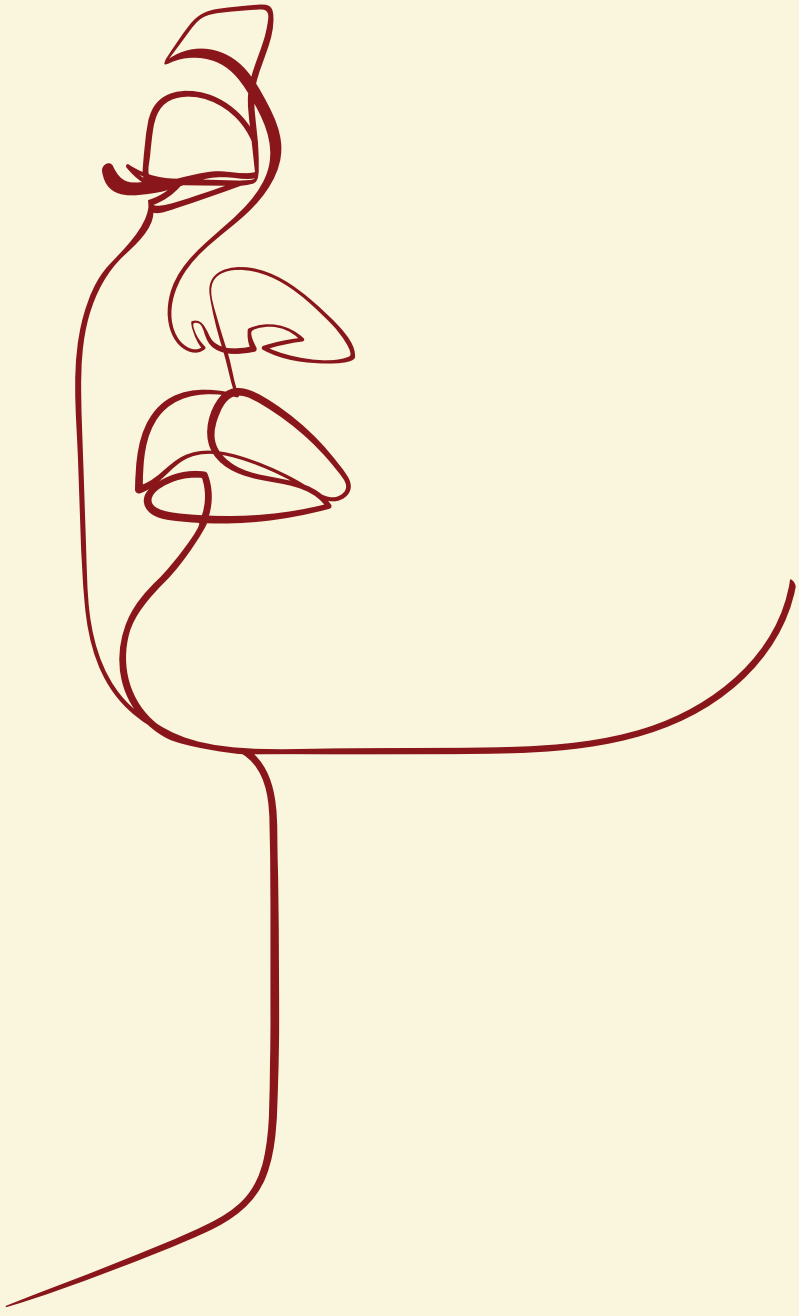

Chapter 4

Relocation of inadequate resection margins in the wound bed during oral cavity oncological surgery: a feasibility study

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4.1 ABSTRACT

Background

Specimen-driven intra-operative assessment of the resection margins provides immediate feedback if an additional excision is needed. However, relocation of an inadequate margin in the wound bed has shown to be difficult.

Methods

During oral cavity cancer surgery, the surgeon placed numbered tags on both sides of the resection line in a pair-wise manner. After resection, one tag of each pair remained on the specimen and the other tag in the wound bed. Upon detection of an inadequate margin in the specimen, the tags were used to relocate this margin in the wound bed.

Results

The method was applied during 80 resections for oral cavity cancer. In 31 resections an inadequate margin was detected and based on the paired tagging an accurate additional resection was achieved.

Conclusions

Paired tagging facilitates a reliable relocation of inadequate margins, enabling an accurate additional resection during the initial surgery.

4.2 INTRODUCTION

Surgery is one of the main treatment modalities for oral cavity cancer. The goal is complete tumor removal with adequate resection margins (i.e., more than 5 mm of healty tissue between tumor border and resection surface) (1). At the same time, healthy tissue should be spared as much as possible to preserve function and esthetics.

Of all oncological prognostic factors (i.e., patient and tumor characteristics), physicians can only influence the quality of resection margins. Inadequate resection margins negatively influence local recurrence, the need for adjuvant therapy and patient survival (2-4). Even the presence of dysplasia of squamous epithelium in the resection margins is associated with a higher risk on local tumor recurrence (1, 5, 6). For that reason, at our institute the resection margins containing severe dysplasia / in-situ carcinoma is considered inadequate as well.

In the oral cavity, an adequate tumor resection is often hard to achieve because of the complex anatomy, the demand for satisfactory remaining function and acceptable physical appearance. During tumor resection the surgeon relies only on his/her eyes and hands, and pre-operative imaging. For oral cavity squamous cell carcinoma (OCSCC) surgery, recent studies show poor results with an adequate tumor resection in only 15% of the cases (2, 3). Evidently, inspection and palpation are not sufficient to distinguish between tumor and the surrounding healthy tissue. In order to control resection margins, intra-operative assessment based on the frozen section procedure is available. Of all surgical disciplines, intra-operative assessment of the resection margins is most often used in head and neck surgery (7). Except for Mohs surgery, the role of the frozen section procedure in other surgical fields is limited. During intra-operative assessment of resection margins by frozen section analysis, suspicious tissue is usually sampled from the wound bed, therefore the method is also called wound bed / defect-driven assessment. In recent years the specimen-driven assessment, in which the surgeon and pathologist together assess the resection margins on the specimen, has been advocated. There is growing evidence that a specimen-driven assessment is superior to wound bed-driven assessment due to better visualization and less sampling error (4, 8-10) (*Smits et al.* 2020). Based on this evidence, the American Joint Committee on Cancer (AJCC) adopted specimen-driven intra-operative assessment as standard of care in the current guidelines (11).

Although intra-operative assessment can be beneficial with both specimen-driven and wound bed-driven, either method lacks an accurate relocation of the inadequate margin. It is known that relocation is particularly difficult in the head and

neck region, and therefore an optimal additional resection is not always achieved (9, 12-19)

Various ideas to solve the problem of relocation of inadequate resection margins have been described, but none of them seems to be efficient. For the wound bed-driven assessment, the use of surgical clips in the wound bed is frequently reported (20-22), as well as systematic cavity shavings, where tissue is sampled for frozen sections by shaving the wall of the surgical cavity (23, 24). For specimen-driven assessment, Mohs' surgery (25, 26) or mapping of the margins (e.g., Breuninger technique) (27, 28), are successfully used in dermato-oncology, which also harbors the problem of relocation. Although it has been described recently for small and simple OCSCC resection specimens, this method is not applicable for all head and neck resection specimens (29).

The main goal of the current study was to report on a reliable and objective method for relocation of the inadequate margins from specimen to the wound bed, based on intra-operative specimen-driven assessment and to assess the ease and accuracy of this method in the surgico-pathological workflow.

4.3 MATERIALS AND METHODS

The study was approved by the Medical Ethics Committee of the Erasmus MC Cancer Institute, Rotterdam, the Netherlands (MEC-2017-1016). In recent years, at the Erasmus MC Cancer Institute we use a paired tagging method for relocation of the inadequate margins from the specimen to the wound bed in oral cavity cancer surgery. Patients with a primary or recurrent tumor of the oral cavity were included for this method. The tags (Premier Farnell Limited BV, Utrecht, the Netherlands), numbered from 0 to 9, were cut to a size of 5 mm x 7 mm x 2 mm. The tags were perforated in order to fix the tag with a suture into the tissue (Figure 1). The tags were sterilized in alcohol 60 minutes before the surgery. During resection, the surgeon fixed the tags with the same number in a pair-wise manner, along both superficial and deep resection lines. In this way, one tag of each pair remained on the resection specimen and the other tag in the wound bed. The tagging procedure is illustrated by Figure 2 (A – C).

A specimen-driven intra-operative assessment of the resection margins followed as standard procedure. The pathologist and the surgeon together assessed the resection specimen by inspection (visually and by palpation) and by incisions

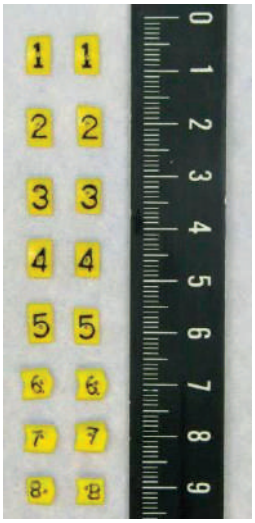

Figure 1. Tags.

perpendicular to the resection plane. If the tumor border could not be clearly identified by visual inspection, the assessment was refined by frozen section histopathology. The resection margins for invasive tumor are defined as adequate; more than 5 mm of healthy tissue between tumor border and resection surface, or inadequate; less than 5 mm of healthy tissue between tumor border and resection surface, in accordance with the guidelines of the Royal College of Pathologists. Moreover, according to our institutional guidelines, resection margins containing severe dysplasia / in-situ carcinoma are also classified as inadequate. In all cases where margins were adequate the tags were removed from the wound bed. If an inadequate margin was found, the numbered tags enclosing this area on the resection specimen indicated its location. Moreover, desirable thickness / depth of the additional resection, to achieve an adequate margin, was also indicated by the pathologist (in millimeters), depending on the initial margin. For example, if the initial margin was 2 mm the pathologist recommended an additional resection of tissue with at least 5 mm thickness. Based on this information the surgeon re-located the corresponding tags in the wound bed and performed an additional resection around these tags with the indicated tissue thickness. The accuracy of the relocation method was checked by comparing the numbers of the tags on the additional resection specimen with the numbers of the tags surrounding the inadequate margin on the main specimen. No intra-operative assessment of the margins in the additional resection was performed. An illustration of the relocation method from the specimen to the wound bed is shown in Figure 2 (D - G). In Figure 3, an example of the tagging method with additional resection, during an

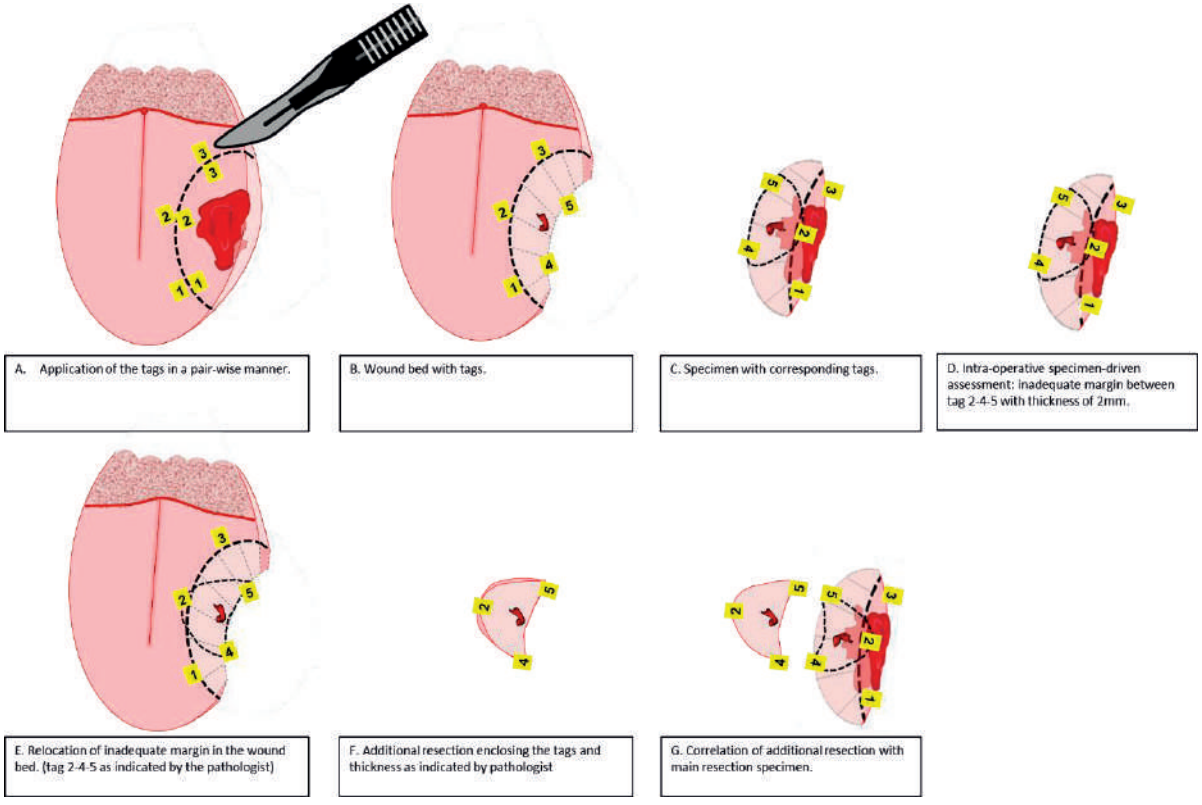

Figure 2. Paired tagging method, overview.

A, Application of the tags in a pair-wise manner. B, Wound bed with tags. C, Specimen with corresponding tags. D, Intra-operative specimen-driven assessment: inadequate margins between tag 2-4-5 with thickness of 2 mm. E, Relocation of inadequate margins in the wound bed. (Tag 2-4-5 as indicated by the pathologist.) F, Additional resection enclosing the tags and thickness as indicated by pathologist. G, Correlation of additional resection with main resection specimen.

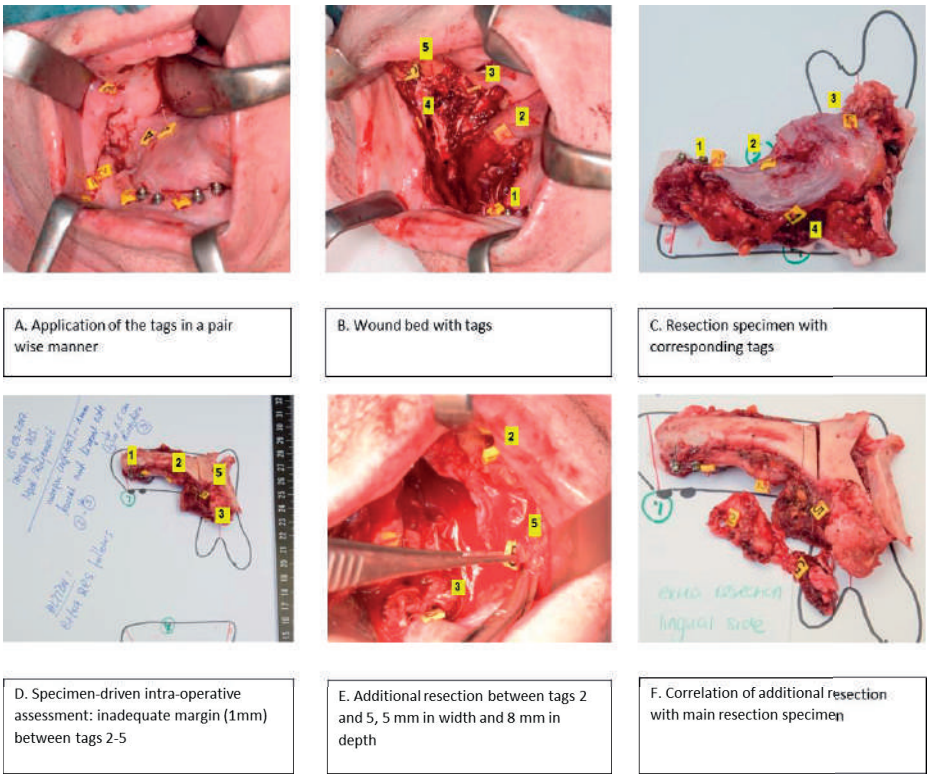

Figure 3. Paired tagging, including intra-operative assessment of the resection specimen and correlation of the additional resection with the resection specimen.
A, Resection of the tumor of the right processus alveolaris with application of the tags in a pair-wise manner. B, Wound bed with numbered tags (superficial and deep). C, Resection specimen with corresponding numbered tags. D, Intra-operative specimen-driven assessment of the resection margins: an inadequate margin was found between tags 2-5. E, An additional resection based on relocation, enclosing the corresponding tags and thickness, as indicated by the pathologist. F, Assessment of the accuracy of the additional resection based on correlation based on with main specimen.

"en bloc" resection with segmental mandibular resection, based on relocation with paired tags is shown, including the correlation of the additional resection with the main resection specimen. After correlation of the additional resection with the main resection specimen, the remaining tags were removed from the wound bed. After completion of surgery, the main specimen and any additional resection specimen followed the standard pathological procedure. Information regarding specimen characteristics, type of surgery, and status of resection margins based on intra-operative assessment were collected. The number of tags used, and their exact location were recorded during each surgery. The time needed for placing the tags was also recorded. In addition, the ease of placing the tags and the ease of relocation of inadequate margins in the surgical wound bed was documented.

The ease and accuracy of the correlation of the additional resection with the main specimen were also recorded.

4.4 RESULTS

From September 2015 until September 2017, the method of paired tagging, as described in the methods section, was applied during 80 surgeries (79 patients) for oral cavity tumors, at the Erasmus MC Cancer Institute. The group comprised of 78 squamous cell carcinomas (SCC) and 2 salivary carcinomas (1 mucoepidermoid carcinoma and 1 adenoid cystic carcinoma). Most of the tumors were early stage carcinomas (20 cT1, 29 cT2). From 80 surgeries there were 30 (37%) local resections, 15 (19%) "en bloc" resections, 16 (20%) "en bloc" resections with segmental mandibular resections, 8 (10%) "en bloc" resections with marginal mandibular resections, 7 (9%) hemiglossectomies, 2 (2.5%) subtotal glossectomies and 2 (2.5%) were partial maxillectomies. In all cases specimen-driven intra-operative assessment of the resection margins was performed. None of the patients had received radiation therapy prior to surgery.

A maximum distance of 5 mm between the two tags of one pair was maintained. For local excisions, 4 to 5 tag pairs were sufficient, with an interval of 1 cm between different tag pairs. In case of large resections, usually all 10 tag pairs were used (numbered 0-9) which were fixed with intervals of approximately 2-3 cm between different tag pairs. The time needed to suture one tag was on average 30 seconds. The surgeons reported an easy relocation of the inadequate resection margin from specimen to the wound bed. They described the use of the tags as easy but time consuming, and therefore interfering with the surgical workflow (September 2015 - September 2017; oral communication; H Mast MD DDS, JAU Hardillo PhD, DA Monserez MD, I ten Hove MD DDS, CA Meeuwis PhD, A Sewnaik PhD, RJ Baatenburg de Jong Prof). The pathologists reported that the tags enabled accurate anatomical orientation of the specimen. Moreover, pairing of the tags on the resection specimen and the additionally resected tissue enabled the pathologists to determine that an as accurate as possible additional resection has been performed. In general, the pathologists did not experience any obstruction of the pathological workflow by this method (September 2015 - September 2017; oral communication; R Verdijk PhD, GJLH van Leenders PhD, S Koljenović PhD). Both the surgeons and pathologists described the method, also referred to as Erasmus MC relocation method, as indispensable. Currently the method has been used as standard of care during head and neck surgery at our institution.

Moreover, there is a great interest in this relocation technique by other centers, nationally and internationally.

During intra-operative specimen-driven assessment, in 43/80 cases an inadequate margin was found for invasive carcinoma (7 tumor-positive margins, 33 close margins) and for severe dysplasia (3 cases with dysplasia-positive mucosal margins). In 31 of these cases an additional resection was performed based on the relocation method: 4 for tumor-positive margins, 24 for close margins, and 3 for severe dysplasia. In the remaining 12 cases (3 tumor-positive margins, 9 close margins) an additional resection was not performed for different reasons: in 11 cases because additional resection interfered with maintenance of function and esthetics (e.g., overlying skin or mandible), and in 1 case it was not possible due to the close relation with the internal carotid artery. The results are summarized in Figure 4.

After additional resection, final pathology confirmed that in 28 out of the 31 cases the status of that specific resection margin was improved: in 25 cases an adequate margin was obtained, in 3 cases the revised margins were improved from 0.1 mm to 2.1 mm, from 1 mm to 4.7 mm, and from 2 mm to 3 mm. In the last 3 cases, the margins remained tumor-positive. These data are shown in Table 1.

Two patients with a second resection because of recurrent disease were included in this study. In both cases the initial margin was inadequate and was improved to adequate after additional resection. Post-operative radiotherapy (PORT) was given based on the following guidelines, with main criteria comprising positive resection margins, lymph node metastases with extra nodal extension or ≥2 positive lymph nodes. Minor criteria are close resection margins, infiltrative growth, perineural growth and pT3/T4. Twelve patients received PORT, based on the above-mentioned guidelines. Two patients had an indication for PORT but refused treatment.

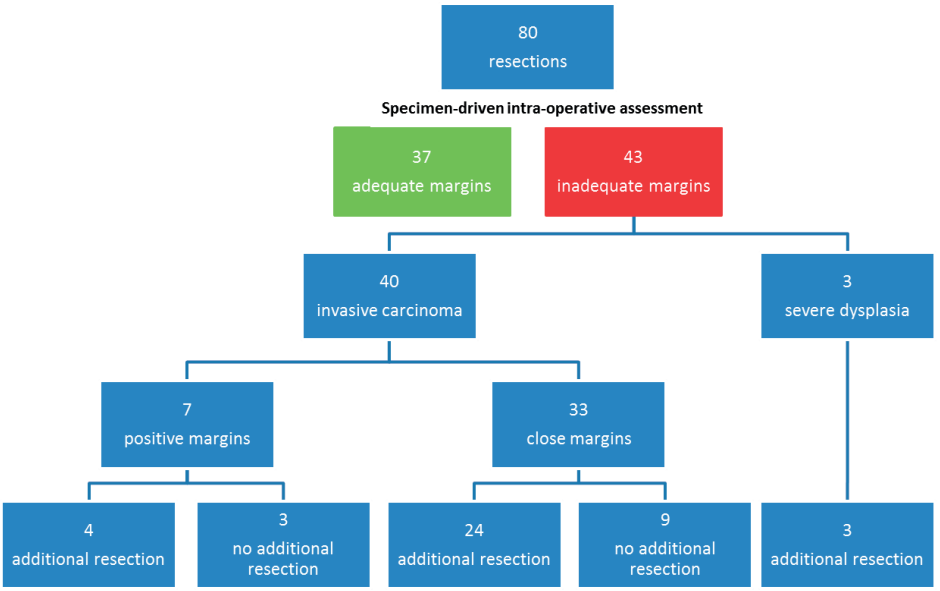

Figure 4. Overview surgico-pathological workflow based on specimen-driven intra-operative assessment of resection margins.

Table 3. Characteristics of resection specimen with revised margins based on Erasmus MC relocation method.

Case number	Location tumor	Type of surgery	Intra-operative assessment: resection margins	Additional resection margin	Accurate additional resection achieved
1	Tongue	Local excision	Dysplasia (3 mm)	Clear (7 mm)	Yes
2	Buccal mucosa	"En bloc" resection with segmental mandibular resection	Dysplasia (4 mm)	Clear (6 mm)	Yes
3	Floor of the mouth	"En bloc" resection with marginal mandibular resection	Dysplasia (4 mm)	Clear (8 mm)	Yes
4	Tongue	Local excision	Close (<5 mm)	Clear (7 mm)	Yes
5	Floor of the mouth	"En bloc" resection	Close (2 mm)	Close (3 mm)	Yes
6	Oropharynx	Local excision	Close (1.5 mm)	Clear (5.5 mm)	Yes
7	Tongue	Subtotal glossectomy	Close (1.8 mm)	Clear (5.8 mm)	Yes
8	Floor of the mouth	Local excision	Close (1 mm)	Close (4.7 mm)	Yes
9	Mandible	"En bloc" resection with segmental mandibular resection	Close (1 mm)	Clear (5.5 mm)	Yes

Table 3. Characteristics of resection specimen with revised margins based on Erasmus MC relocation method. (*continued*)

Case number	Location tumor	Type of surgery	Intra-operative assessment: resection margins	Additional resection margin	Accurate additional resection achieved
10	Floor of the mouth	"En bloc" resection	Close (1 mm)	Clear (5.5 mm)	Yes
11	Tongue	Local excision	Close (1 mm)	Clear (6 mm)	Yes
12	Alveolar process	"En bloc" resection with segmental mandibular resection	Close (2 mm)	Positive (<0.1 mm)	Yes
13	Tongue	Local excision	Close (2 mm)	Clear (6 mm)	Yes
14	Tongue	Local excision	Close (2 mm)	Clear (6 mm)	Yes
15	Floor of the mouth	"En bloc" resection with marginal mandibular resection	Close (2 mm)	Clear (7 mm)	Yes
16	Trigonum retromolare	"En bloc" resection with segmental mandibular resection	Close (2 mm)	Clear (7 mm)	Yes
17	Alveolar process	"En bloc" resection with segmental mandibular resection	Close (3.1 mm)	Clear (5.1 mm)	Yes
18	Tongue	"En bloc" resection	Close (3.5 mm)	Clear (9 mm)	Yes
19	Alveolar process	"En bloc" resection with segmental mandibular resection	Close (3 mm)	Clear (13 mm)	Yes
20	Floor of the mouth	"En bloc" resection with marginal mandibular resection	Close (3 mm)	Clear (6 mm)	Yes
21	Alveolar process	"En bloc" resection with segmental mandibular resection	Close (3 mm)	Clear (8 mm)	Yes
22	Buccal mucosa	Local excision	Close (4 mm)	Positive (0.1 mm)	Yes
23	Tongue	Local excision	Close (4 mm)	Clear (11 mm)	Yes
24	Tongue	"En bloc" resection	Close (4 mm)	Clear (6 mm)	Yes
25	Floor of the mouth	"En bloc" resection	Close (4 mm)	Clear (8 mm)	Yes
26	Tongue	Hemiglossectomy	Close (4 mm)	Clear (8 mm)	Yes
27	Tongue	Local excision	Close (4 mm)	Clear (9 mm)	Yes
28	Buccal mucosa	Local excision	Positive (<0.1 mm)	Close (2.1 mm)	Yes
29	Base of the tongue	"En bloc" resection	Positive (<1 mm)	Positive (<0.1 mm)	Yes
30	Tongue	Hemiglossectomy ("en bloc")	Positive (<1 mm)	Clear (5.6 mm)	Yes
31	Alveolar process	Partial maxillectomy	Positive (<1 mm)	Clear (13 mm)	Yes

4.5 DISCUSSION

Intra-operative assessment of the resection margins is only meaningful if an accurate additional resection is enabled.

McIntosch et al. described that intra-operative control of the resection margins is more frequently performed in head and neck surgery than in other surgical specialties (7). According to the current guidelines of the AJCC specimen-driven intra-operative assessment is the standard of care (11). Although powerful, the impact of intra-operative assessment is negatively influenced by the lack of accurate relocation of inadequate margins for optimal additional resection towards adequate surgery (9, 12-15). As a result, various studies have reported an accurate additional excision for initial tumor-positive margins in only 22.5-50% of the cases (12, 16-18). *Kerawala* and *Ong* performed a study on relocation of the site in the wound bed where tissue was sampled for a frozen section procedure (during wound bed-driven assessment). In this study, the surgeon was asked to indicate the sites of sampling. After 5 minutes, the same surgeon was asked to relocate each site. In 32% (23/71) there was an error of more than 1 cm. The authors concluded that, due to the complex anatomy of the head and neck region, and the three-dimensional structure of the wound bed, it was difficult to relocate the exact place of the inadequate margin, especially in larger resections (15). *Maxwell et al.* found a disappointing high percentage of inadequate resection margins and low local recurrence free survival for patients with an additional resection based on specimen-driven intra-operative assessment. These poor results were explained by the following author's statement "*owing to the challenges of relocating the exact aspect of the relevant margin in the tumor bed, size discrepancy, and uncertain orientation of the additional tissue, it is conceivable that, in some patients, the additional margin may not actually cover the entire residual tumor at the positive margin*" (9). The importance of relocation was also highlighted by *Williams et al.* (13). In this review, the impact of the additional resection was estimated by local control rates. Better local control (LR 13-18%) was found for the surgical resections with adequate margins on initial surgery (where no additional resection was needed), compared to resections in which adequate margins were achieved after additional resection based on specimen-driven intra-operative assessment (LR 22-32%). These authors also concluded that the imprecision of relocation might be a contributing factor to these increased local failures. They stated that another factor, complicating accurate relocation of inadequate margin in the wound bed, is the retraction of the muscle tissue which results in misrepresentation of original anatomical rela-

tionships (13). Also *Hinni et al.* reported that "defect disorientation" can limit an accurate relocation of inadequate margin (10).

The method of paired tagging (with numbered tags) solves the various problems hampering the relocation as mentioned by many authors, such as (muscle) tissue retraction and wound bed deformation, leading to size discrepancy, and the complex anatomy of the three-dimensional structures (9, 10, 13, 15). This relocation method with numbered tags is objective and enables clear communication between pathologist and surgeon. The results of this feasibility study presented here show that by paired tagging an accurate additional resection was performed in all cases where the initial margin was inadequate. In 1 case, the initially tumor-positive margin was revised to close margin. Although the additional resection may not always result in an adequate margin it might have positive impact on the need for adjuvant treatment. A tumor-positive margin is one of the main criteria for PORT, with or without chemotherapy. It is likely, therefore, that the additional resection, guided by the relocation method described here, will have the most impact for patients with pT1-T2 tumors in which other minor criteria for adjuvant therapy are also absent (e.g., positive nodal status, extra nodal extension, perineural growth, and infiltrative growth). For the 2 remaining cases the margin remained close (1 mm to 4.7 mm and 2 mm to 3 mm). However *Nason et al.* describes that each additional millimeter of tumor-free margin may be beneficial for patient outcome (30). Although we present promising results of inadequate margin relocation, at this stage the method has some limitations such as sterility for the use in all head and neck resections, duration of placing the tags, size of the tags, and interruption of the surgical workflow. In order to improve the procedure, we are now developing a prototype instrument for rapid and easy placement of the tags and for tag removal. The goal is to simplify implementation of the procedure, to make the tags 3x4 mm. Finally, we seek a tagging prototype and optimized protocol that can be used by surgeons in all other specialties. We are preparing a retrospective clinical cohort study with matched pair analysis consisting of a larger group of patients and sufficient follow up.

It can be concluded that this simple relocation method enables an accurate additional resection when an inadequate margin is found during intra-operative assessment. It is expected that the implementation of paired tagging will lead to a higher number of adequate tumor resection margins, and thereby will lead to a better patient outcome and/ or reduce adjuvant therapy and the related morbidity.

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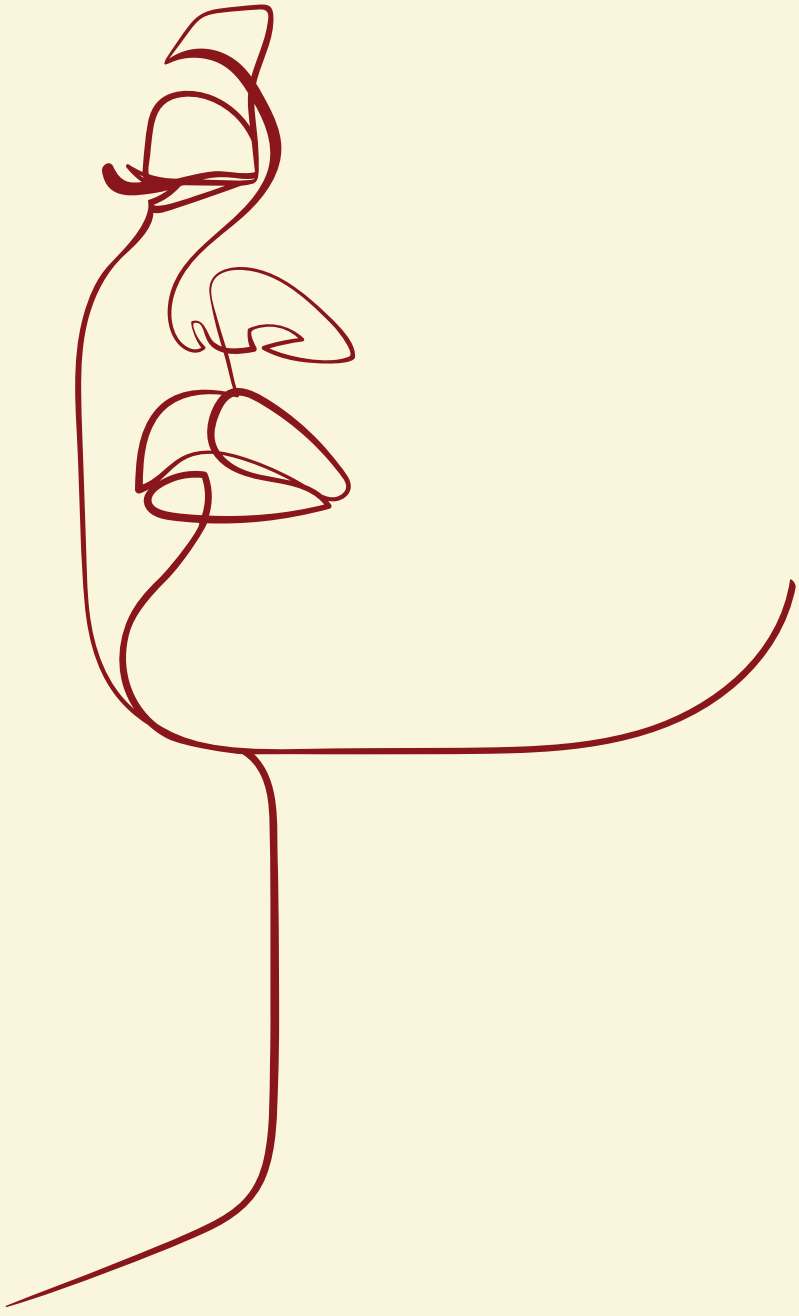

Chapter 5

Improvement of the current relocation method

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5.1 INTRODUCTION

Although powerful, the impact of intra-operative assessment is negatively influenced by the lack of accurate relocation of inadequate margins for optimal additional resection towards adequate surgery. *Kubik et al.* described several reasons (e.g., additional resection at an incorrect location, incorrect orientation of the additional resection, or incorrect dimensions of the additional resection) for inadequate additional resections. It is known that relocation is particularly difficult in the head and neck region, and therefore, achieving an optimal additional resection is not always possible. The spatial relationship between the additional resection and the main specimen is the key factor.

In the previous chapter, we reported on a reliable and objective method for relocating inadequate margins from the specimen to the wound bed, based on specimen-driven intra-operative assessment of resection margins. This method allowed the surgeon to perform an additional resection based on the relocation of the inadequate margin, defined by the tags in the wound bed. For example, if a margin of 2 mm is found between tags 1-2-3, the surgeon performs an additional resection around tags 1-2-3 with a thickness of 4 mm. The effectiveness of this relocation method was demonstrated by the results of the second study in this thesis.

Despite presenting promising results of inadequate margin relocation, the method has some limitations, such as the sterility of the tags, the duration of placing the tags, the size of the tags, and interruption of the surgical workflow. To improve the method, we initiated the development of an instrument for rapid and easy placement of the tags and tag removal. This collaboration involves the Erasmus MC and the faculty of Industrial Design Engineering at Delft University of Technology, with the goal of simplifying the implementation of the relocation method.

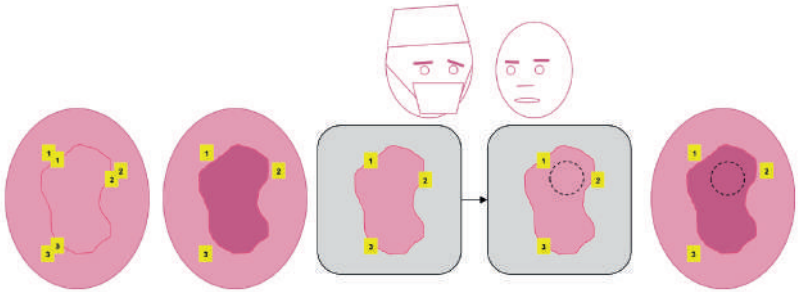

Figure 1. Relocation method.

5.2 SEMI-AUTOMATIC TAG APPLICATOR

The project was executed following the basic design cycle of Pahl and Beitz, which consists of four phases: analysis, conceptualization, embodiment, and evaluation (Roozenburg & Eekels, 1998). For a performance-focused design, this straightforward approach is more suitable than abstract, meaning-centered approaches such as ViP or the ZEN design method. It is important to note that this design process is far from linear, and several cycles of iteration took place. Throughout the process, it became apparent on several occasions that specific design steps yielded valuable insights, necessitating revisiting and revising those steps based on the newfound knowledge.

5.2.1 Analysis

The design process for this product, which includes tags, a semi-automatic applicator, and a remover, commenced with an analysis of the current situation and the formulation of design goals and criteria. The analysis phase aimed to understand the product's intended environment and context of use. It involved conducting a literature analysis, encompassing medical literature, regulations, and market research, as well as a user analysis, which included observations and interviews with surgeons, operating assistants, and sterilization personnel. This comprehensive analysis laid the foundation for the product requirements specification document (appendix) and shaped the design vision for the final prototype.

The primary criteria derived from the product requirements specification are the time required to apply the tags, accuracy, reliability, and ease of use. The development of the tag applicator primarily focuses on addressing the needs of surgeons and operating assistants, who are the direct users of the device. From a usability perspective, ensuring clear communication and non-interference with other actions are also crucial considerations. Aesthetics can play a role in enhancing the user experience and optimizing product performance. The product's design should convey both mechanical robustness and user-friendliness, and visual cues should be employed to inform users of additional functionality.

The project's scope is centered on the European (EU) market, and compliance with the main directive 93/42/EEC concerning medical devices is essential to attain CE compliance (NEN 2016). Market introduction in the EU requires an assessment of compliance with the medical devices directive 93/42/EEC, with the specific procedure determined by the device class. To avoid delays in market launch due

to overly stringent procedures, the product is designed to meet the requirements of class I or class IIa.

In hospital settings, the sterilization process for instruments involves disassembly, cleaning, reassembly, and packaging. The two most common sterilization methods are steam autoclave and EtO gas, with steam sterilization being prevalent at the Erasmus MC. Single-use instruments are increasingly popular due to their advantages in logistics, cost-of-ownership, and infection risks compared to reusable instruments. Additionally, reusable devices are not inherently more reliable than disposable designs, as wear and tear of parts can affect their performance. Sterilization in the hospital may pose more challenges for mechanically complex devices compared to those consisting of only a few parts or mechanically sealed devices. Examples of the latter include blade handles, forceps, electrocautery tools, drills, and reciprocating saws.

5.2.2 Conceptualization

All the collected information has culminated in a comprehensive list of requirements. During the conceptualization phase, these requirements served as input for identifying and resolving design problems. The design process was bound by the essential medical requirements related to safety and reliability. Moreover, potential scenarios involving unusual usage were taken into consideration, acknowledging that tags might need manual application (e.g., suturing) and removal in hard-to-reach locations not accessible by the applicator. In exceptional cases, more than 20 paired tags might be required.

Once the initial concept was generated, the list of requirements played a crucial role in ensuring that the concept aligned with user demands, and it was utilized to evaluate and rate the concept. Taking into account the feedback from previous concepts, the final concept was developed with a specific focus on reducing the size of the tags and applicator. The main priority was to achieve time savings by replacing the suturing of tags, rather than significantly impacting the workflow.

Feedback on the applicator's automatic reloading functionality raised concerns about the lack of control over which tag gets applied, as well as the additional bulk it added to the device. Doubts were also raised about whether the time savings from automatic reloading outweighed those of manual reloading. Consequently, a manual reloading system was considered, as it would offer greater control and flexibility. For instance, it would allow for tag sets with different colors or sets with extended number ranges for more extensive surgeries. To facilitate this, a

disposable cartridge was devised as a convenient way to present the tags. Users can insert the device into the cartridge slot, and the tags will lock themselves into the jaws, requiring only proper insertion technique from the user.

Furthermore, renewed emphasis was placed on applying the tags in pairs, as this would enhance location reliability and improve time savings during the procedure.

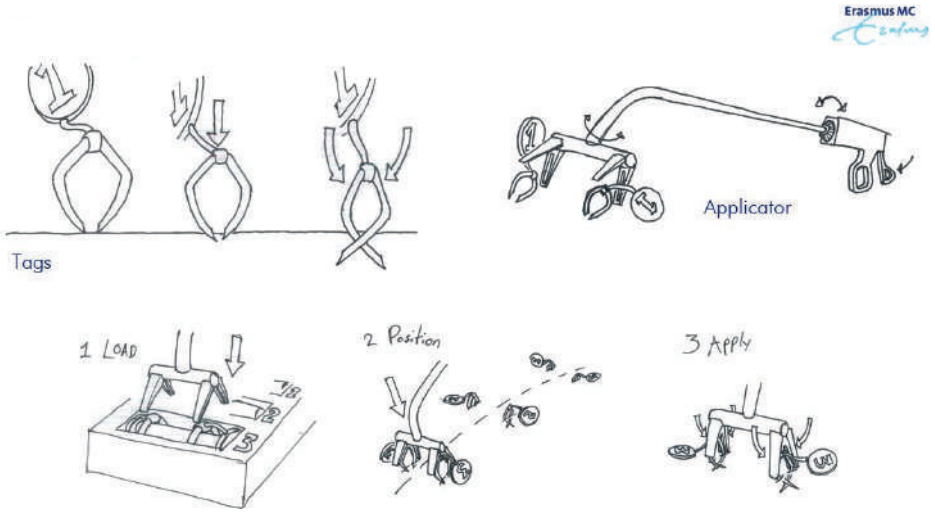

Figure 2. Final concept.

5.2.2.1 Tags

From the previous concepts, the working principle of the staple with the tethered label was retained. Anchoring the tag involves two steps: first, the tag is placed on the desired location and pushed down horizontally, allowing the sharpened prongs to penetrate the tissue. Second, the top ends are pushed towards each other, causing the prongs to penetrate further into the tissue and cross each other inside, creating the most space-efficient configuration. The size of the 'eye' is a compromise between reliability and removability. A smaller opening allows the prongs to travel more distance horizontally, but it becomes more challenging to insert a remover into the eye to pry the tag open.

The dimensions of each part are linked to its functionality. The wire gauge determines the ease with which it deforms, affecting the anchoring strength and ease of application and removal. The anchoring strength is also influenced by the penetration depth and subsurface width. The width and height of the tag part that

protrudes from the tissue determines its visibility to the user, as well as the ease of application and removal. These sizes have been determined based on requirements and experimentation, achieving the best compromise between ease of use, reliability, and minimizing tissue damage. Other dimensions, such as the bevel of the prongs and the angle between top and bottom parts, were chosen intuitively but should be considered during future development. The size of the tag label was also determined in close consultation with prospective users. The smallest size that still allows identification by the naked eye was chosen, and the length of the flexible stem allows the user to move the label out of the way. These dimensions are not critical to the design of the applicator and remover, so they can be easily changed to any convenient size.

As the staple may remain in contact with tissue for prolonged periods, a steel with high corrosion resistance is required, making austenitic grades of stainless steel suitable. Despite being referred to as a 'staple,' its purpose is not load-bearing. To increase ease of application and removal, a smaller diameter, such as 0.4 mm, was selected compared to the 0.6 mm gauge of surgical skin staples. An important requirement for the staple is that it must not be electrically conductive to prevent localized cauterization accidents when touched with an electrocauter. A thin layer of polymer varnish provides electrical insulation, similar to the insulation used for the coils of electromotors and low-voltage transformers.

Tyvek has been chosen as the label material, resembling paper but made from high-density polyethylene. Tyvek is tear-resistant, durable, breathable, and has a superior microbial barrier, making it compatible with common sterilization methods. The color was selected based on the red-green-blue color system. Cyan,

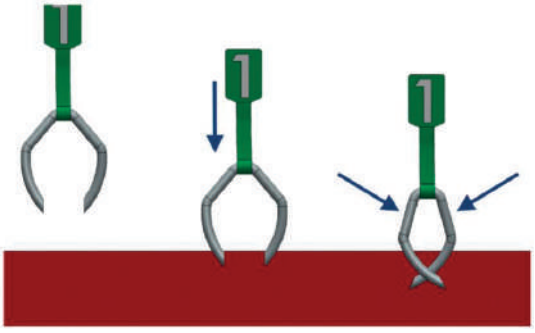

Figure 3. Tags and anchoring method.

although complementary to red (the color of tissue and blood), conflicts with the white lettering. Hence, green was chosen as the next suitable color.

5.2.2.2 Tag applicator

The main purpose of the applicator is to hold a pair of tags and squeeze them closed. Size, location accuracy, and distance within each pair are of crucial importance. The best solution is a pair of jaws, similar to a laparoscopic grasper. These jaws only need to open several millimeters, allowing them to be tiny and seated at the end of a narrow shaft. To ensure ergonomic use, the shaft can be rotated around its axis. The slider at the end of the handle can be retracted to disengage the positioning keys, enabling the shaft to be set in the desired position in 45° steps. Releasing the slider will lock it in place. A rod inside the shaft drives the jaws. To accommodate a bend in the shaft, a section of wire can be used, which should be stiff and as close to the inner diameter of the shaft as possible to reduce backlash. The most important action, closing the jaws, is performed by retracting the rod to minimize backlash. Rotating the jaws with respect to the shaft requires the user to unscrew the retaining collar at the end of the shaft, allowing the piece containing the jaws to be rotated. Fastening the retaining collar will provide the pressure that prevents unintended rotation.

At the users' suggestion, the design will include several different versions of the applicator. This way, multiple and different applicators can be employed during surgery, and the surgeon can choose the most practical reach in any given moment and load the next pair of tags. The surgeon can even alternate between applicators, using one while the other is being reloaded by an assistant. Variations include shaft length (lengths of 200 and 300 mm), shaft bend angle (straight or angled, with an angle of 60° chosen based on other surgical instruments), and single or dual jaw options (an applicator that applies tags in pairs is preferred, but not possible in some situations).

Several handle variants were tried, including straight, outward-curved, and inward-curved grips. The inward-curved grip was chosen for the final design as it conforms better to the natural shape of the hand. The finger loop encompassing both the ring and middle fingers was found to be the most comfortable, providing a better grip for the index finger. A scissor-like variant of the pistol grip was selected as the connection between the shaft and the user's hand. This grip positions the wrist and forearm in a more neutral position when the device is held so that the user's view follows the shaft (similar to aiming a gun), and the thumb-actuated 'trigger' provides plenty of leverage. The dimensions are based on the 95th percen-

tile hand sizes of the Dutch population aged 20 – 60. It can be safely assumed that wearing one or two layers of medical gloves will not significantly affect the hand sizes.

Since the applicator no longer has an automatic reloading function, a device that can be sterilized and reused is the best option. To make the device sterilizable, it must be taken apart beforehand. Most of the device is held together by threads that can be (un)screwed by hand, while the handle has screws that require a small screwdriver. These steps are not uncommon for similar devices, as observed at the Central Sterilization Department.

For a sterilizable design, stainless steel is the material of choice. A stainless steel from the martensitic group can be heat-treated to very high tensile strengths. While there are other high-strength sterilizable materials such as titanium alloys or high-strength plastics, their high costs do not outweigh their benefits. Stainless steel, defined as an iron-based alloy containing at least 10.5% chromium and a maximum of 1.2% carbon, provides corrosion resistance through the formation of a passive oxide surface film that protects the underlying material. Stainless steels are categorized based on properties such as strength and processability, and the international standard ISO 7153-1 covers stainless steels used for standard surgical instruments. Stainless steel allows many structural elements to be smaller or thinner for a given durability. Additionally, stainless steel is machinable, allowing for the design to use stock materials (e.g., tube, bar, sheet), which saves costs compared to injection molding plastic parts. The service life that can be expected for a stainless steel instrument is 15 years, and the spring rails are stamped from sheets of spring-tempered stainless steel, which may be a different grade than used for the other parts.

The applicator also has several components with no high strength requirements, but their geometrical complexity precludes manufacturing from stock metals. Injection molded plastic is the most economical option for these parts. Polypropylene is a medical plastic compatible with EtO sterilization. It is one of the most common plastics, easily injection-molded, and cost-effective. Plastic will be used for the parts that the user will interact with (i.e., head and neck rotator sleeves and handle parts). These non-structural parts, where the effects of wear will not be as significant, make plastic the best choice, and their minimum dimensions for comfortable use further support this decision. Additionally, textures and colors can be easily specified for plastic parts, providing more obvious use cues.

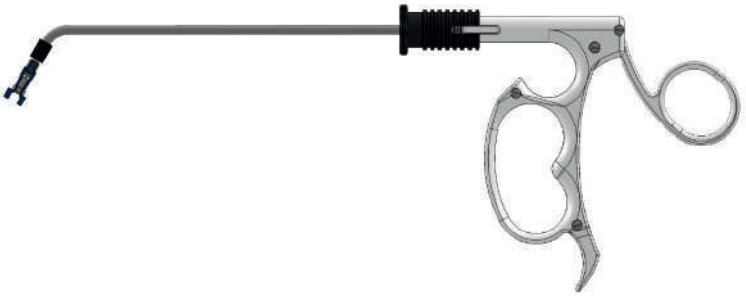

Figure 4. Tag applicator.

Figure 5. Close-up of applicator jaws.

5.2.2.3 Tag remover

Unlike regular scissors, which have two arms stacked on top of each other and held together by a screw, the tag remover consists of an inner and outer arm, held together by a pin fused to the outer arm. This design is in line with most medical reusable hand tools. The tags are removed one by one by pulling the legs apart, in the opposite fashion of the applying process. Accuracy is required when inserting its hooks through the eye. It is important that the remover has the reach to do this and does not drop the tags in the process. To prevent accidentally dropping the tags, the remover includes a latch similar to most forceps. Since only a small movement is needed to spread the tag's legs, a 40 mm travel between the finger holes corresponds to the 4 mm of hook movement needed to release the tag. The total length of the tool is determined by its ability to reach the furthest ends of the wound bed.

The remover will be similar to the applicator's main structural parts, using martensitic stainless steel for durability and a longer lifetime. While contact with corrosive substances is minimal, durability and lifetime remain important factors. The design ensures a more durable construction that is less prone to lateral slack.

Figure 6. Tag remover.

5.2.3 Embodiment

The final concept was further materialized in the embodiment phase, and a prototype was built. All aspects of the design were defined on a concrete level, which demonstrated not only the feasibility of the design but also the most common user interactions. We analyzed the aesthetic design choices and how they stimulated intended usage.

We considered the manufacturing processes involved and calculated an estimate of the manufacturing costs. For estimating the cost price of parts, we used the injection molding and machining estimators of CustomPartNet.

For the applicator and remover, we assumed a batch size of 100,000. Furthermore, we used polypropylene homopolymer for all plastic parts, stainless steel grade 1.4310 (AISI equivalent 301S21) for the spring steel jaws, and grade 1.4021 (AISI equivalent 420S29) for all other steel parts. Stock parts such as screws and springs are also stainless steel, and their prices are derived from suppliers' catalogs. The total cost for the applicator parts came down to an estimate of 12.78 USD. The retail price for consumer products is typically 4 times the manufacturing costs, and for medical products, this factor is 10. Taking into account assembly, packaging, distribution, marketing, etc., we estimate a factor of 20 to 30. Therefore, the end price for the tag applicator becomes 255.60 USD to 383.40 USD.

The remover has a complicated shape, making cost estimation difficult and unreliable. Instrument makers KLS Martin (2015) and Stille AB (2013) suggest manufacturing is largely done by manual labor and consists of many production steps. An indication for the end price can be obtained by comparing with tools of similar construction in the suppliers' catalogs, with prices ranging from 10 – 20 EUR (Quirumed 2016). This seems like a realistic estimate.

For the tag staples, we used stainless steel grade 1.4401 (AISI equivalent 316S31) in the estimate. An estimated factor 2 price increase is assumed for the insulating

varnish. Manufacturing requires two separate machine presses and dedicated molds, the last of which has a complex three-dimensional shape. Operating costs for a small press are approximately one cent per stroke. Since a bed measuring 50x30 cm can theoretically tend to hundreds of tags per stroke, the operating costs become negligible in comparison to the mold costs. The sum of all manufacturing processes is 0.047 USD per tag. The majority of the costs come from ultrasonically welding the label since the tags have to be welded one by one. No guidelines have been found for estimating the cost of pick-and-place machinery for transferring parts from one process to another. Yet this may easily be the highest cost factor, especially given the accuracy required and the sheer quantity of tags. Adding to this the cost of the cartridge, packaging, sterilization, distribution, marketing, and other costs would make a factor of 50 seem conservative. This translates to a sales price of 2.35 per tag USD or 47 USD for a cartridge of 10 tag pairs.

With the tag applicator and remover being reusable instruments, the most logical business model is that of the shaving razor. This consists of selling the applicator and remover at prices that will not yield the most profit, but selling the tags at a price that makes it sustainable. This means that the customer will pay according to their consumption, which presents a low cost of entry. Such an approach is especially favorable at first when a hospital is still new to the pairwise parallel tagging method. However, if the design becomes successful, it is likely that other medical instrument companies will try to copy or produce similar products that may circumvent any applicable patents. In that case, the low cost of entry means that the customer can easily switch to a different brand, making it more difficult to retain customers.

5.2.4 Evaluation

The last phase describes the process used to test various design aspects and evaluate the final design. A proof-of-concept for the anchoring method was used during the development of the design. After finalizing the design, a prototype was constructed from aluminum to demonstrate the size of the applicator and its mechanical principles. The design was evaluated against the list of requirements during a session where users tested the prototype on cadavers. Subsequently, a more sophisticated model was made using stainless steel, intended as a 1:1 scale prototype. However, there were difficulties in achieving this scale for all parts and making it functional due to its small dimensions. The plan for a user evaluation setup was, therefore, canceled. Based on the requirements evaluation and other findings during the design, prototyping, and user tests, the report concludes with a list of recommendations for future developers to pursue development and manufacturing.

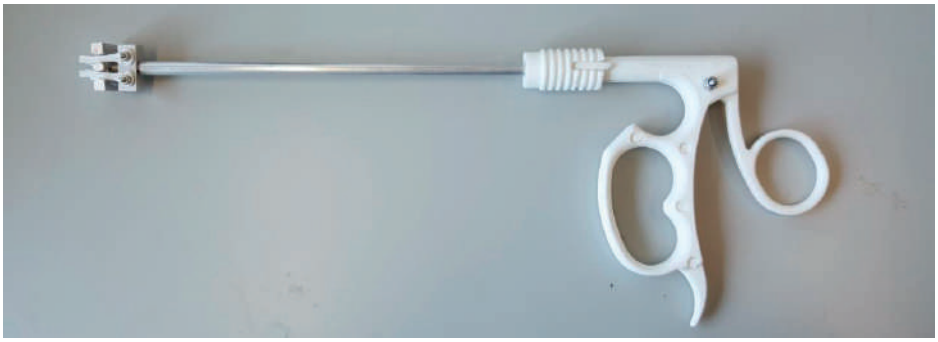

Figure 7. Aluminum prototype.

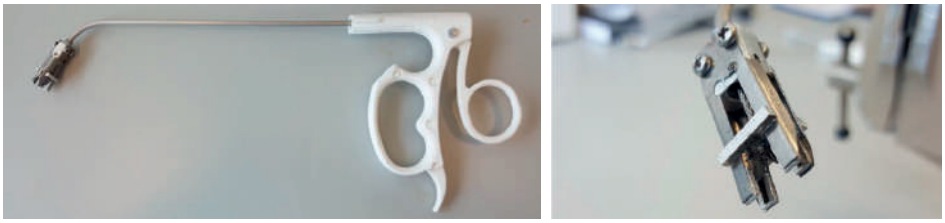

Figure 8. Stainless steel prototype.

5.3 RADIO-OPAQUE TAGS

During the implantation of the relocation method, the surgeons expressed a desire for tags that are biocompatible or soluble and radio-opaque. Tags with these properties can be detected and identified by CT, MRI, or Cyberknife (an image-guided stereotactic dose delivery system used for radiation therapy). This allows the tags to be used during post-operative radiotherapy, enabling a more accurate target volume.

Post-operative radiotherapy (PORT) follows primary surgery with the aim of improving locoregional control and survival. However, it can cause significant acute and late toxicity, which is related to the dose of radiation. For example, in a series of patients treated with either a lower dose (57.6 Gy) or a higher dose (63 Gy) of PORT, confluent mucositis was present in 5% and 47% of the low- and high-dose cohorts, respectively, and tube feeding was required in 12% and 39%, respectively. Additionally, the 5-year rates of late grade 3 and 4 toxicity (such as ulcer, soft tissue necrosis, fibrosis, dysphagia, fistula, osteonecrosis) were 17% and 38% for the low- and high-dose groups. According to the protocol, PORT should be initiated within 4-6 weeks after surgery. The time interval between surgery and initiation of PORT, as well as the duration of treatment, also have an impact on the outcome. In cases

of clear margins in patients with advanced primary lesions (T3/4), PORT doses of 56-60 Gy to the primary tumor bed are recommended. For patients with positive margins or extracapsular extension, a total dose of 66 Gy to the primary tumor bed is recommended.

There are limited data on which to base target volume delineation guidelines in the post-operative setting. Furthermore, outlining after surgery can be difficult due to changes in anatomy, secondary to loss of tissue, post-operative collections, and deformation of adjacent normal structures. The question arises whether the tags from the relocation method can aid in target volume delineation and thereby reduce toxicity. To address this, a new research protocol is developed.

The first research question to be answered is if the target volume can be reduced by marking the wound bed. And if so, what is the effect on toxicity? The following study design will be used: patients with primary head and neck tumors will be included. The relocation method will be performed during all resections, including the specimen-driven intra-operative assessment. Afterwards, the surgeon will replace all tags in the wound bed with radio-opaque markers. Surgical clips are preferred for this purpose, as they are already available in the operation room and visible with Cyberknife. Titanium clips are preferred due to their size, which will not interfere with wound bed closure or reconstruction with any type of flap. Subsequently, the radiotherapist will delineate the target volume according to the current guidelines, based on the surgical clips identified on CT. An analysis will be performed to calculate the differences between the target volume and toxicity.

If there is a safe decrease in target volume and toxicity, the study will proceed to the next step. A randomized control trial will be conducted with two groups: 1. Patients where PORT is administered according to the target volume delineated according to protocol, and 2. Patients where PORT is administered according to the target volume delineated by surgical clips. Patients will be monitored for toxicity and survival and followed up regularly.

In case of positive results, the next step would be to administer PORT only on the positive margins indicated by the surgical clips. The results will be compared to patients where PORT is administered according to the target volume delineated by surgical clips. If toxicity is reduced and survival rates are not negatively influenced, the last step will be to develop radio-opaque tags that are biocompatible or soluble.

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5.5 APPENDIX

Project code "Tag applicator" and "Tag remover"

Version 1.0

Date January 15, 2016

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Product Requirements Specification

Version	Date	Author(s)	Description
1.0	15-01-2016	FvL	After review surgeons EMC

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Revision History

Version 1.0: final version

1.2 Purpose and Scope

Purpose of this specification:
Describe the features of a new product for use in an operating room setting. This specification will be used as input for a design process.

Products application:
The products; “tag applicator” and “tag remover”, are aimed to support surgeons in relocating inadequate surgical margins in the wound bed during tumor surgeries

1.3 Glossary of terms

Not applicable

1.4 References

1. Slootweg PJ, Hordijk GJ, Schade Y, Es van RJJ, Koole R. Treatment failure and margin status in head and neck cancer. A critical view on the potential value of molecular pathology. Oral Oncology 38 (2002) 500–503.
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2 DESCRIPTION OF THE PRODUCT

2.1 Product Description and Rationale

Surgery in the head and neck area is the mainstay of treatment for oral cavity cancer. Removing all of the tumor is crucial for complete cure and survival. Un-

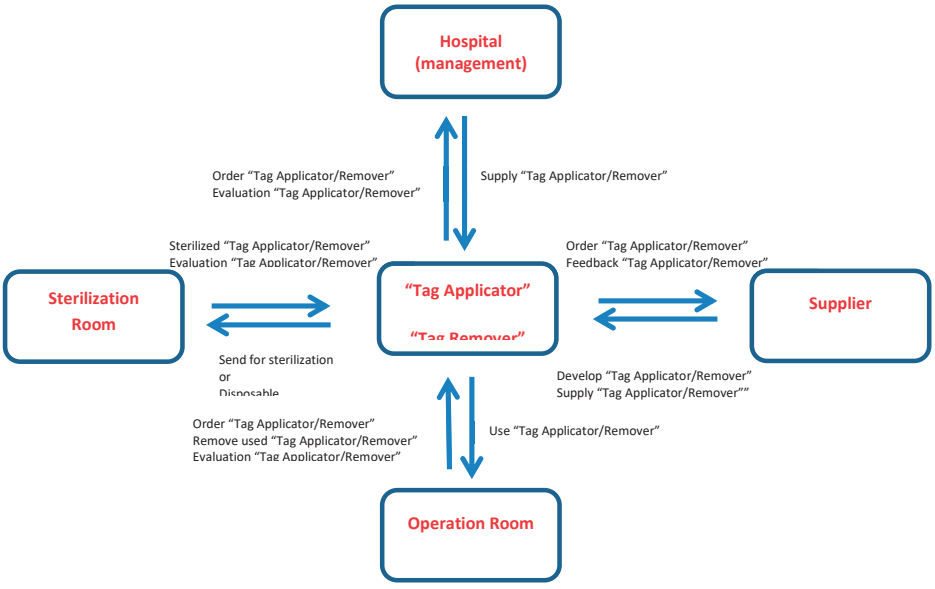
fortunately, full removal of all the tumor is hard to achieve. The hands and eyes of the surgeon are insufficient to warrant a full resection.

Intraoperative inspection of the entire resection margins of a resection specimen is the way forward. In case this inspection reveals that the tumor removal is incomplete, the residual tumor tissue must be located in the wound bed (i.e. in the patient).

The tag applicator and remover will facilitate the relocation of areas with inadequate margins in the wound bed. Provided with this intra-operative information regarding inadequate margins the surgeon then continues to remove more tissue from the wound bed to achieve an adequate surgical result.

2.2 Environment

The products will be used in the operation room of a hospital. This is a sterile environment.



2.3 User Characteristics

The end users will be surgeons and surgical assistants and people working in the sterilization room. Sterility is here the biggest concern. They perform their work in a sterile environment. There is limited time for the use of the "tag applicator" and user.

2.4 General Constraints

The tags must be sterile (single use or disposable) or sterilizable, easily removed, non-toxic to exposed tissues and non-allergenic.

2.5 Assumptions and Dependencies

The products should be developed within six to twelve months. End user price for disposable tag applicator/remover and tags below 100Euro. End user price maybe higher in case of re-usable device.

3 FUNCTIONAL REQUIREMENTS

3.1 Function 1

Before excision the surgeon delineates the cut line. The tag applicator will be used to fix pairs of tags with the same number to the tissue along the surgical cut line. One on each side of the cut line. After the excision, of each pair, one tag will be on the resected tissue and one tag will be in the surgical /wound bed. See appendices for illustration.

3.2 Normal Operation

See above. Up to 20 paired tags will be applied. The distance between two tags of one pair is more than 5 mm. We believe the minimum distance between the tags should be in the order of 5 mm. There has to be enough space between the tags, for the surgeon to cut without being hindered by the tags and without the risk of accidentally detaching tags.

3.3 Abnormal Operation

If the products are not usable due to a defect a new product will be used, please note that more than one of each product needs to be available. If the products are not usable due to difficult conditions in the patient, tags can be applied using sutures or removed using surgical scissors. In exceptional cases (ie larger resections) more than 210 paired tags will be used.

4 PERFORMANCE REQUIREMENTS

"Applicator"
Time: maximum of 5 seconds per tag.
Accuracy: fixed tags should not penetrate more than 3-4mm deep into the human tissue (to minimize damage to the tissue).

Tissue: Applicable to all soft tissues (ie connective tissue, muscle, fat, mucosa). Soft tissues differ in density which may provide difficulty in fixing the tags (example fat).
The product must provide a clear view of the operating site during usage.
The product must be used by right and left handed individuals

"Remover"
Time: maximum of 5 seconds per tag.
The product must provide a clear view of the operating site during usage.

5 DESIGN CONSTRAINTS

5.1 Regulatory Requirements

The products must be sterile.

Note: If the products are re-usable, the products must be sterilization compatible.

US regulations: FDA: Class 1 medical device, FFDCA §513(a)(1)(A).

EU regulations: Directive 93/42/EEC.

Single user operation.

5.2 Other Standards Requirements

There are no other standard requirements.

5.3 Hardware Limitations

The products should not be connected to an electrical power outlet. Preferably manual powered.

5.4 Software Limitations

No software involved.

5.5 Compatibility Requirements

There are no compatibility requirements.

5.6 Configuration Option and Product Family Requirements

There are no configuration options and product family requirements.

5.7 Technology/scientific Constraints

There are no technology/scientific constraints.

5.8 Cost

Maximum cost for the products itself is E100 (if disposable).

6 OTHER REQUIREMENTS

6.1 Attributes

6.1.1 Maintainability

In case of single-use no maintainability is necessary.

In case of re-usage; sterilization is necessary.

6.1.2 Serviceability

No serviceability is necessary. The products have no need to be modified by its users.

6.1.3 Manufacturability

Low cost, depending on disposable or re-usage, large- or small scaled volume.

6.1.4 Safety/Reliability/Availability

The products have to be safe with regard to the user and patient.
The products have to be instantly operational and accessible when one wants to use it.
The product, “tag applicator”, must have a system to prevent jamming of tags.
The product, “tag remover, must have a design preventing tags from breaking.
Failure rate <0.1%.

6.1.5 Usability/Human Factors

The products can be used by specified users to effectively and efficiently achieve the specified goal. These products are applicable in all humans.

6.1.6 Upgradeability (nice to have)

Tags which are biocompatible, soluble and / or radiopaque in human tissue.

6.1.7 Security

No security is necessary.

6.2 Physical Requirements

"Applicator"

Use: one user, manual/handheld, mechanical, single use.
Size: handheld
Weight: lightweight
Design: ergonomic, friendly and clean.
Load: maximum 20 paired tags.

"Tags"

Size: 3-4 mm.
Color: light color, numbers on tags visible with "naked eye".
Material: suitable for use in and on human tissue.

"Remover"

Use: one user, manual/handheld, mechanical.
Size: handheld
Weight: lightweight
Design: ergonomic, friendly and clean.

6.3 Environmental Requirements

No requirements.

6.4 Packaging and Labeling requirements

The products must be sterile and packaged in its totality, the different products can be packaged separately. A packaging label must be present. The label contains at least 'expiration date' and 'content'.

6.5 Documentation Requirements

User manuals are indicated for correct use of the products.

Documentation related to maintenance and sterilization is required if the product is re-usable.

6.6 Database

A database is not applicable.

6.7 Operations

Operations are not applicable.

6.8 Site Adaptation Requirements

Safe storage of the products when not in use.

7 HAZARD AND SAFETY ANALYSIS REQUIREMENTS

Hazard and safety analysis requirements are not necessary.

8 EXTERNAL INTERFACE REQUIREMENTS

8.1 Hardware

Not applicable.

8.2 Software

Not applicable.

8.3 User

Not applicable.

9 APPENDICES

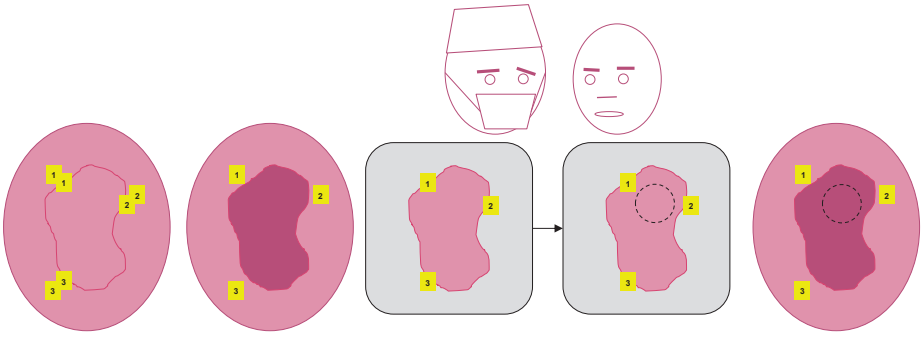
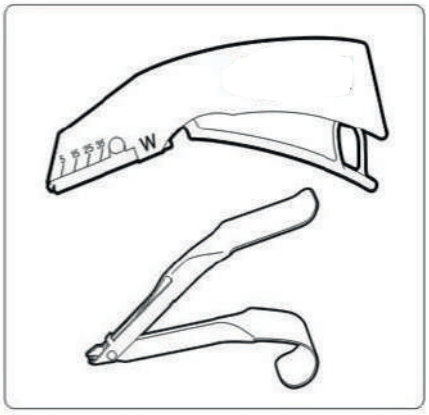
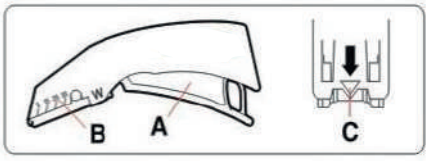
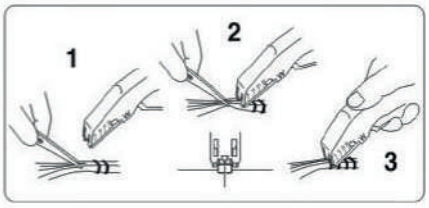
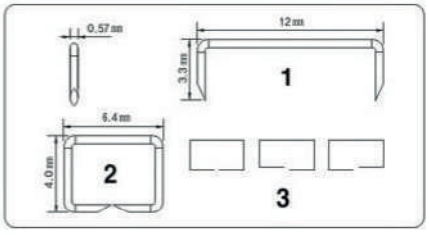


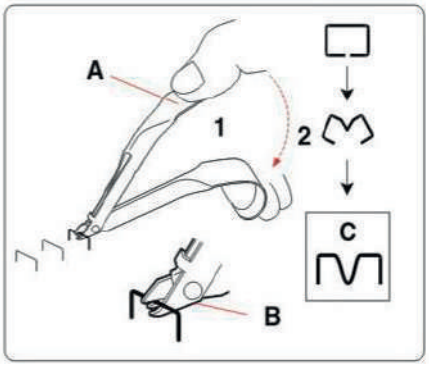
Illustration “Function 1”



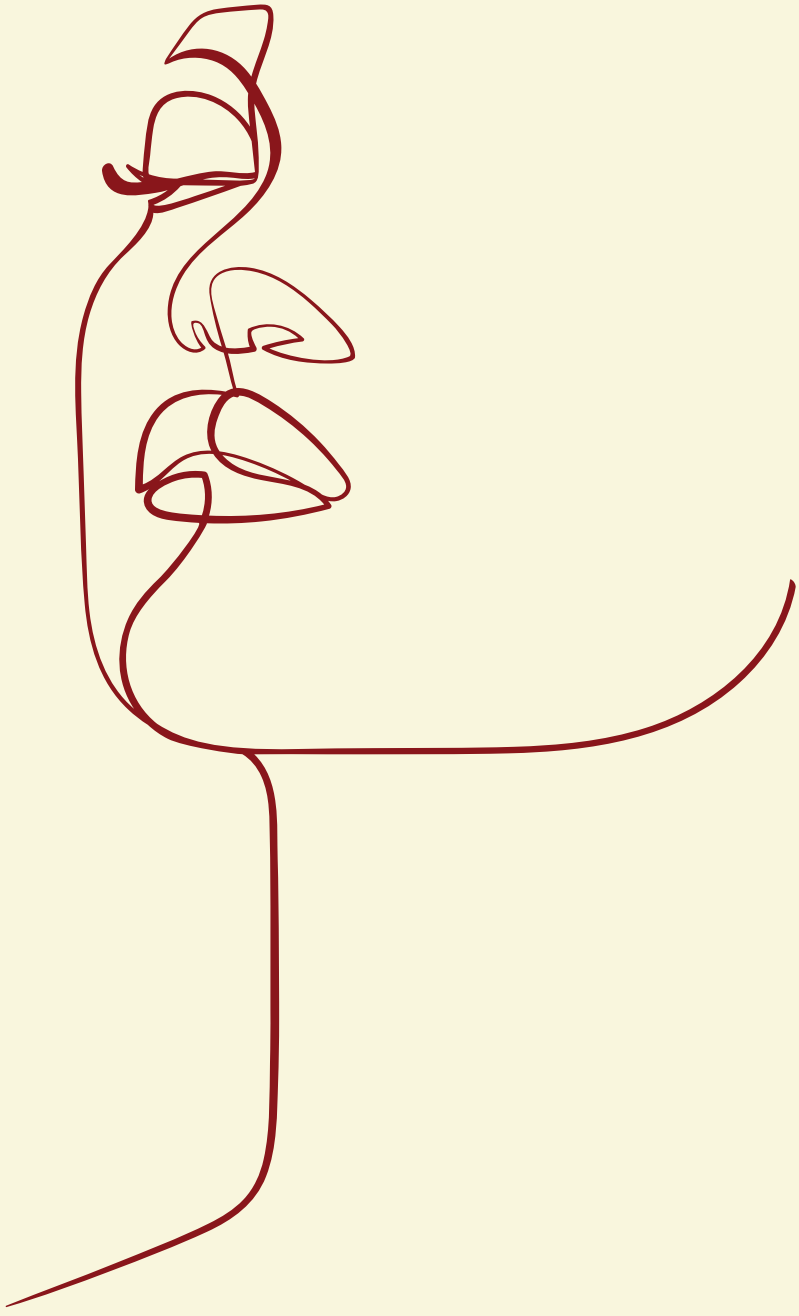








Example surgical (skin) stapler and staple remover



Chapter 6

Depth of invasion in early stage oral cavity squamous cell carcinoma: the optimal cut-off value for elective neck dissection

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Oral Oncology. 2020 Dec;111:104940.

6.1 ABSTRACT

Objectives

Depth of invasion (DOI) is the most important predictor for lymph node metastasis (LNM) in early stage (T1-T2) oral cancer. The aim of this study is to validate the cut-off value of 4 mm on which the decision to perform an Elective Neck Dissection (END) is made.

Materials and Methods

We performed a retrospective study in patients with pathologically proven early stage oral cavity squamous cell carcinoma (OCSCC) without clinical or radiological signs of LNM, who were treated between 2013 and 2018. An END was performed when DOI was ≥4 mm and a watchful waiting protocol was applied in patients with DOI <4 mm.

Results

Three hundred patients were included. END was performed in 77% of patients with DOI ≥4 mm, of which 36% had occult LNM (pN+). Patients in the watchful waiting group (48%) developed a regional recurrence in 5.2% for DOI <4 mm and 24.1% for DOI ≥4 mm. For DOI ≥4 mm, regional recurrence free survival was higher for patients who were treated with END compared to watchful waiting ($p = 0.002$). A Receiver-Operator-Curve -analysis showed that a DOI cut-off value of 4.0 mm was the optimal threshold for the prediction of occult LNM (95.1% sensitivity, 52.9% specificity).

Conclusion

A DOI of ≥4 mm is an accurate cut-off value for performing an END in early stage OCSCC. END results in higher survival rates and lower regional recurrence rates in patients with DOI ≥4 mm.

6.2 INTRODUCTION

For patients with oral cavity squamous cell carcinoma (OCSCC) and clinical cervical lymph node metastasis (LNM), a therapeutic neck dissection is indicated. For patients with no LNM (N0 necks), treatment of the neck is dependent on the risk of occult LNM. If this risk is low, observation of the neck is advisable. Yet, when there is a high risk of occult LNM, elective neck dissection (END) should be performed. Patients that are treated with a neck dissection are exposed to the risks and morbidity that are associated with the procedure (e.g., damage of the accessory nerve, dysfunction of the trapezius muscle and shoulder impairment.) (1, 2). Reliable predictors are needed, for identification of patients with N0 necks, to reduce the number of unnecessary ENDs.

Tumor thickness and depth of invasion (DOI) are predictors for LNM. Many studies have investigated tumor thickness and DOI in OCSCC as predictor for prognosis (3-6). However, in the literature, the definitions of DOI and tumor thickness are often used inconsistently. According to the American Joint Committee on Cancer Classification (AJCC) 8th edition, DOI is measured from the level of the basement membrane of the closest adjacent normal mucosa. A vertical line is drawn from this plane to the deepest point of tumor invasion (7). Therefore, DOI is not the same as tumor thickness and the two are not interchangeable (8). Tumor thickness can be larger than DOI in exophytic tumors, and lower than DOI in an endophytic/ulcerated growth pattern. Tumor thickness has been shown to be less predictive for the risk of LNM (7, 9).

Relevant literature on DOI is scarce due to incorrect use of the definition and incorrect measuring methods. In addition, there is no generally accepted cut-off value for DOI for performing an END. Values ranging from 1.5 mm to 10 mm are mentioned in the literature. Also, some studies differentiate between the different subsites of the oral cavity (3, 10, 11). The various clinical procedures and histopathological measurement methods, together with the lack of sound literature on DOI, undermine the possible role of DOI as predictor of metastasis. In accordance with the guideline of the National Comprehensive Cancer Network (NCCN) an END is performed at our institute based on a DOI ≥4 mm (12).

The aim of this study is to evaluate if 4 mm is indeed the optimal DOI cut-off value to perform an END in oral cancer and to assess the effectiveness of END in OCSCC.

6.3 MATERIALS AND METHODS

Study population: A retrospective cohort study was conducted by the departments of Otorhinolaryngology and Head and Neck Surgery, Oral and Maxillofacial Surgery and Pathology at the Erasmus MC Cancer Institute. All patients with primary OCSCC (pT1 or pT2), who were surgically treated between January 2013 and May 2018, were included. The only exclusion criteria was the presence of a synchronous oral cavity tumor.

Patient characteristics (e.g., age, gender, history on consumption of alcohol, tobacco and drugs) were recorded. Type and indication of adjuvant treatment, recurrence (local, regional, distant) and follow-up time were recorded.

Depth of Invasion: DOI was reviewed and measured according to the AJCC 8th edition. The measurement was taken from the level of the basement membrane of the closest adjacent normal mucosa. A vertical line was drawn from this plane to the deepest point of tumor invasion (7). The patient cohort was categorized into patients with DOI <4 mm and patients with DOI ≥4 mm.

Tumor characteristics: All resection specimen were examined by a head and neck pathologist (S.K.). Pathological TN classification (pTN), tumor growth pattern, vaso-invasion, perineural invasion, and resection margin status were recorded from the pathology reports. The 7th (up to 31 December 2017) and 8th (from 1st of January 2018) editions of the AJCC were used for staging.

Clinical N-classification: A therapeutic lymph node dissection (LND) was performed if there was proof or suspicion of LNM (by physical examination, imaging or aspiration cytology). END (during initial surgery or second surgery) was performed if DOI was ≥4 mm. In case of neck treatment in conjunction with resection of the primary tumor, DOI was measured on biopsy and in case of a second stage neck dissection on the resection specimen from the initial surgery. Patients staged cN0 and with a DOI <4 mm, did not undergo a neck dissection and were kept "under watchful waiting" surveillance. This protocol consisted of a 5-year follow-up with physical examination and regular ultrasound of the neck during the first two years.

Statistical analysis: Statistical analysis was performed using the IBM SPSS Statistics version 24 software (2017). Patient characteristics were compared between the two DOI groups using the independent samples T-test (student T-test) for continuous data and Chi-square test for categorical data. Univariate logistic regression

analysis was used to assess relations between predictor variables (i.e., all tumor characteristics) and lymph node status. A multiple logistic regression model was constructed using forward selection. A Receiver-Operator-Curve (ROC) analysis was performed to determine the optimal cut-off value for predicting LNM using DOI. Follow up was calculated from date of surgery. Regional recurrence free survival (i.e., time until an isolated regional recurrence occurs; RRFS), disease specific survival (i.e., time until death due to disease; DSS) and overall survival (i.e., time until death of patients; OS) were assessed using Kaplan-Meier curves and log rank tests. For all tests a p-value of <0.05 was considered statistically significant.

6.4 RESULTS

Between January 2013 and May 2018, a total of 346 patients with primary pT1/2 OCSCC were treated surgically. Patients with synchronous multiple tumors (n=9) and cN+ patients (n=37) were excluded, resulting in 300 patients for this study. Patient and tumor characteristics are shown in Table 1.

Depth of Invasion

In 139 patients (46.3%) DOI was <4 mm and in 161 patients (53.7%) DOI was ≥4 mm. Adverse histopathological tumor characteristics (such as infiltrative growth, vaso-invasion and perineural invasion) were all associated with DOI ≥4 mm (all p < 0.001). Tumor differentiation was different between the DOI groups (p = 0.001), results are shown in Table 2.

Elective neck dissection versus watchful waiting

One hundred seventy four patients were treated with an END at initial surgery based on pre-operative imaging for 115 patients and on pre-operative biopsy for 32 patients.

Of the 161 patients with a DOI ≥4 mm, 124 patients (77%) were treated with an END at initial surgery. In 38 of these patients, LNM was found at final pathology (30.6%). Eight patients (5%) were treated with an END at second surgery of which one patient (12.5%) had LNM. The remaining 29 patients (18%) with DOI ≥4 mm were not treated with an END, because of poor physical condition (n=6), other malignancies (n=4), patient refusal (n=2), previous LND (n=1) or for unreported/unknown reasons (n=15) and one patient died post-operatively. The other patients were assigned to watchful waiting.

Table 1. Patient characteristics.

	Number (n=300)	%
Sex		
Male	158	52.7
Female	142	47.3
Age (years), median (range)	66.5 (24-94)	
Smoking (n = 267)[a]		
Active smokers	113	42.3
Quit smoking	89	33.3
Non-smokers	65	24.3
Alcohol (n=260)[a]		
Active consumer	163	62.7
Quit alcohol	12	4.6
Non-consumers	85	32.7
pT (7th edition[b]**)**[c]		
T1	197	65.7
T2	90	30.0
pT (8th edition[b]**)**[d]		
T1	5	1.7
T2	8	2.7
Subsite		
Tongue	162	54.0
Floor of the mouth	77	25.7
Buccal mucosa	27	9.0
Lower alveolus and gingiva	12	4.0
Upper alveolus and gingiva	7	2.3
Lip	7	2.3
Retromolar area	5	1.7
Hard palate	3	1.0
Resection Margins		
Clear	79	26.3
Close	146	48.7
Positive	75	25.0
Differentiation (n = 295)*		
Well	50	16.9
Moderate	177	60.0
Poor	68	23.1
Infiltrative growth	179	59.7
Vaso-invasion	28	9.3
Perineural invasion	52	17.3
Tumor diameter (cm), median (range)	1.5 (0.1 – 4.0)	
DOI (mm) median (range)	4.0 (2.2 – 7.0)	

[a] percentages calculated within valid cases; [b] According to the AJCC; [c] treated between 01-01-2013 and 31-12-2017; [d] treated after 31-12-2017

Table 2. Tumor characteristics in depth of invasion groups.

	DOI <4 mm (n=139)	%	DOI ≥4 mm (n=161)	%	p-value
pT					<0.001
T1	123	88.5%	79	49.1%	<0.001
T2	16	11.5%	82	50.9%	<0.001
Subsite					0.04[a]
Tongue	66	47.5%	96	59.6%	0.036
Floor of the mouth	35	25.2%	42	26.1%	n.s.
Buccal mucosa	15	10.8%	12	7.5%	n.s.
Lower alveolus and gingiva	7	5%	5	3.1%	n.s.
Upper alveolus and gingiva	4	2.9%	3	1.9%	n.s.
Lip	6	4.3%	1	0.6%	0.035
Retromolar area	4	2.9%	1	0.6%	n.s.
Hard palate	2	1.4%	1	0.6%	n.s.
Resection margins					0.002
Clear	25	18.0%	54	33.5%	0.002
Close	69	49.6%	77	47.8%	n.s.
Positive	45	33.1%	30	18.6%	0.006
Differentiation (n = 295) [b]					0.001
Well	32	23.7%	18	11.3%	0.005
Moderate	83	61.5%	94	58.8%	n.s.
Poor	20	14.8%	48	30.0%	0.002
Infiltrative growth	55	39.6%	124	77.0%	< 0.001
Vaso-invasion	3	2.2%	25	15.4%	< 0.001
Perineural invasion	5	3.6%	47	29%	< 0.001
Tumor diameter (cm), median (range)[c]	0.9 (0.1 – 3.7)		1.9 (0.5 – 4.0)		< 0.001

[a] Using categories Tongue, Floor of mouth, Buccal mucosa, Lower Alveolus and gingiva and Other; pT: pathologic tumor staging [b] percentages calculated within valid cases; [c] Expressed as mean ± SD

Of the 139 patients with a DOI <4 mm, 116 patients (83.5%) were assigned to watchful waiting. One patient in the watchful waiting group was treated with an END at second surgery, based on the combination of physical examination, imaging results and positive margins at initial surgery. The other 23 patients (16.5%) with a DOI <4 mm were treated with an END at initial surgery based on the physical examination and imaging results (n=22) or as treatment for another head & neck malignancy outside the oral cavity (n=1). In 2 of these patients, LNM was found at final pathology (1.4%).

Independent predictor of lymph node metastasis

Lymph node metastasis were found more often in patients with DOI ≥4 mm (p = <0.001). Using univariate logistic regression analysis, pT, tumor differentiation, infiltrative growth, vaso-invasion, perineural invasion, tumor diameter and DOI (as continuous variable) proved to be predictors for LNM (Table 3). Using a multiple logistic regression model, DOI (OR: 1.1; 95% CI: 1.0 – 1.2. p = 0.008), infiltrative growth (OR: 5.9; 95% CI: 1.7 – 20.6. p = 0.005) and vaso-invasion (OR: 3.4; 95% CI: 1.3 – 8.6. p = 0.010) were independent predictors for LNM (Table 3).

Table 3. Univariate and multiple logistic regression of lymph node metastasis.

		Univariate[a]			Multiple logistic[b]		
		Odds ratio	**95% CI**	**p-value**	**Odds ratio**	**95% CI**	**p-value**
pT							
T1		1					
T2		4.0	2.0 - 7.9	<0.001			
Resection margins							
clear		1		0.582			
close		0.8	0.4 - 1.7				
positive		0.6	0.2 - 1.6				
Differentiation							
well		1		0.012			
moderate		7.3	1.0 - 55.0				
poor		15.1	1.9 - 118.0				
Infiltrative growth							
no		1			1		
yes		10.6	3.2 - 35.2	<0.001	5.9	1.7 - 20.6	0.005
Vaso-invasion							
no		1			1		
yes		6.3	2.7 - 14.6	<0.001	3.4	1.3 - 8.6	0.01
Perineural invasion							
no		1					
yes		5.9	2.9 - 12.1	<0.001			
Tumor Diameter	cm	1.1	1.05 – 1.14	<0.001			
DOI	mm	1.2	1.1 - 1.3	<0.001	1.1	1.0 - 1.2	0.008

[a] Assessed with univariate logistic regression; CI: confidence interval; [b] Assessed with multiple binary logistic regression

Cut-off value

A ROC-analysis was performed to determine the optimal DOI cut-off value for predicting LNM per mm DOI, based on positive predictive value >20%. A cut-off value of 4.0 mm showed a positive predictive value of 24.2% (95% CI: 17.8%-31.6%), with 95.1% sensitivity and 52.9% specificity, for prediction of LNM (Table 4). Area under the curve was 0.82 (95% CI: 0.760 – 0.879. p < 0.001). For patients with DOI <4 mm the probability of LNM is 1.4% (95% CI: 0.2%-5.1%). Stratification by subsite showed an optimal DOI cut-off value of ≥5 mm for tongue tumors (AUC: 79.7%; 95% CI: 0.711–0.882), DOI ≥4 mm for floor of the mouth tumors (AUC: 81.3%; 95% CI: 0.702 – 0.924) and DOI ≥7 mm for buccal mucosa tumors (AUC: 95.0%; 95% CI: 0.850 – 1.000). Stratification for the remaining subsites was not possible due to the small groups.

Table 4. Prediction of lymph node metastasis by depth of invasion cut-off value in cN0.

DOI cut-off (mm)	Sensitivity %	Specificity %	PPV %	NPV %	Group Proportions (n: < vs. ≥)
1	100	3.9	14.1	100	10 : 290
2	100	20.8	16.7	100	54 : 246
3	95.1	35.1	18.8	97.8	93 : 207
4	95.1	52.9	24.2	98.6	139 : 161
5	87.8	64.1	27.9	97.1	171 : 129
6	80.5	70.3	30	95.8	190 : 110

DOI: depth of invasion; PPV: positive predictive value; NPV: negative predictive value

Follow-up and Recurrence

Mean follow-up for the total study population was 23.4, ranging between 0-62 months. The follow-up was not different between a DOI <4 mm and a DOI ≥4 mm, p = 0.554 (24.0 ± 16.4 months; 22.9 ± 16.3 months, respectively).

Tumor recurrence was seen in 51 patients (17.0%); of which local recurrence in 25 patients (8.3%), regional recurrence in 21 patients (7.0%), loco regional recurrence in 3 patients (1%) and distant recurrence in 2 patients (0.7%). Regional recurrence was ipsilateral in 21 cases (7.0%) and contralateral in 3 cases (1%).

Tumor recurrence was 12.9% for a DOI <4 mm and 20.5% for a DOI ≥4 mm, p = 0.083. Regional recurrence was higher for DOI ≥4 mm (11.2% vs. 4.3%, p = 0.029). For the group with DOI <4 mm and watchful waiting, 5.2% developed regional recurrence. For the group with DOI <4 mm and END, no regional recurrence occurred (0%). For the group with DOI ≥4 mm and watchful waiting, 24.1% developed regional recurrence, of which one contralateral (3.4%). For the group with DOI ≥4

mm and END (for both initial surgery (n=124) and second surgery (n=8)) regional recurrence occurred in 11 patients (8.3%). Of these, 2 regional recurrences were contralateral (1.5%) and 3 bilateral (2.3%). In the latter group, regional recurrence was on the side of END and in the resected lymph node levels (i.e., in field). The remaining 6 patients (4.5%) had ipsilateral in field regional recurrence. The END of these patients (for both initial and second surgery) had shown LNM in 7/11 patients (72.7%). In the watchful waiting group, regional recurrence was reported more often for DOI ≥4 mm (p = 0.008). Results are shown in Table 5.

Table 5. Lymph node dissection and outcome.

	DOI <4 mm (n=139)	Regional Recurrence	DOI ≥4 mm (n=161)	Regional Recurrence
Watchful waiting	115 (82.7%)	6 (5.2%)	29 (18%)	7 (24.1%)
END (initial surgery)	23 (16.5%)	0 (0%)	124 (77%)	11 (8.9%)
pN0	21 (91.3%)	0 (0%)	86 (69.4%)	3 (3.5%)
pN+	2 (8.7%)	0 (0%)	38 (30.6%)	8 (21.1%)
END (second surgery)	1 (0.7%)	0 (0%)	8 (5%)	0 (0%)
pN0	1 (0.7%)	0 (0%)	7 (87.5%)	0 (0%)
pN+	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)

DOI: depth of invasion; END: elective neck dissection

Survival

Regional Recurrence Free Survival (RRFS) was lower for a DOI ≥4 mm compared to a DOI <4 mm (5-year RRFS 85.6% vs. 94.0%, logrank test p = 0.028). For a DOI ≥4 mm, RRFS was higher for patients who were treated with END compared to those without END (5-year RRFS 89.0% vs. 69.9%, logrank test (p = 0.008)) (Figure 1). For a DOI <4 mm, no difference in RRFS between watchful waiting and END was found (5-year RRFS 92.7% vs. 100%, logrank test p = 0.261) (Figure 2).

The 5-year DSS for OCSCC was 80.1% for a DOI ≥4 mm compared to 91.3% for a DOI <4 mm (logrank test p = 0.097). No difference in DSS was seen for both DOI groups, irrespective of treatment with END (DOI ≥4 mm: END 77.3% vs. watchful waiting 89.1%, p = 0.531, DOI <4 mm: END 90.9% vs. watchful waiting 91.4%, logrank test p = 0.881).

The 5-year OS was 70.8% for a DOI ≥4 mm and 69.7% for a DOI <4 mm (logrank test p = 0.404). Similar to DSS, no difference in OS was found, irrespective of treatment (DOI ≥4 mm: END 70.9% vs. watchful waiting 68.5%, p = 0.418. DOI <4 mm: END 68.7% vs. watchful waiting 70.1%, logrank test p = 0.760).

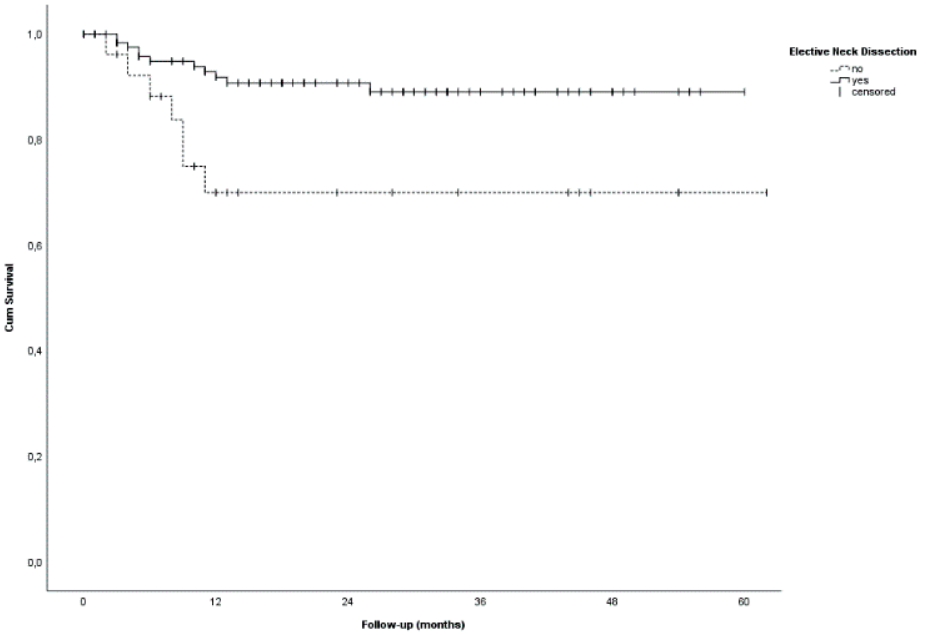

Figure 1. Regional recurrence free survival for patients with depth of invasion ≥4 mm treated with or without elective neck dissection (p = 0.008).

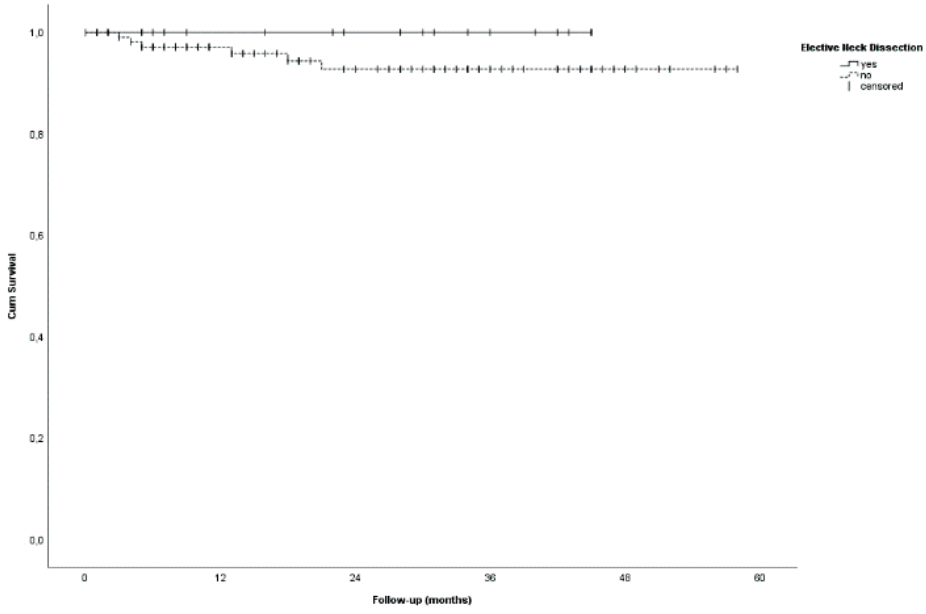

Figure 2. Regional recurrence free survival for patients with depth of invasion <4 mm treated with or without elective neck dissection (p = 0.261).

6.5 DISCUSSION

Lymph node metastasis are an important prognostic factor for patients with OCSCC (13-17). END is recommended based on the risk of occult metastasis (12). For this risk, DOI is regarded as an independent parameter (3-5, 18), as confirmed in this study ($p < 0.001$).

At our institution, an END is performed at a DOI cut-off value of ≥4 mm. The results of this study show that an optimal cut-off value is indeed 4.0 mm (sensitivity 95.1%, specificity 52.9%). In literature, DOI cut-off values are often not optimized to sensitivity and specificity (19). The few studies that explicitly mention that DOI measurements were performed from the level of the basement membrane, showed similar DOI cut-off values of 4 mm (20, 21). *Kane et al.* reported an increased risk of LNM at DOI ≥5 mm, which was not supported by statistical analysis ($P = 0.101$) (6). *Giacomarra et al.* reported a correlation of >3 mm and occult LNM, which was also not statistically significant ($p = 0.29$) (22). A subgroup analysis within a prospective randomized control trial on cT1-2N0 OCSCC demonstrated an increased LNM when DOI increased from 3 mm (5.6%) to 4 mm (16.9%). They suggest a 3 mm DOI cut-off value for END. However, this cut-off value was not maximized to sensitivity and specificity (18).

In our study, regional recurrence was 8.9% for patients with DOI ≥4 mm that had been treated with an END. This can be explained by contralateral recurrence, which was seen in five patients. An important predictor for contralateral recurrent disease is a tumor in proximity to/ or crossing the midline. Also, other histological features of the tumor can be of influence to regional recurrence. In this study, an OR for LNM of 15.1 (95% CI 1.9-118) was found for poor tumor differentiation and 7.3 (95% CI 1.0 - 55.0) for moderate differentiation. In literature, moderate or poor tumor differentiation were found to be a strong predictor for locoregional recurrence and occult metastasis (both $P < .001$) (23, 24). An incidence of occult metastasis of 17.4% for moderate differentiated and 28.5% for poor differentiated cT1N0 OCSCC is reported (24). Likewise, an OR of 5.9 (95% CI 2.9 – 12.1) for perineural invasion was found in this study. Studies describe perineural invasion too to be a predictor for regional recurrence (25-28). These data suggest that an END, regardless of DOI, should be considered in case of perineural invasion or moderate / poor tumor differentiation.

The survival analysis supports a DOI cut-off value of ≥4 mm for performing an END. The 5-year RRFS is worse for patients with DOI ≥4 mm in the watchful wait-

ing group compared to patients that were treated with an END ($p = 0.008$). No significant difference was seen for patients with a DOI <4 mm between the two treatment modalities ($p = 0.261$), suggesting that watchful waiting is sufficient.

The strength of this study is that DOI was measured in all cases according to the definition of the AJCC 8th edition (7). The literature differs in cut-off values for DOI ranging from 1.5-10 mm (3). These variations can be ascribed to ambiguous definition or description of the measuring methods resulting in the measurement of tumor thickness instead of tumor DOI.

It is now believed that tumor thickness underestimates the aggressive potential of the tumor (6-8, 10). The extent of tumor DOI is associated with invasion of the cortical bone and of blood- and/or lymphatic vessels (3, 8, 29, 30). In our study, the aggressive tumor characteristics (including tumor differentiation grades and perineural invasion) were associated with DOI. These characteristics were significantly different between tumors with DOI <4 mm and DOI ≥4 mm.

Recent literature shows difference in DOI cut-off values for the several tumor subsites of the oral cavity because of the variation in frequency of regional metastasis and differences in prognosis (12, 31, 32). For example, 20-30% occult LNM is reported for tongue cancer in comparison to 15.4% for buccal mucosa, 41.7% for floor of the mouth and infrequent involvement for alveolar ridge and hard palate (12, 32). These variations can be explained by the difference in tumor distance to lymphatic vessels and by the different caliber of the vessels present at the different subsites (3, 33).

Although the patient groups are small, we report significant DOI cut-off values for tongue 5 mm and floor of the mouth 4 mm. This is not in accordance with literature, where 2 mm - 7.25 mm DOI for tongue and 3 mm DOI for floor of the mouth is reported (19, 20, 34, 35). However, the study population of these articles included cT3-4 tumors, patients with cN+ or all patients treated with LND, which must be taken into account. Other studies only report on tumor thickness for the different subsites which hampers comparison (36-40).

Unfortunately, DOI is usually determined only days after initial surgery based on final pathological evaluation. Hence, in some cases, a second surgery is needed for an END. There is no reliable method of estimating DOI before or at the time of initial surgery (18). DOI can be measured from diagnostic biopsies but is often not representative due to sampling error. At present, determining DOI with a

pre-operative MRI is not accurate in tumors with DOI <5 mm (41-43). Measuring DOI during an intra-operative assessment for early stage OCSCC with a cN0 status could be of great value. This would enable the surgeon to always perform an END at initial surgery if DOI ≥4 mm. Only one study analyzed the accuracy of frozen section analyses for measurement of DOI intra-operatively. The authors report a high correlation between the measurement on frozen section and HE-stained section (44). A specimen-driven intra-operative assessment of the resection margins is recommended as standard of care by the AJCC 8th edition (7). Implementation of DOI measurements during this intra-operative assessment can therefore be easy. *Brockhoff et al.* implemented intra-operative assessment of DOI and reported its success in assisting with final decisions but also limitations (20). Of course, there is a logistical downside on reserved OR time for an END that often will not be performed. It is inefficient use of available resources and time, which entails high costs. Also, the implantation is not always feasible, particular in hospitals where the facilities are missing.

Sentinel lymph node biopsy (SLNB) could be another option to identify occult LNM at initial surgery. According to the NCCN guidelines, if technical expertise and experience of this procedure is available, SLNB is the best predictor for LNM and should be used to guide decision making on END (12). Sentinel lymph node detection rates of 95% are reported (45-47) with a 0.93 sensitivity and negative predictive value of 0.88-1 (18, 46-50). Caution is however advised to use SNLB as an alternative to the END. Especially for floor of the mouth tumors were accuracy is lower (45, 46), or tumors at the upper gingiva and hard palate which are not suited for this procedure (12).

Our research group is currently investigating the possibility to develop a Raman spectroscopic method for preoperative DOI determination, based on previously reported results. We have demonstrated that Raman spectroscopy can discriminate OCSCC from surrounding healthy tissue with high accuracy showing the capability of this optical technique to be implemented during oral cavity cancer surgery (51, 52).

6.6 CONCLUSION

Depth of invasion correlates with occult lymph node metastasis for patients with early stage oral cavity squamous cell carcinoma. Depth of invasion of ≥4 mm is an accurate cut-off value for performing an elective neck dissection. The regional recurrence free survival is worse for patients with depth of invasion ≥4 mm, who are not treated with an elective neck dissection. Because accurate preoperative assessment of depth of invasion is currently impossible, intra-operative assessment of the depth of invasion should be considered as an option to determine the necessity of performing an elective neck dissection at initial surgery. An elective neck dissection should also be considered in case of perineural invasion or moderate / poor tumor differentiation.

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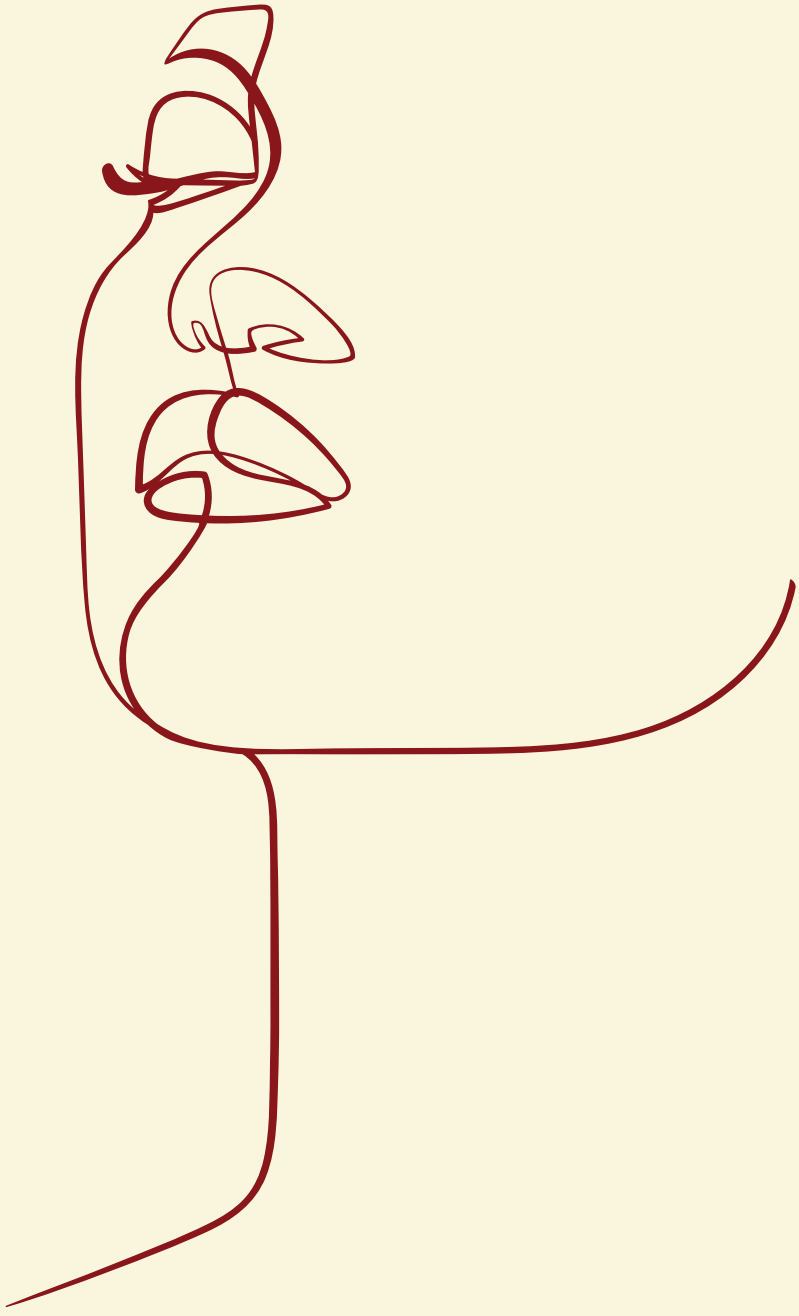

Chapter 7

Management of the neck in T1 and T2 buccal squamous cell carcinoma

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7.1 ABSTRACT

Buccal squamous cell carcinoma (SCC) appears to behave more aggressively than other oral cavity subsites, in particular with regards to regional disease at presentation or regional recurrence. Adequate management of the neck is of upmost importance but still subject to debate. We performed an international multicenter retrospective review of patients treated for T1-T2 buccal. Of 101 cases, 24 were staged clinical node positive (cN+) and underwent therapeutic neck dissection (TND); while 77 were staged node negative (cN0), with 32 undergoing elective neck dissection (END), with an occult nodal metastasis rate of 28%. Of the 77 cN0 patients, END was associated with a non-significant improvement in regional recurrence observation (8.9% versus 6.3%, $p = 0.67$). Regional recurrence was also more common in the pN+ (24%) and undissected group (8.9%) compared to the pN0 patients (0%, $p = 0.01$) and was associated with a depth of invasion (DOI) of >5 mm. Regional recurrence resulted in a reduction in overall survival (24 vs. 93 months, $p = 0.01$). In the pT2cN0 group, END resulted in improved overall survival (123 vs. 26 months, $p < 0.01$). We suggest END be performed in patients with cT2N0 buccal SCC, particularly for those tumors with DOI of more than 5 mm.

7.2 INTRODUCTION

Buccal squamous cell carcinoma (SCC) appears to have a more aggressive behavior than other subsites in oral cavity, in particular with regards to regional disease at presentation or regional recurrence. Patients with a clinically positive neck are treated with a therapeutic neck dissection (TND). Treatment of the clinically negative neck (cN0) varies from observation to elective neck dissection (END) due to an unclear risk of occult disease and regional recurrence. Adequate treatment of the cN0 neck in buccal cancer is important for prognosis, but still subject to debate. No large series exist on the clinical outcomes and risk of regional recurrence in buccal cancer, especially in cohorts where betel (areca) nut chewing is not the primary etiological factor. Currently, treatment of the cN0 neck in buccal cancer varies from careful observation to sentinel lymph node mapping END (1).

Buccal SCC represents around 10% of the oral carcinomas (2) and is distinct from tongue SCC because of the high rate of submucous fibrosis related to betel nut and pan chewing and lichenoid lesions. Some of these risk factors are endemic practices and therefore prevalent in certain geographical areas, including the Indian subcontinent and South East Asia (2,3). In Western populations however, buccal SCC may represent a different pathological entity (2,3).

There is need to elucidate the risk of regional disease in T1 – T2 buccal SCC in a Western population. This multicenter retrospective study is one of the largest series of T1-T2 buccal SCC in a Western population and aims to describe the rate of regional recurrence and the impact on survival at the Royal Melbourne Hospital (RMH), Melbourne, Australia, and the Erasmus University Medical Center Cancer Institute (EMC), Rotterdam, The Netherlands.

7.3 MATERIALS & METHODS

Patient Selection

Approval was obtained from RMH (QA2017143) and EMC (MEC-2017-336) medical ethics committees. Retrospective review was performed of patients treated for buccal SCC between January 2007 and December 2017. The analysis included patients with pathologically staged T1 or T2 tumors who underwent surgical resection with curative intent. Exclusion criteria were carcinoma in-situ, synchronous head and neck tumors, recurrence of previous buccal tumors, buccal tumors managed with primary radiotherapy, palliative intent or patients who declined treatment. Forty-four cases of buccal SCC from RMH and 57 cases from EMC were included.

The Adult Comorbidity Evaluation-27 (ACE-27) scoring system was utilized to analyze the severity of a patient's comorbidities and therefore stratify each into a pre-operative class- none (0), mild (1), moderate (2) or severe (3). ACE-27 has been validated to provide prognostic information in prospective tumor registries (4,5). Smoking behavior was documented as current smoker, ex-smoker (ceased at least three months prior) or non-smoker. Alcohol consumption was documented as current alcohol drinker, ex-alcohol drinker (ceased for at least six months) or non-alcohol drinker. Social drinking was considered to be two units per week while sporadic alcohol consumption of less than one unit per week was considered equivalent to non-alcohol consumption.

Clinical evaluation

Clinical evaluation of the primary tumor and neck was performed in accordance with National Comprehensive Cancer Network (NCCN) guidelines using computed tomography (CT) and/or magnetic resonance imaging (MRI). The neck was further assessed using ultrasound-guided fine needle aspiration (USS-FNA) in all cases at EMC and in select cases at RMH where the CT or MRI findings were equivocal. Distant sites were selectively assessed using chest x-ray, positron emission tomography (PET-CT) or chest CT. Patients were staged cN0 if there was no evidence of cervical nodal metastases clinically and radiologically or FNA result was negative.

Tumor Characteristics

Clinical and pathological staging was in accordance with the American Joint Committee on Cancer (AJCC) TNM staging classification 7th edition (6), the contemporary staging system during the study period. Collected tumor data included tumor size, tumor grade, lymphovascular invasion (LVI), perineural invasion (PNI), surgical margin, clinical and pathological lymph node status and presence of extra-capsular extension (ECE). Local recurrence was defined as a recurrence in the buccal site within 2 cm of the index lesion and within five years of primary treatment. Regional recurrence was defined as relapse in the cervical nodes while distant metastases were defined as recurrent SCC outside the mucosa or cervical lymph nodes.

Clinical Management

Surgical management

Treatment involved surgical excision of the buccal tumor. Elective ND was performed simultaneously if the resultant defect was likely to require free flap reconstruction or if the primary was clinically- or radiologically assessed as a high-risk tumor. Therapeutic ND was performed on all cN+ cases.

Histopathological analysis

Standard histopathological analysis was performed using formalin-fixed, paraffin embedded tissue with 2 mm, hematoxylin and eosin stained tissue section per block. Smaller nodes <5 mm were submitted whole while 5–15 mm nodes were bisected and >15 mm nodes were serially sectioned. One section of each macroscopically involved node was submitted, including areas of possible ECE. For all tumor specimens (both RMH and EMC), depth of invasion (DOI) was assessed and reviewed by both specialized head and neck pathologists from each center. The measurement was taken from the level of the basement membrane of the closest adjacent normal mucosa. A vertical line was drawn from this plane to the deepest point of tumor. The cases in which there was discrepancy between the assessments of both pathologists were jointly reviewed to reach consensus.

Adjuvant therapy

The adjuvant dose of radiotherapy was 60Gy in 30 fractions and in the setting of close margins (1-5 mm), positive margins (<1 mm) or ECE. The at-risk site was boosted to 66Gy in 33 fractions. At RMH, all patients with pathological cervical nodal metastases (pN+) or close margins were recommended for adjuvant radiotherapy, while presence of PNI and DOI >5 mm were considered relative indications. The RMH cohort had their adjuvant treatment coordinated at The Peter MacCallum Cancer Center, where the institutional policy included covering both the tumor bed and the neck, either adjuvant or prophylactically. At EMC, radiation was recommended in cases that met one major criteria (positive margins or ECE) or two minor criteria (close margins, PNI or 'spidery growth'). At both institutions, cisplatin was utilized as the adjuvant chemotherapy agent for positive margin or ECE. At RMH, this was recommended in suitably fit patients while at EMC only those patients less than 70 years of age were considered for chemotherapy.

Follow-up

At RMH, follow-up consisted of regular surveillance for five years with clinical examination and CT, MRI or PET-CT 12 months after treatment and subsequently as clinically indicated. Similarly, at EMC, regular clinical surveillance was performed for five years with US-FNA of cervical nodes four times per year during the first year for patients who did not undergo neck dissection.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, IBM Corporation., Chicago, IL, USA) software version 25. Chi-square test

with p-value <0.05 was used to indicate significance and Kaplan-Meier curves were used to assess survival.

7.4 RESULTS

Demographic Data

A total of 101 cases of stage pT1 (67) or pT2 (34) tumors were included: 44 from RMH and 57 from EMC. Mean follow-up time was 48 months (range 0-145). The patient and tumor characteristics and treatment approaches are outlined in Table 1, with no differences between the two institutions except a higher rate of poorly differentiated tumors at EMC and a higher rate of clear surgical resection margin at RMH.

Management of the neck

A total of fifty-six patients (55%) underwent neck dissection, 32 elective and 24 therapeutic, with institutional variation regarding the indication (Figure 1).

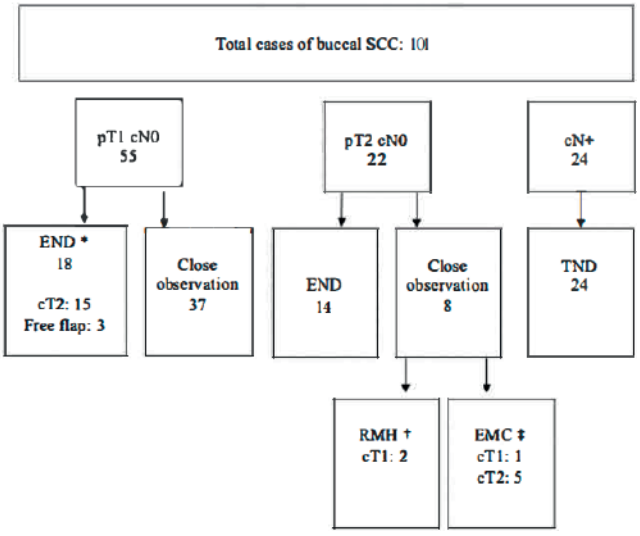

Figure 1. Management of the neck.
Management of the neck in 77 cN0 cases (pT1 = 55, pT2 = 55) and 24 cN+ cases of buccal SCC.
*Of the patients found to be pathologically pT1, END was indicated due to clinical T2 staging in 15 cases and due to requirement for free flap reconstruction in three cases.
† At RMH, two patients underwent staged END following histopathological up-staging from cT1 to pT2. Two patients with pT2 disease were deemed unsuitable to undergo staged END because of advanced age (n=1) or concurrent radiation for a synchronous lung primary (n=1). Both patients subsequently developed a local buccal recurrence within two months.
‡ At EMC, one cT1 patient was upstaged to pT2 but did not undergo staged neck dissection due to post-operative death. Among the cT2 patients, one was unfit for neck dissection and the others were considered “low risk”.

Table 1. Patient, tumor and treatment characteristics.

Characteristic	RMH (n = 44)	EMC (n = 57)	Total (n = 101)	p-value
Sex				0.49
Male	25 (57%)	28 (49%)	53	
Female	19 (43%)	29 (51%)	48	
Age – mean, range	68, 41 – 90	72, 41 – 93	71, 41 – 93	0.53
ACE27[4]				0.08
0	11 (25%)	8 (14%)	19	
1	21 (47.7%)	20 (35%)	40	
2	7 (15.9%)	23 (40.4%)	30	
3	5 (11.3%)	5 (8.8%)	10	
Unknown	0	1 (1.8%)	1	
Smoking history				0.20
Smoker	23 (52.3%)	37 (64.9%)	60	
Non-smoker	21 (47.7%)	20 (35.1%)	40	
Alcohol use > 2 units per week	14 (32.6%)	26 (60.5%)	40	0.16
T-stage				0.04
pT1	34 (77.3%)	33 (57.9%)	66	
pT2	10 (22.7%)	24 (42.1%)	34	
N-stage				0.39
pN0	17 (38.7%)	14 (24.6%)	30	
pN1	6 (13.6%)	8 (14%)	14	
pN2	3 (6.8%)	8 (14%)	11	
cN0	18 (40.9%)	27 (47.4%)	25	
Tumor grade				0.04
Well differentiated	14 (31.8%)	16 (28.1%)	27	
Moderately differentiated	28 (66.6%)	28 (49.1%)	54	
Poorly differentiated	2 (4.5%)	13 (22.8%)	15	
Primary tumor resection margin				< 0.01
Clear >5 mm	36 (81.8%)	19 (33.3%)	55	
Close 1– 5 mm	7 (15.9%)	20 (35.1%)	26	
Involved <1 mm	1 (2.3%)	18 (31.6%)	19	
High-risk histological features				
Lymphovascular invasion	9 (20.5%)	12 (21%)	14	0.94
Perineural invasion	3 (6.8%)	6 (10.5%)	9	0.52
Extra-capsular extension	1 (2.3%)	7 (12.3%)	8	0.12
Neck dissection	26 (59.1%)	30 (52.6%)	55	0.52
Management of cN0 neck				0.96
(RMH = 31, EMC = 46)				
Neck dissection	13 (41.9%)	19 (41.3%)	31	
Close observation	18 (58.1%)	27 (58.7%)	45	
Adjuvant radiation	7 (5.92%)	16 (28.1%)	23	0.25

Comparison of the clinical and pathological nodal staging is outlined in Table 2. Thirty-two (41.6%) of the 77 patients with cN0 necks were treated with END with nine patients having nodal metastases on histopathological analysis, indicating an occult lymph node metastases rate of 28.1%. Eight of these cases were upstaged to pN1 and one to pN2. The three patients with occult nodal metastases from RMH were all pT1 and all underwent pre-operative CT and MRI and one case underwent pre-operative PET. The six patients with occult nodal metastasis at EMC were all pT2 and underwent pre-operative US-FNA.

Table 2. Clinical vs. pathological staging after neck dissection.

	pN stage			
	pN0	**pN1**	**pN2**	**Total**
cN stage				
cN0	23 (71.9%)	8 (25%)	1 (3.1%)	32
cN1	3 (21.4%)	5 (35.7%)	6 (42.9%)	14
cN2	5 (50%)	1 (10%)	4 (40%)	10
Total	31 (55.4%)	14 (25.5%)	11 (19.6%)	56

Depth of invasion of primary tumor

DOI was determined in 82 cases (81.2%) for which pathology slides were available. The mean DOI was 6.9 mm (range 0.5 – 19 mm), with a mean DOI of 4.26 mm for the pT1 cases and 7.81 mm for pT2 cases. There was a significant difference in mean DOI between pN0 and pN+ (5.6 mm vs. 8.4 mm respectively, $p = 0.046$) and only one pN+ case had DOI <4 mm. DOI was analyzed for all nine cases of occult nodal disease, with a mean DOI of 7.87 mm (range 2.5 – 12.6 mm). The DOI for the six pT1 tumors with occult nodal disease were 2.5, 4, 5.6, 7.6, 11 and 11 mm, with a mean DOI of 7 mm. The DOIs for three pT2 tumors with occult nodal disease were 7.3, 9.2 and 12.6 mm, with a mean DOI of 9.7 mm. A DOI ≤5 mm was associated with a significantly lower rate of cervical nodal metastases of 20% vs. 80% ($p = 0.046$) and for DOI ≤4 mm this was 15% vs. 85% ($p < 0.001$). For DOI >5 mm, there was no difference in cervical metastases rates (35% vs. 65%, $p = 0.07$), Table 3.

Table 3. Primary tumor depth of invasion compared to pathological nodal status, $p = 0.046$.

	Pathological nodal status		
	pN0 (23)	**pN+** (20)	**Total**
Depth of invasion			
<5 mm	10 (43.5%)	4 (20%)	14
5–10 mm	12 (52.2%)	10 (50%)	22
>10 mm	1 (4.3%)	6 (30%)	7
Mean DOI	5.6 mm	8.4 mm	6.9 mm

Adjuvant radiotherapy

At RMH, eight patients received adjuvant radiotherapy for management of cervical nodal metastases (n=6) or involved margin (n=2). Two patients with pN+ did not receive radiation- one declined and one died post-operatively. At EMC, 16 patients received adjuvant radiotherapy to the neck for cervical metastases (n=1), to the primary site for involved or close margins (n=5) or both indications (n=10). All but one patient who underwent radiotherapy to the primary site alone had undergone ND, confirming pN0 disease. Six patients with pN+ disease did not undergo radiotherapy- in two cases, adjuvant treatment was not recommended due to either pN1 disease without ECE or patient frailty; two patients declined, and one died postoperatively. Four patients with close or involved margins did not undergo adjuvant treatment- three decided for close observation and one died post-operatively.

Recurrence

Twenty-four patients (23.8%) developed recurrent disease: 13 local (12.9%), eight regional (7.9%), two locoregional (2%) and one isolated distant metastasis (1%). Secondary to their local and/or regional recurrence, four patients later developed distant metastases. Regional recurrence usually occurred ipsilateral in levels I and V, with only two occurring bilaterally and one in level II.

Among the 56 patients who underwent ND, there were six cases of regional recurrence (10.7%), compared to four among the 45 patients who were surveilled (8.9%, p = 0.74). In the cN0 group, there was no difference in the rate of regional recurrence between the END group compared to observation (6.3% vs. 8.9%, p = 0.67, Table 4). Overall, regional recurrence was more common in pN+ and surveilled patients than in pN0 patients (24% vs. 8.9% vs. 0% respectively, p = 0.01, Table 5).

Table 4. Neck dissection and regional recurrence among cN0 patients, p = 0.67.

	Regional recurrence		
	Yes	**No**	**Total**
Neck dissection			
No neck dissection	4 (8.9%)	41 (91.1%)	45
Neck dissection	2 (6.3%)	30 (93.7%)	32
Total	6 (7.8%)	71 (92.2%)	77

Table 5. Tumor characteristics and regional recurrence.

	Regional recurrence			
	Yes	**No**	**Total**	**p-value**
N-status				
pN0	0	31 (100%)	31	0.01
pN+	6 (24%)	19 (76%)	25	
cN0	4 (8.9%)	41 (91.1%)	45	
Tumor grade				
Well differentiated	1 (3.6%)	27 (96.4%)	28	0.05
Moderately differentiated	5 (8.8%)	52 (91.2%)	57	
Poorly differentiated	4 (26.7%)	11 (73.3%)	15	
Perineural invasion				
Present	2 (22.2%)	7 (77.8%)	9	0.20
Absent	8 (8.8%)	83 (91.2%)	91	
Lympho-vascular invasion				
Present	6 (28.6%)	15 (71.4%)	21	< 0.01
Absent	4 (5.1%)	75 (94.9%)	79	
Extra-capsular extension				
Present	3 (37.5%)	5 (62.5%)	8	0.12
Absent	2 (11.1%)	16 (88.9%)	18	
Depth of invasion				
<5 mm	3 (6.5%)	43 (93.5%)	46	<0.01
5–10 mm	3 (10.7%)	25 (89.3%)	28	
>10 mm	4 (50%)	4 (50%)	8	

The relative risk of regional recurrence for cN0 observation compared to END was 1.42 (95% CI 0.27 – 7.30). In the pT1cN0 group (n = 56), regional recurrence in the END group occurred in 5.3% vs. 5.4% in the observation group (p = 0.98). Comparatively, among the pT2cN0 patients (n = 21), regional recurrence rate was 7.7% vs. 25% (p = 0.27), with a relative risk of 3.25 (95% CI 0.35 – 30.31). The mean time to regional recurrence was 10 months (range: 2 - 30 months), with only one patient recurring after two years. LVI was associated with an increased risk of regional recurrence (p < 0.01). DOI more than 5 mm, and particularly more than 10 mm, was associated with a higher risk of regional recurrence (6.5% vs. 10.7% vs. 50%, p = 0.002, Table 4). Only two cases of regional recurrence were salvageable. In those who had undergone ND, four occurred in the previously treated surgical field, while two developed recurrence out-of-field and these were managed palliative due to advanced age and associated distant metastases respectively.

Survival

Mean overall survival (OS) for all 101 cases was 48 months from date of surgery (range 0 – 145). Disease-free and regional recurrence-free survival rates are outlined in Table 6. Regional recurrence was associated with a reduction in mean OS (24 vs. 93 months, $p < 0.01$, Figure 2). In the cN0 patients, mean OS was 103 months in the END group versus 77 in those managed with close observation ($p = 0.16$). In the pT1cN0 group, there was no difference in OS between END and close observation (70 vs. 86 months, $p = 0.65$). However, among pT2cN0 patients, END was associated with an OS benefit compared to observation (123 vs. 26 months, $p = 0.01$, Figure 3). If regional recurrence occurred in a previously treated neck, there was worse OS compared to recurrence in a dissected neck (10 vs. 39 months, $p = 0.08$). Patients with occult nodal disease had a trend towards higher mean OS compared to those with clinically overt nodal metastases (80 vs. 40 months, $p = 0.74$).

Table 6. Overall, disease-free and regional recurrence-free survival.

Time frame	Overall survival	Disease-free survival	Regional recurrence-free survival
2 years	84%	61%	61%
3 years	74%	49%	51%
5 years	69%	28%	30%

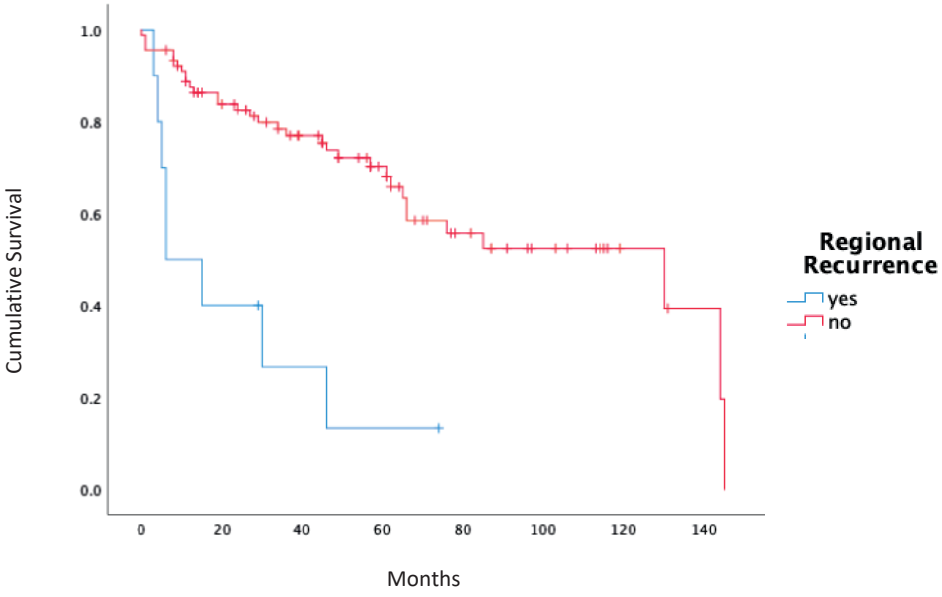

Figure 2. Overall survival: regional recurrence vs. no regional recurrence, $p < 0.01$.

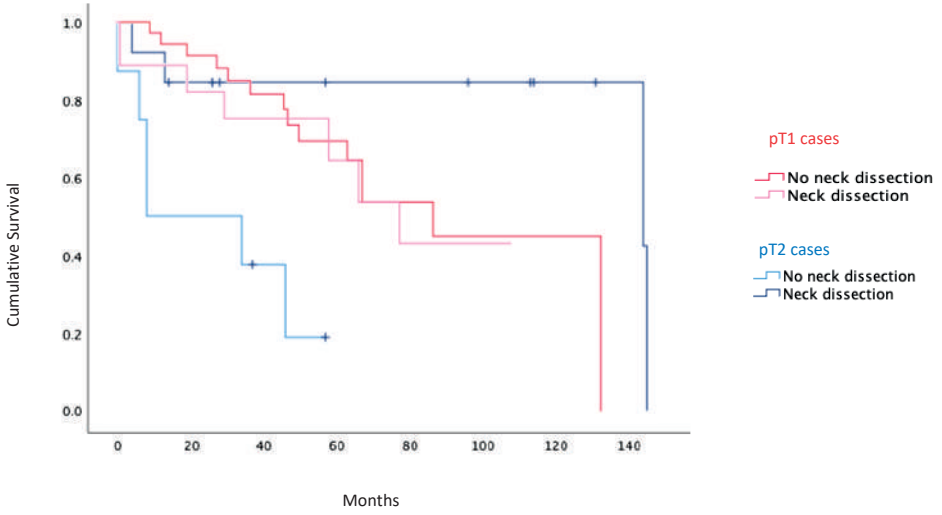

Figure 3. Overall survival of pT1cN0 and pT2cN0 patients undergoing neck dissection vs. clinical observation (p = 0.65 and p = 0.01 respectively).

Candidates contribution

The candidate contributed to gathering, analyzing, and interpreting data. Additionally, the candidate was actively involved in drafting the article, revising it critically for significant intellectual content, and contributing to the final revision of the version intended for publication.

7.5 DISCUSSION

Buccal SCC forms a subsite of the oral cavity cancers, with most studies agreeing that it is characterized by invasive tumor behavior, high incidence of locoregional recurrence and therefore poorer prognosis (3, 8, 9). Our multicenter, retrospective study represents one of the largest cohorts of T1 – T2 buccal SCC in a Western population, demonstrating an aggressive entity with overall 2-year survival of 84% and 5-year survival of 69%. Lymph node status is an important prognostic factor and regional recurrence is rarely salvageable. Occult metastases are a significant issue and elective neck dissection affords a survival benefit, particularly for pT2 tumors or DOI >5 mm.

While a selective neck dissection is standard of care for cN+ necks, treatment of the clinically negative neck can vary from END to close clinical observation or even sentinel lymph node biopsy (1,10). Some studies report reduced rates of

regional recurrence and improved survival in patients who undergo END (11-13), whereas others consider the evidence to be inconclusive (14). A survival benefit has been shown in patients with early-stage oral tongue SCC who undergo END rather than close observation (1). The role of END for early-stage buccal SCC, however, remains controversial, with few large-scale studies in a Western population. In this series, occult metastases occurred at a rate of 28%, in keeping with the cited rate of 26–38% (2, 9, 15, 16).

Staging of cervical lymph node metastases based on clinical palpation alone has been shown to be inaccurate (17). To avoid unnecessary neck dissection, preoperative staging sensitivity needs to reduce the risk of occult nodal disease to less than 20%, or a negative predictive value of more than 80% (18-20). A meta-analysis by *Liao et al.* in 2012 did not show any significant difference in reliability between CT, MRI, PET-CT or ultrasound. PET-CT, being a more expensive imaging modality, does not seem to improve sensitivity and negative predictive value (NPV) (10). Some studies demonstrate sensitivity and NPV for US-FNA of up to 90% and 95% respectively, with specificity being up to 100%, although false positive results can occur in patients with previously irradiated necks. Insufficient aspirate can occur in up to 20% of cases but can be reduced by immediate cytological evaluation by a pathologist (21-23). Overall, our study did not achieve the suggested occult metastasis rate of less than 20%.

In the oral cavity, there is a strong correlation between the DOI and nodal metastasis (24-27). Accordingly, *Diaz et al.* (2) suggested that buccal tumors with 3 mm DOI should warrant END, while *Ahmed et al.* (28) recommended a 2 mm DOI cutoff. These suggestions, amplified by the results of the 2014 multicenter study by *Ebrahimi et al.* (29) ensued the inclusion of DOI as a staging criteria in the AJCC 8th edition (7). Similarly, our study supports an association between DOI and chance of nodal disease, with DOI $\geq$5 mm having an increased risk of nodal disease of greater than 20%, suggesting END be performed in these tumors. This depends upon reliable radiological assessment of DOI, although staged neck dissection can also be performed once pathological DOI has been determined. It is important to acknowledge that utilization of the current AJCC 8th edition, and its attendant emphasis on DOI, would have changed the management of the neck in some included patients in our study. All but two of the patients with occult nodal disease had DOI >5 mm and all three of the patients who were managed with surveillance who went on to develop regional metastases within 24 months of treatment had DOI $\geq$10 mm. Both institutions now have accepted protocols that would have indicated an END be performed for these patients.

This study provides long-term data regarding buccal SCC recurrence rates, with regional recurrence in the neck occurring in 10%. Regional recurrence occurred on average 10 months after surgery with only one transpiring after 24 months, suggesting occult nodal disease may contribute to regional recurrence. Most of the Western literature reports similar regional recurrence rates occurring within 12 months of diagnosis (2, 30, 31). Regional recurrence was significantly more common in pN+ and undissected necks compared to pN0 patients, where there were no cases of regional recurrence. There was a trend towards reduced regional recurrence in patients who underwent END, particularly in pT2 cases, but this was not statistically significant. Regional recurrence was rarely salvaged surgically due to associated local or distant metastases or advanced patient age and was associated with a reduction in overall survival. END was associated with improved overall survival among pT2 but not pT1 cases. Although the patients with occult nodal disease had double the overall survival compared to those with clinically overt nodal disease, this was not statistically significant.

Most data that is available on regional recurrence and survival in early-stage buccal SCC is derived from South East Asian populations. *Huang et al.* and *Lin et al.* examined early-stage cases in Taiwan and found that END resulted in improved disease-free survival (13, 32). A contemporary Western series by *Dillon et al.* examining 98 cases showed that END resulted in improved locoregional recurrence rates and overall survival, however, there were only 66 cases of T1 and T2 disease (15).

Our multicenter, retrospective study represents one of the largest cohorts of T1 and T2 buccal SCC in a Western population, demonstrating an aggressive entity. Despite following the diagnostic guidelines of the NCCN, we report a high risk of occult nodal disease (28%). Moreover, cervical nodal metastases are a reliable predictor of future regional recurrence. Regional recurrence had a negative effect on survival, while END resulted in improved overall survival in pT2 patients. There is a strong argument for performing an END in patients with T1 – T2 buccal tumors, particularly T2 tumors with DOI greater than 5 mm.

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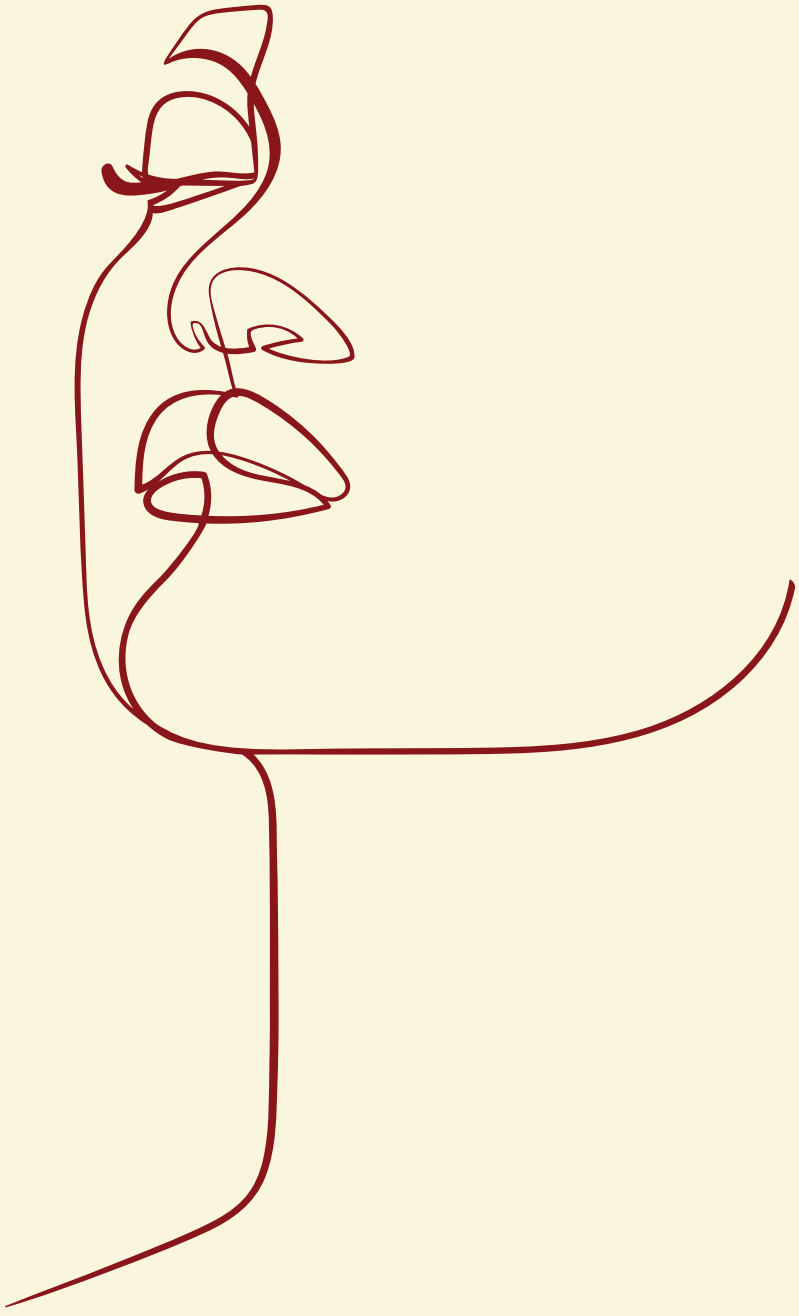
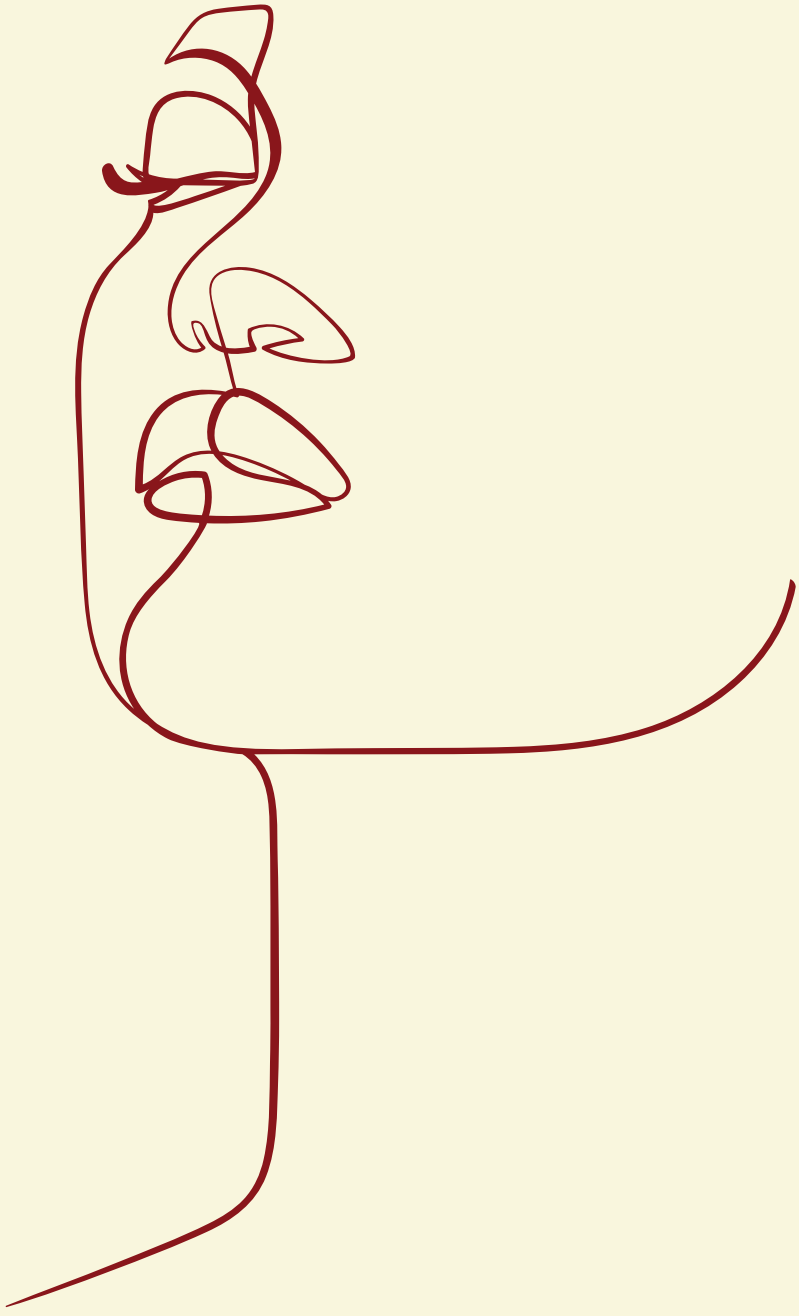


Larynx and hypopharynx



Chapter 8

A new proposal for adequate resection margins in larynx and hypopharynx tumor surgery

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8.1 ABSTRACT

Objective

Resection margins are an important prognostic factor for patients with head and neck cancer. Generally, a margin of >5 mm is recommended by the Royal College of Pathologists. However, this cannot always be achieved during laryngeal and hypopharyngeal surgeries. This study aimed to investigate whether a margin of >5 mm is feasible or whether an alternative guideline is needed.

Methods

The head and neck surgeons and a pathologist from Erasmus MC performed total laryngectomy and total laryngopharyngectomy on intact head and neck specimens designated for research. The primary objectives were to identify the resection surfaces and subsequently measure the maximum feasible margins per subsite. Finally, the clinical relevance of these maximum feasible resection margins was analyzed.

Results

For total laryngectomy, resection margins of >5 mm were not feasible for the ventral and dorsal resection surfaces. For total laryngopharyngectomy, resection margins of >5 mm were not feasible for the ventral, dorsal, and lateral resection surfaces.

Conclusion

Clear resection margins, defined as a margin >5 mm, are not always feasible in laryngeal and hypopharyngeal surgery because of the anatomy and location of the tumor. However, striving for the maximum feasible margin is the primary goal. This study proposes a new guidelines for maximum feasible yet adequate resection margins in laryngeal and hypopharyngeal tumor surgery.

8.2 INTRODUCTION

Laryngopharyngeal squamous cell carcinoma is one of the most common types of head and neck cancers (1). Surgery remains an important treatment modality for these tumors, followed by organ-preserving (chemo) radiation techniques. The goal of surgery is to achieve complete tumor removal with clear margins, which is a crucial prognostic factor for head and neck cancer (2, 3). Resection margins (i.e., the distance between the tumor border and resection surface) are defined by the Royal College of Pathologists (RCP) as follows: clear, >5 mm; close, 1-5 mm; and positive, <1 mm. Margins with severe dysplasia or in situ carcinoma were classified as positive (4). The RCP guidelines, which provide textual guidance and reporting preformats, are created to assist pathologists and facilitate accurate cancer staging (4).

Although >5 mm margins can mostly be achieved during tongue excisions, obtaining such margins can be a challenge for laryngeal and hypopharyngeal resections. The anatomy is complex, and resection margins are limited by the surrounding critical structures such as the skin, prevertebral structures, and vital neurovascular structures. The larynx and hypopharynx can be seen as tubular organs surrounded by musculature, cartilage, and fascia. Tumors grow towards the lumen, into the wall of the tubular organ to the surrounding structures, or cranially and caudally from the starting point of the tumor. Thus, resections are constrained by the anatomy and limited thickness of different tissue layers. A balance between achieving >5 mm margins and sparing healthy tissue to maintain function and aesthetics is essential.

There is limited evidence supporting the clinical relevance of resection margins as defined by RCP in laryngeal and hypopharyngeal cancer. Moreover, published studies did not show a significant association between margins >5 mm and overall survival (OS) ($p = 0.93$) nor disease-free survival (DFS) ($p = 0.71$) (5). In addition, resection margins were not an independent predictor of OS (($p = 0.11$) (6) or recurrence in univariate or multivariate analysis (7). However, surgeons always strive for clear margins because a positive margin worsens the prognosis (8, 9). Although a margin of >5 mm (according to the RCP) is the goal of larynx/hypopharynx surgery, the question is whether this margin is always feasible. This study aimed to investigate whether a margin >5 mm is feasible per subsite.

8.3 MATERIALS AND METHODS

Diagnostic panendoscopy of a random patient was recorded using film and photographs to identify the different resection surfaces of the larynx and hypopharynx. A head and neck surgeon (A.S.) and two researchers identified different resection surfaces per site and tumor location. Subsequently, a team of five head and neck surgeons and a dedicated pathologist (S.K.) performed a total laryngectomy (TL) and laryngopharyngectomy (TLP) on one intact freshly frozen head and neck specimen, specifically available for research, at the Erasmus MC skills lab. This procedure was also recorded using films and photographs. During resection, the resection surfaces per site and the tumor location were described and measured (Figure 1). The maximum feasible resection margins (MFM) were defined as resection margins limited to 1-5 mm based on the anatomy and limited thickness of the different tissue layers at the resection surface. MFM was determined according to the tumor location and subsite in the larynx (supraglottic, glottis, and subglottic) and hypopharynx (piriform sinus, postcricoid and posterior pharyngeal wall). Subsequently, the transcripts of the resections and the agreed MFM were discussed with the same team of head and neck surgeons and pathologist. This study was approved by the Medical Ethics Committee (MEC-2017-336) of the Erasmus Medical Center.

8.4 RESULTS

Resection surfaces and maximum feasible margins for a total laryngectomy (tumors located supraglottic, glottic and subglottic).

The cranial resection surface includes the suprahyoid muscles (m. digastricus, m. geniohyoideus, m. mylohyoideus, m. stylohyoideus, m. styloglossus) and base of the tongue. A resection margin of >5 mm was feasible for all subsites.

The caudal resection surface is the trachea. A resection margin of >5 mm was feasible for all subsites.

The ventral resection surface includes the thyroid cartilage, strap muscles (thyrohyoid, cricothyroid, and sternohyoid), the superficial layer of the deep cervical fascia, and the skin. A resection margin >5 mm is feasible for endolaryngeal tumors. In cases of invasion of the thyroid cartilage a >5 mm margin is not feasible given the thickness of the strap muscles (4 mm measured intra-operatively), fas-

cia, and skin. Additional skin resection must be performed in cases of clinical skin invasion.

The dorsal resection surface includes the mucosa extending from the arytenoids to the postcricoid and esophageal inlet. A resection margin of >5 mm is not feasible for all subsites because of the thickness of the mucosa (2 mm measured intra-operatively). Dorsal to this resection surface is the lumen of the hypopharynx and esophagus, and thus, air.

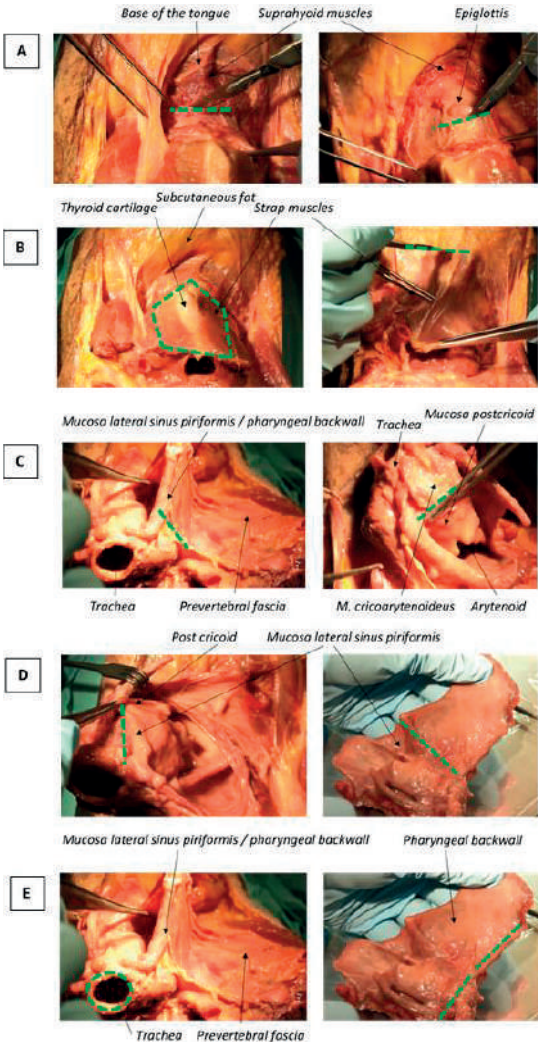

Figure 1. The resection surfaces per subsite and tumor location.
A. Cranial resection surface. B. Ventral resection surface C. Dorsal resection surface D. Lateral resection surface E. Caudal resection surface.

The lateral resection surface included the mucosa of the piriform sinus. A resection margin >5 mm is feasible for all subsites.

Resection surfaces and maximum feasible margins for a total laryngopharyngectomy (tumors located in the piriform sinus, postcricoid or posterior pharyngeal wall)

The cranial resection surface includes the mucosa of the lateral and posterior oropharyngeal walls, suprahyoid muscles outside the larynx, and the base of the tongue. For all subsites, a resection margin of >5 mm was feasible.

The caudal resection surface is the esophagus. For all subsites, a resection margin of >5 mm was feasible.

The ventral resection surface included the thyroid cartilage, strap muscles (4 mm measured intra-operatively), the superficial layer of the deep cervical fascia, and skin. This is of significance for anterior, medial, and lateral piriform sinus tumors. Agreement regarding the resection margins for the ventral resection surface in a TL also applies. Posterior pharyngeal wall tumors do not have a ventral resection surface because the lumen of the hypopharynx is on the ventral side. Postcricoid tumors have the (endo) larynx as the ventral resection surface.

The dorsal resection surface includes the hypopharyngeal mucosa, m. prevertebralis, prevertebral fascia, and the vertebral column. It is relevant for tumors of the lateral wall sinus piriformis or posterior pharyngeal wall. A resection margin >5 mm is not feasible because of the thickness of the mucosa (<1 mm measured intra-operatively) and prevertebral fascia. Invasion through the prevertebral fascia renders the tumor inoperable. A resection margin of >5 mm is feasible for anterior wall sinus piriform tumors. Medial wall piriform sinus and postcricoid tumors do not have a dorsal resection surface because of the lumen of the hypopharynx and esophagus.

The lateral resection surface comprises the mucosa, m. constrictor pharyngeus, and carotid space with its own layer of the deep cervical fascia. It is only important for lateral and anterior piriform sinus tumors. A resection margin >5 mm is not feasible because of the thickness of the mucosa (3 mm measured intra-operatively) and vital vascular structures directly laterally. Encasement of the carotid artery renders the tumor inoperable. A medial wall piriform sinus tumor does not have a lateral resection surface because of the lumen of the hypopharynx on the lateral

side. A resection margin >5 mm is feasible for posterior pharyngeal wall and postcricoid tumors.

Table 1 provides an overview of the resection surfaces per tumor location. Table 2 provides an overview of the resection surfaces in which margins of >5 mm are not feasible.

Table 1. Resection surfaces per tumor location from inside to outside.

Tumor location	Surgery	Cranial	Caudal	Ventral	Dorsal	Lateral
Larynx - supraglottic - glottic - subglottic	TL	suprahyoid muscles -> base of the tongue	trachea	thyroid cartilage -> strap muscles -> superficial fascia -> skin	mucosa postcricoid and lumen esophagus	mucosa piriform sinus
Piriform sinus - lateral (L) - anterior (A) - medial (M)	TLP	lateral oropharyngeal mucosa -> suprahyoid muscles -> base of the tongue	esophagus	thyroid cartilage -> strap muscles -> superficial fascia -> skin	- L: hypopharyngeal mucosa - A: medial and lateral piriform sinus -> lumen hypopharynx - M: lumen hypopharynx (no resection surface)	- L: m. constrictor pharyngeus -> internal jugular vein -> carotic artery - A: m. constrictor pharyngeus -> internal jugular vein -> carotic artery - M: lumen hypopharynx (no resection surface)
Postcricoid	TLP	(endo) larynx	esophagus	(endo) larynx	lumen hypopharynx (no resection surface)	mucosa piriform sinus
Posterior pharyngeal wall	TLP	posterior oropharyngeal mucosa	esophagus	lumen hypopharynx (no resection surface)	m. prevertebralis -> pre-vertebral fascia -> vertebrae	lateral hypopharyngeal mucosa

TL: total laryngectomy. TLP: total laryngopharyngectomy.

Table 2. Resection surfaces where margins >5 mm are not feasible in total laryngopharyngectomy.

Tumor location	
Larynx *(*supraglottic, glottic, and subglottic)	Ventral (4 mm measured intra-operatively): in cases of cartilage invasion. In cases of skin invasion, an additional resection is needed Dorsal**:** lumen of the hypopharynx and esophagus
Piriform sinus Lateral (L)	Ventral (4 mm measured intra-operatively): in cases of cartilage invasion. In cases of skin invasion, an additional resection is needed Dorsal (<1 mm measured intra-operatively): prevertebral fascia Lateral (3 mm measured intra-operatively): carotid space
Anterior (A)	Ventral (4 mm measured intra-operatively): in cases of cartilage invasion. In cases of skin invasion, an additional resection is needed Lateral (3 mm measured intra-operative): blood vessels
Medial (M)	Ventral (4 mm measured intra-operatively): in cases of cartilage invasion. In cases of skin invasion, an additional resection is needed Dorsal: lumen hypopharynx
Postcricoid	Dorsal: lumen hypopharynx
Posterior pharyngeal wall	Ventral: lumen hypopharynx. Dorsal (<1 mm measured intra-operatively): prevertebral fascia

8.5 DISCUSSION

Although resection margins are an important prognostic factor for head and neck cancer, the question arises as to whether the RCP guidelines can be applied to the complex anatomical areas of the larynx and hypopharynx. The RCP guidelines for mucosal malignancies of the larynx describe how to record a histopathology report, whereby the diameter, depth of invasion, cartilage invasion, invasion of the deep tissue planes (paraglottic and pre-epiglottic spaces), and differentiation grade must be documented. The resection margins (mucosal and deep) are defined as clear (>5 mm), close (1-5 mm), and positive (<1 mm) and are only briefly discussed. The required orientation (ventral, dorsal, etc.) and measurement of these margins are not mentioned. Only the following is described: “deep resection margins may be inapplicable unless the tumor extends into the soft tissue of the neck or close to the base of the tongue” (4). It is unclear whether resection surfaces or margins are implied. In addition, the RCP guidelines are not organ-specific. The authors explained that incomplete resection or the presence of dysplasia at the margin is associated with a significantly increased risk of local recurrence. However, they referred to four articles that described the clinical relevance of resection margins for all head and neck subsites (4). There is a lack of evidence regarding resection margins of the larynx and hypopharynx. As mentioned earlier, the anatomy of the subsites in the head and neck area is different, and the clinical importance of a >5 mm margin per subsite cannot be compared.

The question remains: is a margin of >5 mm feasible in the larynx and hypopharynx? Several studies have asserted that the anatomy of the head and neck region restricts resection margins owing to the limited thickness of the different tissue layers (10-12). In this study, we showed that a >5 mm margin is not always feasible because of the complex anatomy and surrounding structures. We suggest that a margin of 1-5 mm should be accepted in specific cases. This needs to be justified by oncological outcome data, which will be the aim of our next study.

The ventral resection surface of laryngeal tumors is first discussed. In cases of invasion of the thyroid cartilage, a >5 mm margin is not feasible because the strap muscles are <5 mm thick. We do not perform additional skin resection because of the related morbidity. In addition, the superficial layer of the deep cervical fascia ventral to the strap muscles is a natural barrier against tumor spread. In cases of clinical skin invasion, we recommend resection of the skin, which is also recommended in the literature (13). The second resection surface to be discussed is the dorsal surface of laryngeal tumors. In this case, the resection surface is the mucosa extending from the posterior commissure to the arytenoids and postcricoid, but the mucosa is <5 mm thick and the hypopharyngeal lumen is dorsal to the larynx. We recommend performing an additional resection of the mucosa extending to the esophageal inlet (caudal) only if the postcricoid mucosa is invaded. In cases without tumor invasion, additional resection of the postcricoid mucosa is not necessary. Sparing the pharyngeal mucosa makes the primary closure of the pharynx feasible.

The lateral resection surface is challenging for hypopharyngeal tumors because the constrictor muscle is <5 mm in size. Additionally, the carotid artery and internal jugular vein are lateral to these muscles. In case of encasement of the carotid space, we consider the tumor inoperable. The dorsal resection surface for posterior pharyngeal wall, lateral piriform sinus, and anterior piriform sinus tumors are limited by the thin mucosa of the posterior pharyngeal wall, m. prevertebralis, and the vertebral column. The prevertebral fascia is a natural tumor barrier. If there is invasion of the prevertebral fascia, we consider the tumor to be inoperable. At our institute, anatomical restrictions are respected with regard to patient morbidity. Extensive resections, for example, removal of the skin with an associated reconstruction, result in increased morbidity with worse functional and aesthetic outcomes. The question is whether removal of healthy tissue is necessary for better oncological outcomes. We always strive for wide resection margins. However, if there is less than 5 mm of space, a margin of >5 mm cannot be achieved.

Adequate resection differs between surgeons and pathologists. The surgeon strives for complete resection with maximum feasible margins and preservation of the normal tissue and function. The pathologist prefers complete resection with margins of >5 mm. Communication between the surgeon and the pathologist is necessary to understand which resection surfaces are crucial. False-negative resection margins could result in under-treatment (i.e., missing adjuvant therapy), and false-positive resection margins could result in overtreatment and unnecessary concerns. We propose performing an intra-operative assessment of resection margins on the specimen, where the surgeon and pathologist together assess the resection specimen visually, by palpation, and by making incisions perpendicular to the resection plane to accurately record the resection surfaces of importance and determine the MFM.

Moreover, we designed a template for structured registration of the margins of the larynx and hypopharynx resection specimens during surgicopathological evaluation (Table 3). In addition, for other head and neck subsites, such as the oropharynx and mandibular or maxillary regions, it can be difficult to achieve clear margins (>5 mm) (14) and the same consideration applies to these tumor locations.

In this study, we performed measurements during diagnostic panendoscopy and on freshly frozen specimens to obtain the most accurate measurements of the resection margins. In the literature, shrinkage of head and neck cancer specimens and margin dimensions owing to intrinsic tissue properties and formalin effects have been described. Therefore, immediate measurement after resection or during intra-operative assessment is recommended to avoid underestimation of the resection margins (15-17). As mentioned previously, our recommendation is that the surgeon and pathologist carefully document the measurements of the resection margins during intra-operative assessment.

Currently, it is unclear whether there is a universal approach following the guidelines of the RCP or if resection margins of 1-5 mm, based on anatomical restraints, are accepted. An extensive literature search in the Medline, Embase, and Cochrane Collaboration databases showed a lack of studies regarding the clinical relevance of resection margins in the larynx and hypopharynx. Twelve studies on resection margins during TL/TLP were identified. A single article followed the RCP guidelines (9). Two studies defined close margins as <5 mm and positive margins as tumor at the resection surface (18, 19). The remaining nine articles used descriptive definitions for margin status instead of the exact value of the resection mar-

gin, such as 'positive', 'microscopically positive', 'tumor at the resection surface', 'negative', 'safe margins' or 'no invasive tumor at the resection surface'. Therefore, it is not possible to clarify the clinical relevance of the resection margins in the larynx and hypopharynx. *Saraniti et al.* confirmed this by stating that, 'To reach a unanimous agreement regarding the prognostic value of resection margins, it would be necessary to carry out meta-analyses on studies sharing definition of resection margin, methodology and post-operative therapeutic choices' (7). To introduce MFM for laryngeal and hypopharyngeal cancer into the guidelines of the RCP and implement it as a standard of care, it is important to investigate its clinical relevance. Our next study will focus on recurrence rates and survival data regarding the resection margins.

Table 3. Pathology report with resection margins in millimeters.

Laryngeal tumors	**Cranial** *Suprahyoid muscles – tongue base*	**Caudal** *Trachea*	**Ventral**[1] *Thyroid cartilage – strap muscles – fascia – skin*	**Dorsal**[2] *Mucosa arytenoids to postcricoid*	**Lateral** *Mucosa piriform sinus*
Supraglottic	… mm	… mm	… mm**	… mm*	… mm
Glottic	… mm	… mm	… mm**	… mm*	… mm
Subglottic	… mm	… mm	… mm**	… mm*	… mm
Hypopharyngeal tumors	**Cranial** *Mucosa oropharynx – suprahyoid muscles – tongue base*	**Caudal** *Esophagus*	**Ventral**[1] *Larynx*	**Dorsal**[2] *Mucosa hypopharynx – m. prevertebralis – fascia – vertebral colum*	**Lateral** *Mucosa – m. constrictor pharyngeus – vessels*
Medial wall piriform sinus	… mm	… mm	… mm**	n.a. (lumen hypopharynx)	n.a. (lumen hypopharynx)
Anterior wall piriform sinus	… mm	… mm	… mm*	… mm	… mm*
Lateral wall piriform sinus	… mm	… mm	… mm*	… mm*	… mm*
Postcricoid	… mm	… mm	… mm	n.a. (lumen hypopharynx)	… mm
Posterior pharyngeal wall	… mm	… mm	n.a. (lumen hypopharynx)	… mm*	… mm

** >5 mm not possible in case of invasion of the thyroid cartilage

Describe: [1] Extralaryngeal growth and/or resection of the skin performed? [2] Invasion of m. cricoarytenoideus / mucosa postcricoid?

* >5 mm not possible due to anatomy

8.6 CONCLUSION

To the best of our knowledge, this is the first study to investigate and describe the maximum feasible resection margins for all resection surfaces in total laryngectomy and laryngopharyngectomy. This study challenges the RCP guidelines after showing that resection margins of >5 mm are not always feasible at every subsite in the larynx and hypopharynx. We advocate maximum feasible margins of >1 mm instead of >5 mm to enable an adequate resection. The focus of our next study is to justify this proposal by using oncological outcome data.

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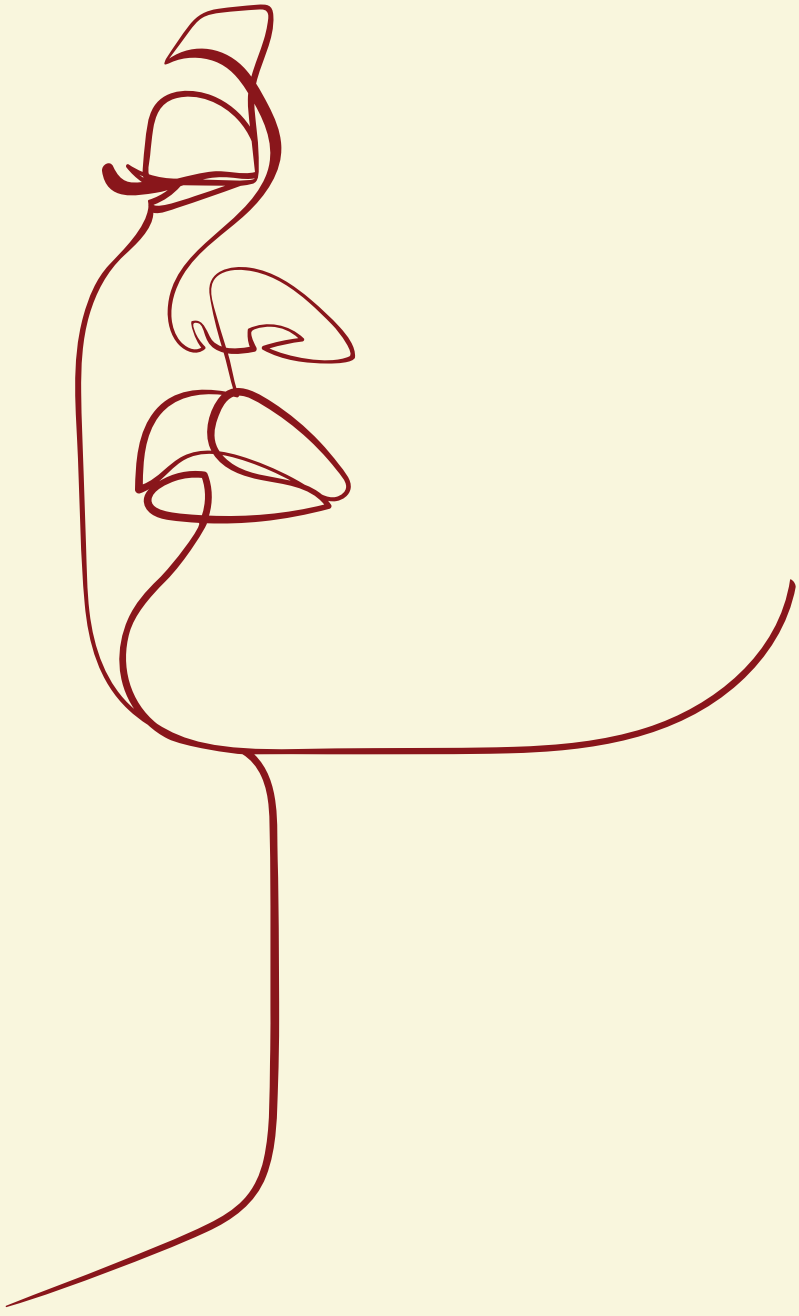

Chapter 9

Clinical relevance of resection margins in patients with total laryngectomy or laryngopharyngectomy

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9.1 ABSTRACT

Objective

Resection margins are an important prognostic factor. However, the anatomy of the larynx and hypopharynx is complex, and resections are constrained. There is limited evidence regarding the clinical relevance of resection margins in laryngeal and hypopharyngeal cancers. This study aimed to investigate the clinical relevance of resection margins in laryngeal and hypopharyngeal surgery.

Methods

This retrospective cohort study included patients treated with total laryngectomy (TL) or laryngopharyngectomy (TLP) for laryngeal or hypopharyngeal squamous cell carcinoma (LSCC resp. HSCC). Resection margins were defined according to the Royal College of Pathologists guidelines. The study population was divided into primary LSCC, recurrent LSCC, primary HSCC and recurrent HSCC groups. The relationships between resection margin status, recurrence, and survival rates were investigated, within each group.

Results

Hundred Seven primary LSCC, 100 recurrent LSCC, 45 primary HSCC and 16 recurrent HSCC were included. Positive resection margins were found in 54% for primary LSCC, 29% for recurrent LSCC, 62% for primary HSCC and 44% for recurrent HSCC cases. For primary and recurrent LSCC, there was a linear association between the total recurrence and narrowing margins ($p = 0.007$ resp. $p = 0.008$). Multivariate survival analysis for primary and recurrent LSCC showed significantly worse disease-free and disease-specific survival in cases with positive margins than in those with clear margins.

Conclusion

Resection margins of >5 mm are not possible for every subsite in the larynx and hypopharynx owing to anatomical limitations. This study showed similar survival rates for close and clear margins for recurrent LSCC and primary HSCC. This may suggest that a margin >5 mm is not clinically relevant in terms of survival. Therefore, a margin of 1-5 mm should be accepted for certain subsites. Margins <1 mm are associated with significantly worse outcomes and should be avoided.

9.2 INTRODUCTION

Surgery is an important treatment modality for head and neck squamous cell carcinoma next to (chemo) radiation. The goal of surgery is to achieve adequate resection margins (i.e., the distance between the tumor border and resection surface), as this is a crucial prognostic factor (1, 2). For head and neck cancer, resection margins are defined by the Royal College of Pathologists (RCP) as follows: clear, >5 mm, close, 1-5 mm and positive, <1 mm (3). However, achieving margins of >5 mm is challenging in the larynx and hypopharynx because resections are constrained by their complex anatomy. A balance between achieving adequate margins for better outcomes in terms of recurrence and survival versus sparing healthy tissue to maintain function and aesthetics is essential.

There is limited evidence for the clinical relevance of resection margins, as defined by the RCP, in laryngeal and hypopharyngeal cancer. Moreover, the published studies did not show an association between clear margins (>5 mm) and overall survival (OS) ($p = 0.286$) (4), disease-free survival (DFS) ($p = 0.11$) (5), nor did resection margins appear to be an independent predictor for disease-specific survival (DSS) or recurrence in univariate or multivariate analysis (5, 6). However, surgeons always strive to prevent a positive margin (<1 mm) because this impairs prognosis (7, 8). In an earlier study, we determined the resection surfaces and described the maximum feasible resection margins for the larynx and hypopharynx per tumor location. In that study, we reported that a >5 mm margin is not always feasible for all resection surfaces because of the anatomy and limited thickness of the different tissue layers. A margin of >1 mm should be accepted for specific tumor subsites in the larynx and hypopharynx (9).

The aim of current study was to retrospectively assess the clinical relevance of resection margins defined by the RCP, in total laryngectomy (TL) and total laryngopharyngectomy (TLP).

9.3 MATERIALS AND METHODS

Inclusion criteria. Based on the medical records, a retrospective cohort study was performed at the Erasmus MC Cancer Institute, Netherlands (EMC). Patients treated with TL or TLP for primary or recurrent squamous cell carcinoma between January 2008 and July 2017 were included. Patients were excluded if they had an

additional simultaneous head and neck tumor. This study was approved by the Medical Ethics Committee (MEC-2017-336).

Patient and tumor characteristics. A database was created based on patient characteristics (e.g., age, and sex), tumor characteristics (i.e., location, c/pTNM, primary treatment, histological characteristics including differentiation grade, infiltration pattern, perineural growth, and angio-invasion), resection margin status, outcome data on tumor recurrence (location and date), and the last date of follow-up or date of death. The last follow-up was defined as the last date on which the patient was confirmed alive and ended in February 2022. Follow-up time was measured from the date of treatment (i.e., surgery or (chemo) radiation) until the last follow-up. The resection margins were recorded from the final pathology report (in millimeters) with respect to all resection surfaces (cranial, ventral, lateral, dorsal, and caudal). Total recurrence (TR) was recorded as the sum of local recurrence (LR) (i.e., around the stoma, in the neopharynx/ esophagus/ base of the tongue), regional recurrence (i.e., neck lymph nodes), and/or distant metastasis. Resection margins were defined according to the RCP guidelines as follows: clear >5 mm, close 1-5 mm and positive <1 mm (3).

Statistical analyses. Statistical analyses were performed using IBM SPSS Statistics, version 21.0 for Windows, version 25 (IBM Corp., Armonk, NY, USA). A significance level of 5% was considered to be statistically significant. The study population was divided into four groups: primary laryngeal squamous cell carcinoma (LSCC), recurrent LSCC, primary hypopharyngeal squamous cell carcinoma (HSCC), and recurrent HSCC. Patient and tumor characteristics were compared using Pearson's chi-square test for categorical variables and ANOVA for age. Univariate statistical analyses were performed separately for each group. Linear-by-linear association tests were used to determine the relationship between the resection margins and (local) recurrence. Disease-specific survival (DSS) and disease-free survival (DFS) were analyzed using Kaplan-Meier estimates, and differences in survival with respect to margin status were tested using the log-rank test. DSS was defined as the percentage of patients who did not die from LSCC or HSCC. DFS was defined as the time (months) after treatment without (recurrent) disease. Multivariate Cox survival analysis was performed for LSCC (primary and recurrent) and HSCC (primary and recurrent). In these models, confounders (candidate confounders: age, primary/recurrent tumor, pT, pN, and postoperative adjuvant treatment) were selected using the mean squared error method. Next to this retrospective cohort study, we performed an extensive literature search in the Medline, Embase, and

Cochrane Collaboration databases regarding the clinical relevance of resection margins in the larynx and hypopharynx in oncologic surgery.

9.4 RESULTS

In total 268 patients were included in the study: 107 with primary LSCC, 100 with recurrent LSCC, 45 with primary HSCC, and 16 with recurrent HSCC. The clinicopathological characteristics are shown in Table 1. The resection margins of these four different tumor groups) were analyzed according to the RCP guidelines. Clear, close, and positive resection margins were identified for each resection surface (cranial, ventral, lateral, dorsal, and caudal), and are reported in Table 2. The results regarding recurrence and survival are summarized in Table 3 and Figures 1-3.

Table 1. Patient and tumor characteristics.

Characteristics	Laryngeal primary tumor N = 107	Laryngeal recurrent tumor N = 100	Hypopharyngeal primary tumor N = 45	Hypopharyngeal recurrent tumor N = 16	P-value*
Age (years), mean (SD)	65 (10)	65 (11)	65 (9)	62 (7)	0.67
Sex					0.86
Male	87 (81)	80 (80)	35 (78)	14 (88)	
Female	20 (19)	20 (20)	10 (22)	2 (12)	
Tumor location					0.29
Supraglottic	50 (47)	36 (36)			
Glottic	53 (50)	59 (59)			
Subglottic	4 (3)	5 (5)			
Piriform sinus			30 (67)	11 (69)	0.95
Posterior pharyngeal wall			4 (9)	1 (6)	
Postcricoid			11 (24)	4 (25)	
pT classification					<0.001
pT1&2	5 (5)	32 (32)	5 (11)	7 (44)	
pT3&4	102 (95)	68 (68)	40 (89)	9 (56)	
pN classification					0.002
pN0	68 (64)	81 (81)	25 (56)	14 (87)	
pN+ (pN1&2)	39 (36)	19 (19)	20 (44)	2 (13)	
Type of surgery					<0.001
TL	99 (93)	93 (93)	9 (20)	2 (13)	
TLP	8 (7)	7 (7)	36 (80)	14 (87)	
Perineural invasion					0.20
No	65 (66)	51 (53)	24 (53)	7 (47)	
Yes	34 (34)	46 (47)	21 (47)	8 (53)	
Unknown	8	3	-	1	
Angio-invasion					0.55

Table 1. Patient and tumor characteristics (continued).

Characteristics	Laryngeal primary tumor N = 107	Laryngeal recurrent tumor N = 100	Hypopharyngeal primary tumor N = 45	Hypopharyngeal recurrent tumor N = 16	P-value*
No	59 (60)	60 (66)	23 (54)	9 (56)	
Yes	39 (40)	31 (34)	20 (46)	7 (44)	
Unknown	9	9	2	-	
Infiltrative growth pattern					0.73
No	13 (19)	11 (14)	5 (14)	1 (8)	
Yes	55 (81)	66 (86)	31 (86)	11 (92)	
Unknown	39	23	9	4	
Cartilage invasion					0.001
No	28 (26)	44 (44)	13 (29)	11 (69)	
Yes	79 (74)	56 (56)	32 (71)	5 (31)	
Preoperative therapy					**
RT	-	77 (77)	-	5 (31)	
CRT	-	18 (18)	-	10 (63)	
TLM	-	3 (3)	-	-	
TLM + RT	-	2 (2)	-	1 (6)	
Postoperative therapy					<0.001
None	16 (15)	96 (96)	16 (36)	16 (100)	
Yes***	91 (85)	4 (4)	29 (64)	0 (0)	

TL: total laryngectomy, TLP: total laryngopharyngectomy, RT: radiotherapy, CRT: chemo radiotherapy, TLM: transoral laser microsurgery

For categorical variables numbers and percentages of valid cases in each category are presented

** P-value for difference between four or two groups*

*** Too small amount*

***** RT, CRT, RT and hyperthermy or proton therapy

Table 2. Percentage in resection margin status for each resection surface.

	Cranial	Ventral	Lateral	Dorsal	Caudal
Laryngeal SCC (n = 207)					
Clear	53%	32%	25%	10%	94%
Close	12%	29%	7%	7%	4%
Positive	8%	31%	2%	7%	2%
Unknown	27%	7%	66%	76%	0%
Hypopharyngeal SCC (n = 61)					
Clear	52%	28%	22%	0%	90%
Close	12%	28%	12%	9%	7%
Positive	11%	34%	3%	12%	3%
Unknown	24%	10%	68%	75%	0%

SCC: squamous cell carcinoma

Table 3. Total and local recurrence for resection margins according to the Royal College of Pathologists guidelines.

Margins	Total	Recurrence	Local recurrence
Primary LSCC (n = 107)			
Clear	19% (20)	5% (1)	5% (1)
Close	27% (29)	24% (7)	0% (0)
Positive	54% (58)	36% (21)	16% (9)
Recurrent LSCC (n = 100)			
Clear	37% (37)	35% (13)	14% (5)
Close	34% (34)	38% (13)	21% (7)
Positive	29% (29)	69% (20)	45% (13)
Primary HSCC (n = 45)			
Clear	7% (3)	33% (1)	33% (1)
Close	31% (14)	29% (4)	7% (1)
Positive	62% (28)	54% (15)	18% (5)
Recurrent HSCC (n = 16)			
Clear	25% (4)	50% (2)	25% (1)
Close	31% (5)	60% (3)	60% (3)
Positive	44% (7)	71% (5)	43% (3)

LSCC: laryngeal squamous cell carcinoma. HSCC: hypopharyngeal squamous cell carcinoma.

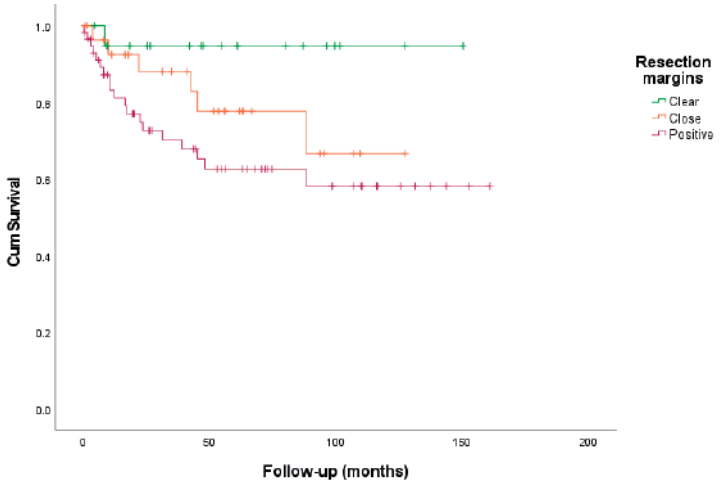

Figure 1. Relation between disease-specific survival and resection margins for primary laryngeal squamous cell carcinoma.

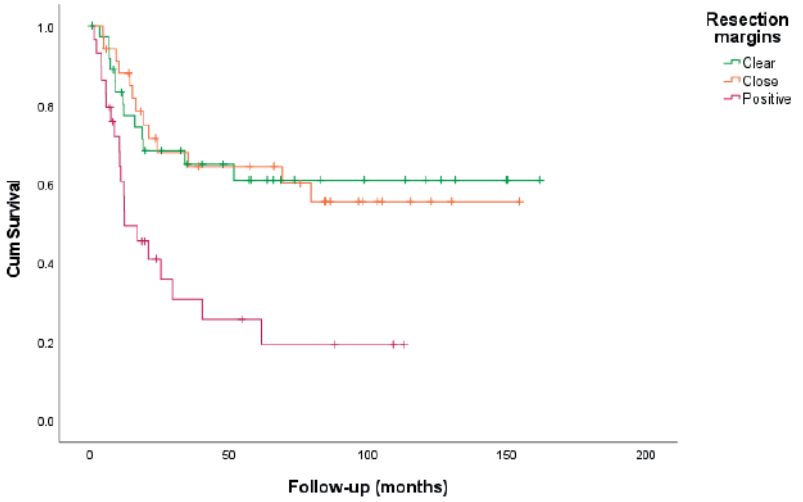

Figure 2. Relation between disease-specific survival and resection margins for recurrent laryngeal squamous cell carcinoma.

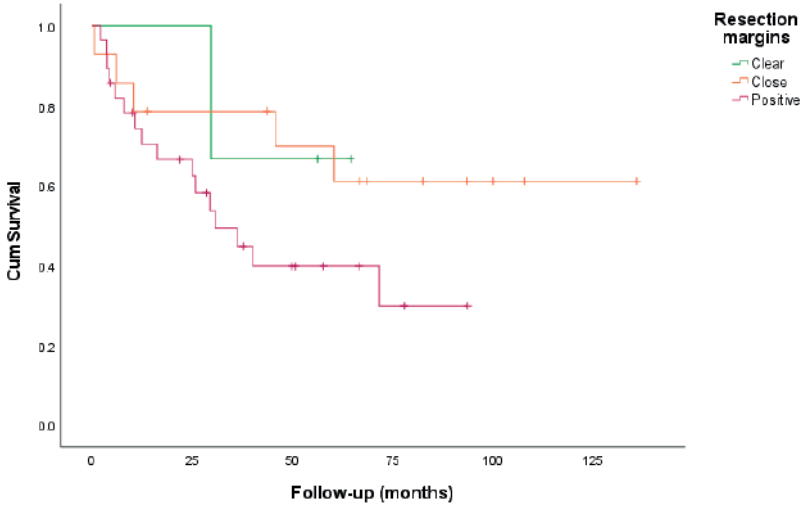

Figure 3. Disease-specific survival by resection margin in primary hypopharyngeal squamous cell carcinoma.

Primary LSCC. One hundred and seven patients underwent TL or TLP. The resection margins were clear (>5 mm) in 19%, close (1-5 mm) in 27%, and positive (<1 mm) in 54% of cases, with TR of 5%, 24%, and 36%, respectively. A linear association was observed between TR and narrowing margins (linear-by-linear association, $p = 0.007$). LR was found in 5% of clear, 0% of close, and 16% of positive margins. There was no increase in the LR rate for narrowing margins (linear-by-linear association, $p = 0.058$). The 5-year DSS rates for clear, close, and positive margins

were 95%, 78%, and 63%, respectively (log-rank test, p = 0.041). The 5-year DFS rates for clear, close, and positive margins were 55%, 45%, and 39%, respectively (p = 0.776).

Recurrent LSCC. One-hundred patients underwent TL or TLP. The resection margins were clear in 37%, close in 34%, and positive in 29% of the cases, with TR of 35%, 38%, and 69%, respectively. A linear association was observed between TR and narrowing margins (p = 0.008). LR was found in 14% of clear, 21% of close, and 45% of positive margins. There was an increased LR rate for narrowing margins (p = 0.004). The 5-year DSS rates for clear, close, and positive margins were 61%, 64%, and 26%, respectively (p = 0.002). The 5-year DFS rates for clear, close, and positive margins were 40%, 43%, and 10%, respectively (p = 0.002).

A multivariate survival analysis for primary and recurrent laryngeal tumors (n=207) with confounders including age, primary or recurrent tumor, pT1&2 or pT3&4, pN0 or pN+, and postoperative adjuvant treatment showed a worse DFS (hazard ratio (HR) 1.7, 95% CI 1.1 to 2.8, p = 0.020) and DSS (HR 1.7, 95% CI 1.0 to 2.7) p = 0.041) in cases with positive margins compared to those with clear margins. There were no differences between close and clear margins (DFS HR 0.9, 95% CI 0.6 to 1.5; DSS HR 0.9, 95% CI 0.5 to 1.4).

Primary HSCC. Forty-five patients underwent TL or TLP. The resection margins were clear in 7%, close in 31%, and positive in 62% of the cases, with TR of 33%, 29%, and 54%, respectively. There was no linear association between TR and narrowing margins (p = 0.165). LR was found in 33% of clear, 7% of close, and 18% of positive margins. There was no increase in the LR rate in the case of narrowing margins (p = 0.942). The 5-year DSS rates for clear, close and positive margins were 67%, 70%, and 40%, respectively (p = 0.207). The 5-year DFS rates for clear, close, and positive margins were 33%, 50%, and 18%, respectively (p = 0.030).

Recurrent HSCC. Sixteen patients underwent TL or TLP. The resection margins were clear in 25%, close in 31%, and positive in 44% of the cases, with TR of 50%, 60%, and 71%, respectively. There was no linear association between TR and narrowing margins (p = 0.486). LR was found in 25% of clear, 60% of close, and 43% of positive margins, resulting in no difference in LR rates (p = 0.678). Survival analysis was not performed because the number of patients was too small.

Multivariate survival analysis in both HSCC groups (n=61) with confounders, age, primary or recurrent tumor, pT1 and 2 or pT3 and 4, pN0 or pN+, and postoperative

adjuvant treatment, did not show an association between resection margin status and survival (for positive vs. clear margins, DFS HR 2.3, 95% CI 0.7 to 7.0, and DSS HR 2.4, 95% CI 0.8 to 7.4).

9.5 DISCUSSION

The larynx and hypopharynx have a complex anatomy and the achievement of >5 mm resection margin is limited. In this study, the percentage of positive resection margins was remarkably high for both primary and recurrent LSCC and HSCC (54% for primary LSCC, 29% for recurrent LSCC, 62% for primary HSCC, and 44% for recurrent HSCC). Here, we discuss the resection margins of different resection surfaces and their anatomical limitations. Resection margins in relation to the cranial and caudal surfaces were analyzed for LSCC and HSCC without distinguishing between primary and recurrent tumors. Caudal resection surfaces showed low numbers of positive margins for LSCC (2%), HSCC (3%), and close margins (LSCC 4% and HSCC 7%) (Table 2). At the caudal site (trachea or esophagus), resection margins of >5 mm for both LSCC and HSCC are always feasible because of the anatomy. The percentages of positive margins for cranial resection surfaces in this cohort were higher: 8% for LSCC and 11% for HSCC. In addition, 12% had close margins for both LSCC and HSCC. However, at the cranial site, a margin of >5 mm should always be feasible because at that location an additional mucosal resection could be performed when needed. Resection margins in relation to the other surfaces (ventral, dorsal, and lateral) were analyzed separately for LSCC and HSCC owing to their delicate anatomy (Table 2). For the ventral resection surface, a positive margin of 31% and a close margin of 29% were found in LSCC. In cases of extra-laryngeal tumor growth and/or cartilage invasion, ventral margins of >5 mm cannot be achieved because the strap muscles are <5 mm thick. Skin resection should only be performed in cases of skin involvement because of the morbidity associated with reconstruction. Therefore, a close ventral resection margin should be accepted in patients without skin involvement. For HSCC, only piriform sinus and postcricoid tumors with endolaryngeal invasion have a ventral resection surface. The ventral resection margin was positive in 34% of cases and close in 28% of cases. For the dorsal resection surface, the positive and close margins were both 7% in LSCC. The dorsal resection surface in LSCC is confined only by the postcricoid mucosa because the adjacent lumen of the hypopharynx and esophagus are not resection surfaces. Achieving a resection margin >5 mm in this area is not feasible because the thickness of the dorsal laryngeal tissue (mucosa and submucosa) is only 2 mm. For HSCC, a positive dorsal resection margin of 12%

and a close resection margin of 9% were observed. The dorsal resection margin for HSCC only exists for anterior and lateral wall piriformis sinus tumors or posterior pharyngeal wall tumors. However, only the thickness of the tissue (mucosa and submucosa) of the anterior wall of the piriform sinus allows for a resection margin of >5 mm. Tumors at the medial wall of the piriform sinus and postcricoid do not have a dorsal resection surface because of the adjacent lumen of the hypopharynx and esophagus. For LSCC, a positive lateral resection surface was found in 2% of the cases and close in 7% of the cases. Lateral margins of >5 mm should always be feasible because of the piriform sinus anatomy. For HSCC, a positive lateral resection surface was found in 3% of the cases and close in 12% of the cases (Table 2). For lateral and anterior piriform sinus tumors, this resection surface is relevant, but a margin of >5 mm is not feasible because of the limited thickness of the mucosa and submucosa and its direct relationship with vital vascular structures. A resection margin of >5 mm is feasible only for tumors of the posterior pharyngeal wall and the postcricoid.

Furthermore, we assessed the clinical prognostic relevance of resection margins by analyzing the recurrence and survival rates. Owing to the different tumor characteristics, primary and recurrent LSCC and HSCC were analyzed separately in the four groups. For LSCC, we found a significant association between TR and narrowing margin for primary ($p = 0.007$) and recurrent tumors ($p = 0.008$). In addition, narrowing of the margin was associated with an increased LR rate for recurrent LSCC ($p = 0.004$). In contrast, for both primary and recurrent HSCC, narrowing margin showed no association with TR or LR. It can be argued that resection margins mostly influence LR and not regional recurrence or distant metastasis. *Basheeth et al.* found a significant association (univariate analysis, $p < 0.001$) between positive margins and LR in the neopharynx (base of the tongue/pharynx) compared to LR around the stoma ($p = 0.45$) for primary and recurrent LSCC (10). In this study, we only found a higher LR rate (mucosal neopharynx or stomal recurrences) for positive margins in patients with recurrent LSCC ($p = 0.004$), but not for primary LSCC. This is probably because patients with primary LSCC received postoperative therapy after TL or TLP. Positive margins were associated with a significantly worse 5-year DSS for primary ($p = 0.041$), and recurrent LSCC ($p = 0.001$). This was also the case for the 5-year DFS in patients with recurrent LSCC ($p = 0.002$) and primary HSCC ($p = 0.030$). In contrast to positive resection margins, the survival rates (DSS or DFS) for close and clear margins were comparable between primary recurrent LSCC and primary HSCC, which could imply that close and clear margins are similar in terms of survival. This may suggest that a clear margin is not always feasible because of the anatomy of the larynx and hypopharynx, and that a clear margin is

not clinically relevant in terms of survival. Multivariate survival analysis for LSCC (primary and recurrent) showed that positive margins were independent negative predictors of DFS and DSS. Histological tumor characteristics (e.g., differentiation grade, perineural growth, and angio-invasion) were not consistently reported and were therefore not included in this analysis. For HSCC, positive margin status was not found to be an independent prognostic factor. However, firm conclusions could not be drawn because of the small number of patients.

Unfortunately, in retrospective studies, it is not possible to determine whether pathological assessment was performed consistently and according to the RCP. Next, the resection margins for each resection surface were not always available in the pathology reports (particularly the dorsal and lateral resection margins, which were often unknown). The RCP guidelines for the larynx describes how to record a histopathology report, whereby the resection margins are defined as clear >5 mm, close 1-5 mm, and positive <1 mm. How the pathological examination should be performed, such as the required orientation of the different resection surfaces or how to measure these margins, is not mentioned. The importance of anatomical orientation of the resection specimen, accurate identification of different resection surfaces, and measurement of resection margins is crucial (9). A standardized pathological assessment and report is needed before a definite statement on patient prognosis can be made for LSCC and HSCC. Clear communication and collaboration between the pathologist and surgeon are key.

A literature search revealed a lack of studies regarding the clinical relevance of resection margins in laryngeal and hypopharyngeal oncologic surgery. Only 12 studies on resection margins during TL/ TLP have been found. Only one study has followed the RCP resection margin guidelines for recurrent LSCC and HSCC (8). The authors reported 72% clear, 18% close, and 9% positive margins, and a 5-year DFS of 55% and DSS of 55% for the total population, regardless of the margin status. Compared to our study of patients with recurrent LSCC and HSCC, the percentage of close and positive margins was higher (35% clear, 34% close, and 31% positive margins), and the 5-year DFS (31%) and DSS (49%) were lower. DFS in another study (not according to the RCP guidelines) was 63% for primary and 47% for recurrent LSCC and HSCC (11). The DSS rates in other studies (not according to the RCP guidelines) were 58% (primary and recurrent LSCC) (10), 80% (recurrent LSCC) (12), 52% (primary HSCC) (13), 46% (primary HSCC; TL or RT as treatment) (14), 63% (primary LSCC; TL) (15) and 58% (primary LSCC; TL and RT) (15). Two studies defined close margins as <5 mm and positive margins as tumor at the resection surface (13, 15). These studies reported 77-81% clear, 9-14% close,

and 5-11% positive margins. LR was reported in 18-24% and 5-year DSS in 52-63%, regardless of margin status. The remaining 9 articles used descriptive definitions for margin status, such as 'positive', 'microscopically positive', 'tumor at the resection surface', 'negative', 'safe margins' or 'no invasive tumor at the resection surface'. These studies reported clear margins in 70-100% and positive margins in 0-30% (10-12, 14, 16-20). The results of our survival data cannot be compared with those in the literature because diverse patient cohorts and descriptive definitions for margin status have been used in different studies. The patient cohorts varied in tumor location, TNM classification, and pre- and postoperative treatment. *Saraniti et al.* confirmed that a comparison of the literature is not possible, and to investigate the prognostic value of resection margins, a meta-analysis should be performed with identical definitions of resection margins, methodology, and postoperative treatment (6). This study is unique because we analyzed separate groups (primary/ recurrent and LSCC/ HSCC) and used the RCP guidelines. Despite the lack of a meta-analysis, it is needless to say that margins of <1 mm should be strictly avoided, while a margin of 1-5 mm could be accepted in specific cases, as shown in this study.

9.6 CONCLUSION

To the best of our knowledge, this study is the first to analyze the relationship between resection margins of different resection surfaces and the prognostic value in distinctive groups: primary and recurrent LSCC and HSCC. For primary and recurrent LSCC, significantly more (local) recurrences were found in cases with narrowing margins, and positive margins were an independent predictor of worse DFS and DSS in a multivariate survival analysis. The survival rates for close and clear margins were comparable for recurrent LSCC and primary HSCC, implying that close and clear margins are similar in terms of survival. This may suggest that a margin >5 mm is not clinically relevant in terms of survival. Therefore, a margin of 1-5 mm should be accepted for certain subsites. Margins <1 mm should be avoided, particularly in salvage surgery. Histopathological assessment of laryngeal and/or hypopharyngeal resection specimen should be universal to draw definitive conclusions about the influence of resection margins on patient outcomes.

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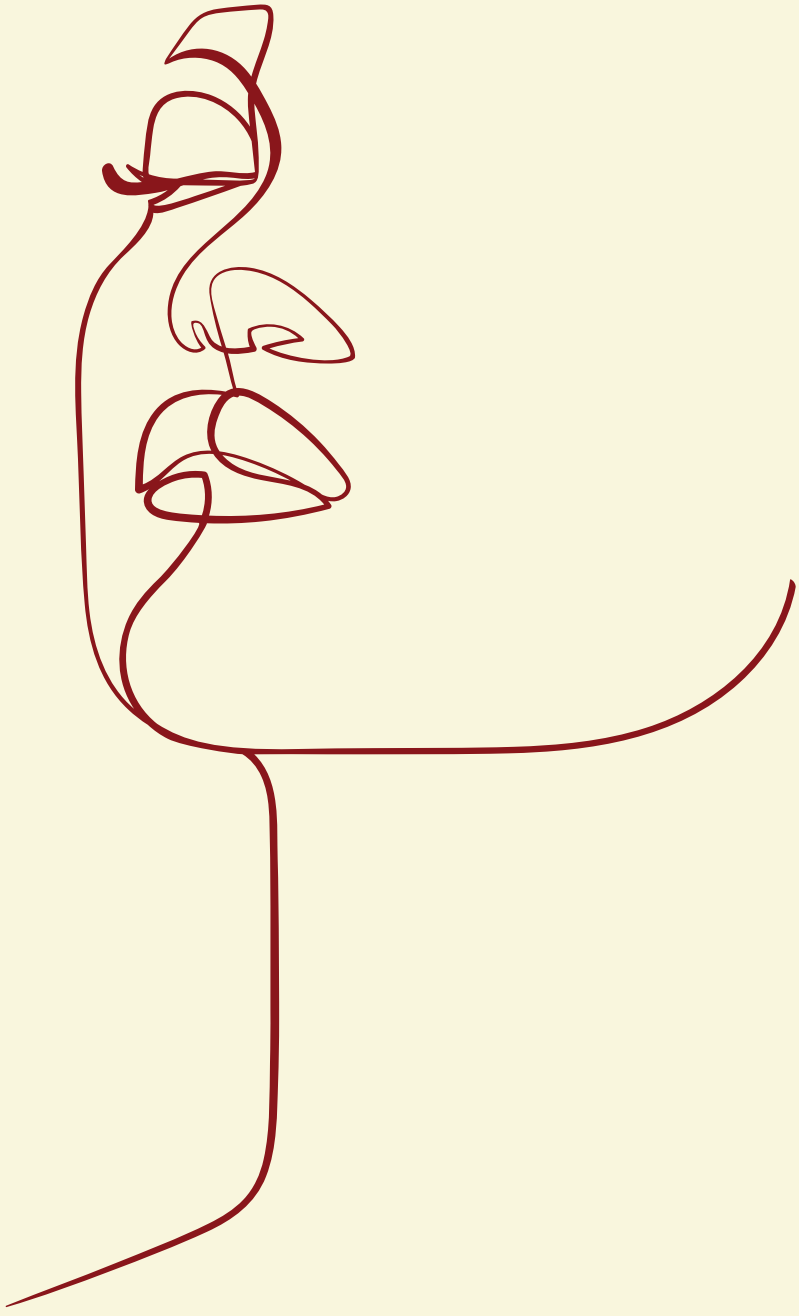

Chapter 10

Raman spectroscopy to discriminate laryngeal squamous cell carcinoma from non-cancerous surrounding tissue

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10.1 ABSTRACT

Purpose

As for many solid cancers, laryngeal cancer is treated surgically, and adequate resection margins are critical for survival. Raman spectroscopy has the capacity to accurately differentiate between cancer and non-cancerous tissue based on their molecular composition, which has been proven in previous work. The aim of this study is to investigate whether Raman spectroscopy can be used to discriminate laryngeal cancer from surrounding non-cancerous tissue.

Methods

Patients surgically treated for laryngeal cancer were included. Raman mapping experiments were performed *ex vivo* on resection specimens and correlated to histopathology. Water concentration analysis and CH-stretching region analysis were performed in the high-wavenumber range of 2500-4000 cm^{-1}.

Results

Thirty-four mapping experiments on 22 resection specimens were used for analysis. Both laryngeal cancer and all non-cancerous tissue structures showed high water concentrations of around 75%. Discriminative information was only found to be present in the CH-stretching region of the Raman spectra of the larynx (discriminative power of 0.87).

Conclusion

High wavenumber region Raman spectroscopy can discriminate laryngeal cancer from non-cancerous tissue structures. Contrary to the findings for oral cavity cancer, water concentration is not a discriminating factor for laryngeal cancer.

10.2 INTRODUCTION

Surgical treatment with adequate resection margins is an important prognostic parameter for patients with laryngeal squamous cell carcinoma (LSCC) (1-3). *Van Lanschot et al.* described a significantly worse survival for positive margins in LSCC compared to those with close and clear. Yet, avoiding positive margins is difficult, due to the anatomy of the larynx (4). To guide the surgeon towards an adequate resection, intra-operative assessment with frozen section analysis is often performed (4, 5), especially in cases where preservation of speech and swallowing is possible (6, 7). However, this method is time-consuming and prone to sampling error (4, 5). A rapid and objective technology would be of added value for the intra-operative assessment of resection margins.

Various optical techniques have been investigated for this important clinical oncological need. These optical techniques have opened new perspectives because of their ease of use, high speed, and real-time and objective tissue characterization (8-11). Narrow-band imaging (NBI), autofluorescence (AF), contact endoscopy (CE), optical coherence tomography (OCT), fluorescence imaging (FLI) and Raman spectroscopy are examples of these techniques. NBI, AF, CE and OCT are established tools for early detection of laryngeal cancer, but their intra-operative function is limited by the inability to image beyond the (sub-) mucosa. These techniques are only useful for mucosal margin assessment and unsuitable for deep margin assessment. Fluorescence imaging has potential for assessment of deeper margins because of the possibility to image at considerable depths and tissues other than the mucosa. Unfortunately, research on FLI for margin assessment is sparse. Raman spectroscopy has important advantages allowing for margin assessment in the deep margin planes. Raman spectroscopy is a technique based on inelastic scattering of light in tissue and is able to characterize all different tissue types (11, 12). Many studies have shown the potential of Raman spectroscopy to discriminate between cancer and non-cancerous tissue (11). Increased accuracy for cancer diagnosis with Raman spectroscopy-based biopsy is reported with sensitivities between 73% - 100% and specificities of 66% - 100% (11).

Although promising, for laryngeal tissue there are only a few feasibility studies performed on cancer detection with Raman spectroscopy. Different research groups determined that Raman spectra can be obtained rapidly and can reveal differences between non-cancerous tissue and LSCC (13-17). These findings are based on the global spectral differences between cancerous and non-cancerous tissue, and the exact differences in discrimination between tumor and different

surrounding tissue structures like connective tissue, gland, cartilage, muscle, and necrotic tissue were not analyzed.

In the literature mostly the fingerprint region is used, in this study it is chosen to use the high wavenumber region (2500 - 4000 cm^{-1}) because of the stronger Raman signals and reduced fluorescence background, and because it enables *in vivo* application using simple single fiber optical probes (18).

In previous work, it was demonstrated that high wavenumber Raman spectroscopy can discriminate oral cavity squamous cell carcinoma (OCSCC) from surrounding non-cancerous tissue with 99% sensitivity and 92% specificity based on water concentration. Based on this information, the tumor border could be determined, with a decrease in concentration from 76% inside the tumor to 54% at 5 mm from the tumor border (19). The aim of this study was to investigate the spectral differences between tumor and the different tissue structures and to determine whether high wavenumber Raman spectroscopy can be used to discriminate laryngeal cancer from surrounding non-cancerous tissue.

10.3 MATERIAL AND METHODS

Medical ethical approval: The study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Center Rotterdam (MEC-2013-345). Informed consent was obtained from all patients before treatment.

Study population: Patients surgically treated (e.g., laryngectomy) between December 2015 and January 2019 for LSCC were included in this study. The experiments were performed *ex vivo* on resection specimens of the patients directly after surgery.

Tissue sampling: The time for experiments was limited to 60 minutes for optimal preservation of the tissue for the final pathological evaluation. After surgery, the specimen was brought to the cutting room of the pathology department. A dedicated head and neck pathologist (author) and the surgeon performed intraoperative assessment of the resection margins, as described by *Aaboubout et al* (20). After this assessment, a tissue section was cut from the specimen for the Raman experiments (hereafter referred to as Raman section), without interfering with the standard pathological evaluation of the specimen. All steps handling the resection specimen were recorded. The Raman section was rinsed from blood

with physiological salt solution (0.9% NaCl) and patted dry. Afterwards, the tissue section was inserted in a closed cartridge of which the upper side consists of a fused silica window. The fused silica window was in contact with the tissue section. The maximum tissue area that could be scanned was determined by the size of the cartridge which was 3x3 cm^2. If possible, both sides of the Raman section were scanned. After the experiment, the Raman section was added to the main specimen for fixation in formalin, for routine histopathologic evaluation.

Raman instrumentation and experiments: a confocal Raman microscope was used to perform experiments. The setup is described in detail in an earlier study (19). The equipment contains a 671nm laser (CL671-150-SO, CrystaLaser), a charge-coupled device (CCD) camera fitted with a back-illuminated deep depletion CDD-chip (Andor iDus 401, DU401A BR-DD, Andor Technology Ltd.) and a multichannel Raman Module (HPRM 2500, RiverD International B.V). The Raman Module was coupled to the microscope (Leica DM RXA2, Leica Microsystems Wetzlar GmbH) and a computer-controlled sample stage (Leica DM STC). The cartridge, with the Raman section, was fixed on the stage and the surface of the tissue was mapped in a grid. The step size of the grid varied between 250 µm x 250 µm to 1000 µm x 1000 µm depending on the size of the tissue section and the maximum time to perform the experiment. Laser light (80 mW) was focused through the microscope objective (0.4 numerical apertures) with a 1.1 mm working distance (NPLAN 11566026, Leica Microsystems B.V.). The laser was focused in the tissue, 50-70 µm below the fused silica window. The signal acquisition time per spectrum was 1s. Spectral information was collected in the high-wavenumber range of 2500-4000 cm^{-1} with a resolution <5 cm^{-1} and a depth resolution of 40 µm.

Calibration and processing data: Software for calibration and processing the collected spectra was developed with MATLAB (R2017b). Spectra were calibrated on the relative wavenumber axis and corrected for wavelength-dependent detection efficiency of the setup, according to instructions of the spectrometer manufacturer (RiverD International B.V.). Preprocessing of the spectra was performed by removal of cosmic ray events and subtraction of signal background generated in the optical path of the setup (21). The background signal, caused by tissue autofluorescence, was estimated as a 3rd-order polynomial and subtracted from the preprocessed spectra (19). For all spectra, the average intensity over the range of 2700-3100 cm^{-1} was used as a measure of signal intensity. The lowest quality spectra (with an average intensity <5% of the overall average intensity) were excluded from the analysis.

Histopathology: Histopathologic evaluation of the Raman section was performed by a head and neck expert pathologist (author) on a hematoxylin and eosin (HE)-stained section. The HE-stained section was digitized, and the pathologist delineated different tissue types (e.g., tumor, connective tissue, gland, cartilage, muscle, necrotic tissue) on the digitized section.

Data analysis: Data analysis was performed in MATLAB; R2017b. Data analysis consisted of 1) analysis of the water concentration, 2) analysis of the CH-stretching region by performing principal component analysis (PCA), and linear discriminant analysis (LDA).

1. Water concentration analysis: The water concentration in the Raman section was calculated for each measurement point according to the method developed by *Caspers et al.* (19). Spectra with water percentages >88% were considered outliers and discarded from analysis. Raman maps were created by plotting the water concentration as a 2D map with color codes representing the range in water concentration. The Raman map was averaged (convoluted with a 3x3 average filter) to obtain a representative water concentration (reducing noise in the image), and better visualization of the difference in water concentration between tumor and non-cancerous tissue (22). The delineated images of the tissue sections were projected over the corresponding Raman maps. For each Raman map, areas were selected with unambiguous histological annotation. The Raman data from these areas were used for data analysis. The precision of the annotation was limited by the resolution of the Raman map (pixel size varying from 250 μm x 250 μm to 1000 μm x 1000 μm). Afterwards, the water concentrations were separated into two groups: water concentrations from non-cancerous tissue and water concentrations from tumor. A Wilcoxon rank sum test was used to test whether the water concentration distributions were significantly different at the 0.05 confidence level. The discriminatory power for tissue classification based on water concentration was determined by measuring the area under the ROC-curve (Receiver Operating Characteristic curve).

2. CH-stretching region analysis: For analysis of the CH-stretching Raman signal, the spectral region between 2800 and 3100 cm^{-1} was used, which is independent of, and complementary to, the water concentration analysis. All spectra were scaled using an extended multiplicative scatter correction (EMSC) procedure (using water as spectral interferent) (23) to eliminate spectral interference of varying water contributions. This method has been used in earlier studies and ensures the CH-stretching region analysis is independent of the water signal (24). For each Raman

map, a color map based on a PCA on the CH-stretching data was made. The scores on the first 3 (most significant) principal components (PCs) were used as input data for the red, blue, and green channels of the color map. In this way the most important signal variance in CH-stretching region is displayed as an image and can be compared to histology. PCA on the whole dataset was performed to reduce the dimensionality of data before LDA modeling. The spectra were first filtered with a Savitzky-Golay filter (order 3, window size of 11 points) to reduce the influence of noise on the PCA result. To separate tumor spectra from non-cancerous spectra, LDA was used to find the direction in PCA space that maximizes the ratio between the inter-group and intra-group variance (25, 26). The scores on the first (most significant) PCs were selected as input parameters for the LDA. The optimal number of PCs for the PCA-LDA model was determined by leave-one-map-out validation on a model data set with the spectra of 25 maps from 17 patients. The PCA-LDA model was validated with an independent test data set with the spectra of 9 maps from 5 patients. ROC-curve analysis was used to determine the discriminative power of the LDA analysis.

10.4 RESULTS

Forty-seven *ex vivo* Raman experiments were performed on laryngectomy specimens from 27 patients.

For seven experiments the registration of the measured region was not reliable and therefore correlation with histopathology was not performed. Six experiments were excluded because the general spectral quality was insufficient. Finally, thirty-four experiments were included for further analysis.

Figure 1 shows an overview of a single mapping experiment.

Figure 2 shows the histograms of the water concentration for the different tissue types with the mean and standard deviation (std) of the distribution. Although the water concentration distributions of tumor and all different non-cancerous tissue structures are highly overlapping, the distributions of tumor and non-cancerous are significantly different (Wilcoxon rank sum test at the 0.05 confidence level: $p < 0.01$). Because of the high degree of overlap, the discriminative power of the classification is low (area under ROC-curve: 0.56)

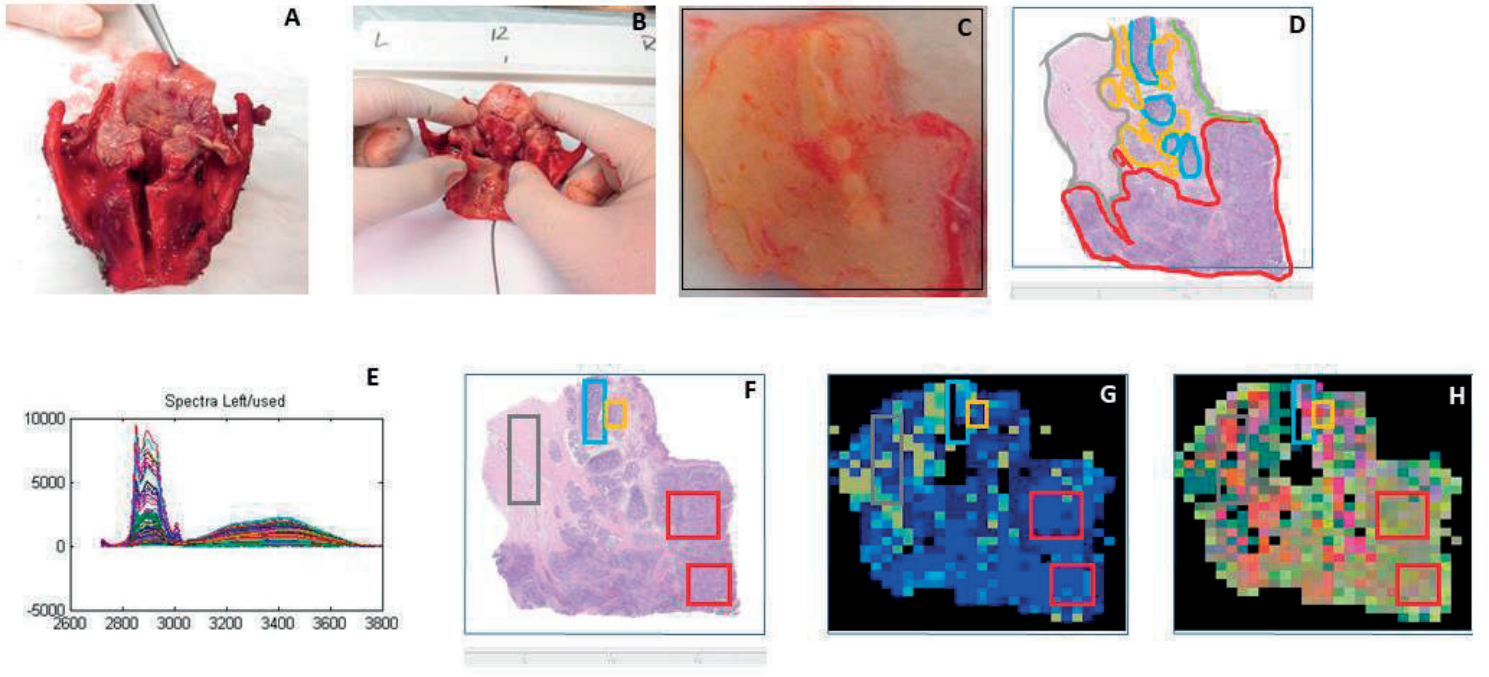

Figure 1. an overview of a single mapping experiment.

A: The resection specimen was cut open for intra-operative assessment of the resection margins B: A tissue section with tumor and non-cancerous surrounding tissue was obtained for the Raman experiment C: This Raman section was inserted into a cartridge and the whole area was measured D: The HE-stained Raman section with annotation of the different tissue types. E: The Raman spectra measured for the Raman section F: Areas with unambiguous histopathology were selected for Raman analysis G: Raman water map with water distribution and projected histological annotation. Black pixels correspond to absence of tissue or to spectra with low Raman signal quality. H: CH-stretching region PCA map and projected histological annotation. Black pixels correspond to absence of tissue or to spectra with low Raman signal quality.

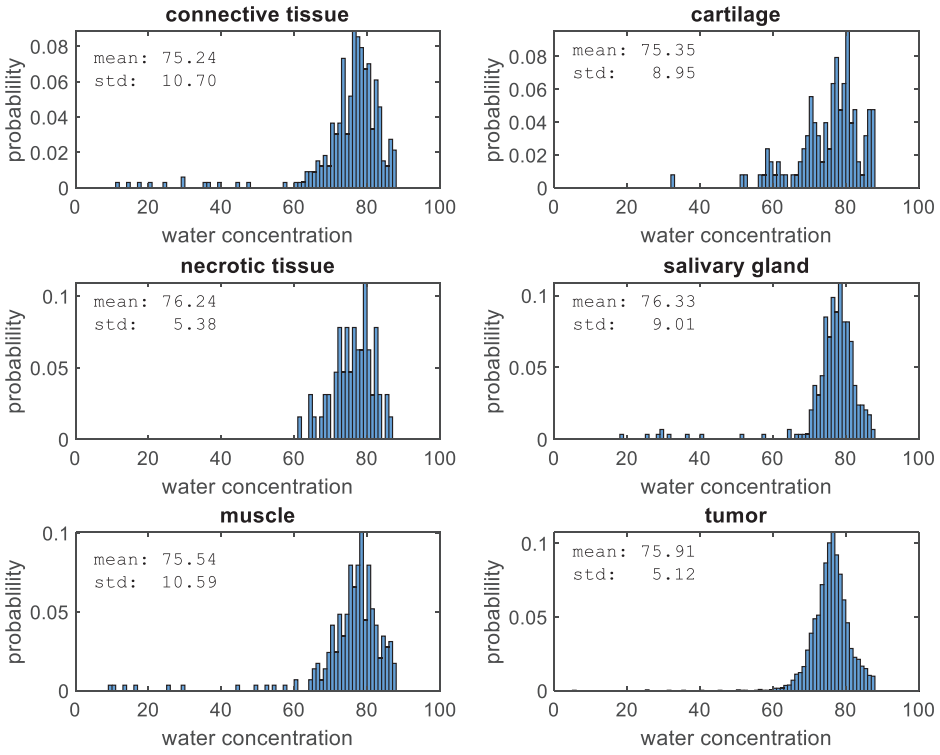

Figure 2. Histograms of the water concentration for the different tissue types.

Considering the low discriminative power of the water concentration analysis, the CH-stretching region (2800-3100 cm^{-1}) was investigated whether it contained more discriminative information. The spectra were EMSC-scaled and filtered as described in the Material & Methods section. The left panel of Figure 3 shows the mean spectra of the different tissue types in this region. The spectral differences between the mean spectra of tumor and of non-cancerous tissue types are shown in the right panel of Figure 3.

A PCA-LDA model was built using the model data set of 25 maps from 17 patients. The optimal number of input PCs for the PCA-LDA model was first determined by leave-one-map-out validation on the model data set. The discriminating power of the PC-LDA model (area under the ROC-curve) was determined for different amounts of input PCs. The best number of input PCs, yielding the highest discriminating power (0.90) was found for a PCA-LDA model built on the scores of the first 4 PCs as input. The left panel of Figure 4 shows the ROC-curve of a leave-one-map-out validation of this PCA-LDA model. The PCA-LDA model was validated with an independent test data set of 9 maps from 5 patients. The right panel of Figure 4

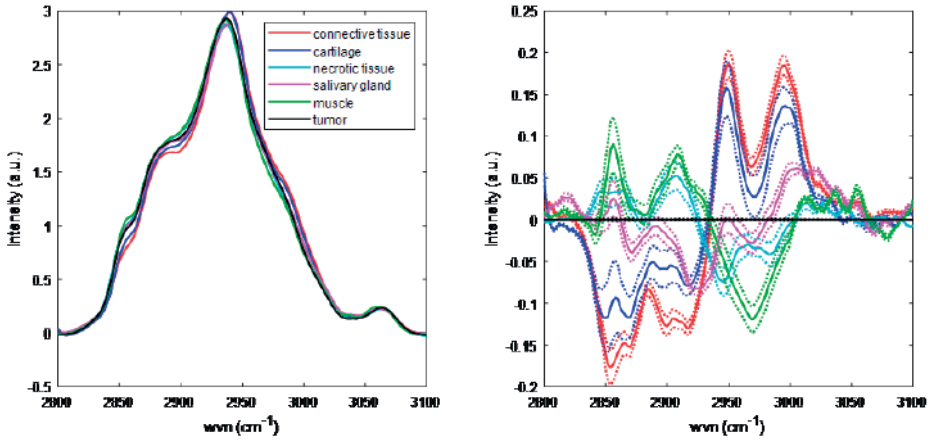

Figure 3. CH-stretching region of the spectra used for linear discriminant analysis.
Left panel: mean spectra per tissue type. Right panel: the solid lines denote the differences with tumor for different tissue types and the dotted lines denote the +/- standard error of the mean for the different tissue types.

shows the ROC-curve of the validation with the independent data set. As can be seen from the figure, the ROC-curves for the model data set and the independent test data set give similar results, showing that there is consistent discrimination information present in the CH-stretching region of the Raman spectra of the larynx.

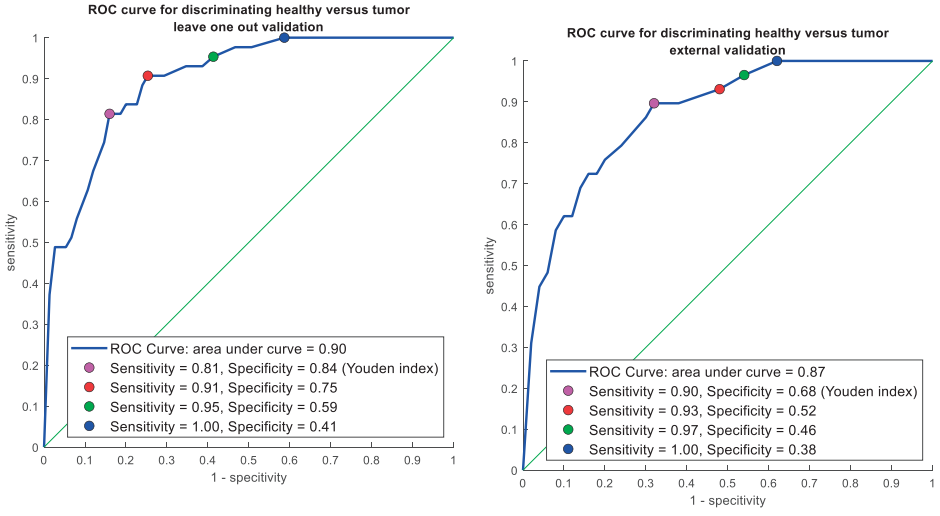

Figure 4. Left panel: ROC curve of leave one map out validation on the model set; right panel: ROC curve of external validation using the independent data set.

At the Youden index (optimal combination of sensitivity and specificity), the validation set showed a sensitivity of 0.90 and a specificity of 0.68. All false positives were analyzed to investigate which non-cancerous tissue structures are falsely identified as tumor. Forty percent of all necrotic tissue spectra were identified as tumor spectra, followed by the salivary gland (36%), connective tissue (18%), and cartilage (9%). Spectra from muscle were all identified as non-cancerous tissue spectra.

10.5 DISCUSSION

Therapeutic surgery guidance (i.e., facilitating the achievement of adequate resection margins) is an important clinical need during surgical oncological procedures. The aim of this study was to determine whether high wavenumber Raman spectroscopy can be used to discriminate laryngeal cancer from surrounding non-cancerous tissue.

Using the CH-stretching region only, it was able to classify tumor from non-cancerous tissue with a discriminative power of 0.87, validated on an independent dataset. Analysis of the false positive predictions shows that mostly necrotic tissue and salivary gland were identified as tumor. The mean spectra of these two tissue types also showed the smallest difference with the mean spectrum of tumor. The high false positive rate for the salivary gland is in line with earlier observations for oral cavity tissue where the salivary gland was also wrongly identified as tumor in 35% of the cases (24). The discriminative power is lower than the 0.97 reported by *Lin et al.* in a study where they used a trans-nasal Raman spectroscopy technique integrated with an endoscope-based fiber-optic Raman probe to collect spectra of cancerous and non-cancerous laryngeal tissue in the CH-stretching region (16).

The results of this study show high water concentrations in both LSCC and all surrounding non-cancerous tissue structures. Despite the significant shape difference in the water concentration distributions for tumor and surrounding non-cancerous tissue, the discriminative power of 0.56 is low. This confirms the results of *Lin et al.* who reported a low diagnostic difference for the OH-stretching band around 3400 cm^{-1} to discriminate cancer from non-cancerous tissue in the larynx (17).

These findings are different from the results obtained in previous studies for the oral cavity, where consistently higher water concentration was found for OCSCC

compared to surrounding non-cancerous tissue (19). The water concentrations measured in non-cancerous laryngeal tissue are significantly higher than in non-cancerous oral cavity tissue.

Surprisingly, for all tissue types in the larynx, the mean water concentration is higher than in OCSCC. First, a literature search was performed to find an explanation for the higher water concentration such as physiological, anatomical, or molecular differences in the larynx compared to the oral cavity. Only for the vocal cords it has been shown that they have a high water concentration (27). However, the vocal cords are a small part of the larynx, and no publication was found mentioning a generally higher water concentration in healthy laryngeal tissue compared to healthy oral cavity tissue.

Second, to confirm these surprising findings, water concentration measurements were performed on freshly excised tissue using a fiber-optic needle probe setup that was developed for the assessment of oral cavity cancer based on water concentration. The same experiments were performed, as described above, with this new Raman set-up for the larynx. The results from the Raman setup with the cartridge were confirmed with a high water concentration of +/- 75% in all tissue types in the larynx. The details are described in the supporting material S1.

There are two limitations in the study design that may have contributed to the relatively low discriminative power of the classification model. The first limitation is related to the fact that to build a good classification model based on Raman spectroscopy, a large spectral database is needed with accurate histopathologic annotation. For the current study, it was only possible to retrieve small tissue sections from the resection specimen to not interfere with the standard pathological evaluation of the specimen. As explained by *Barroso et al.*, with a laser spot size of 4 µm it is not always feasible to translate the exact position of the laser to the HE-stained section, resulting in only a limited number of spectra with an accurate annotation per tissue sample (19). The second limitation is the signal-to-noise ratio of the measurements. In this study, a signal acquisition time of one second was used. The selection of this acquisition time was based on earlier work. Due to the high water concentrations encountered, this was too short to obtain a sufficiently high signal-to-noise ratio in the CH-stretching region for optimal discrimination, given the small differences between tumor and non-cancerous tissue structures.

For this study, the high wavenumber region (2500-4000 cm^{-1}) was chosen because of the stronger Raman signals, the reduced fluorescence background, and the easy *in*

vivo implementation using a handheld Raman spectroscopy probe (i.e., single fiber probe without filters). Currently, biomedical Raman research in diagnosing cancer is mostly centered on the fingerprint region (i.e., 800-1800 cm^{-1}) that contains rich biochemical information about the tissue. The advantage of the fingerprint Raman spectroscopy technique stems from its capability to uncover specific information about backbone structures of proteins, lipids and nucleic acid assemblies in cells and tissue. The fingerprint region has shown successful results for the differentiation of LSCC and non-cancerous tissue, with a 69-92% sensitivity and 90-94% specificity in previous studies (13-15). *Teh et al.* identified 21 Raman features related to the biochemical and biomolecular changes (e.g., proteins, lipids, nucleic acids, and carbohydrates) that are associated with LSCC. They developed a random forests algorithm and observed significant differences in Raman spectra between tumor and non-cancerous tissue with an overall accuracy of 89.3%, a sensitivity of 88.0%, and a specificity of 91.4% (15). Some limitations are associated with the implementation of the Raman spectroscopy fingerprint region, especially if used *in vivo*. This region is hampered by the strong signal background generated by the optical fiber, requiring complicated probe designs with multiple fibers and filters which makes them expensive and difficult to reproduce (16, 17, 28). Also, the signal intensity in the fingerprint region is relatively low with relatively large fluorescence backgrounds, which may cause long signal integration times making it impractical for clinical use (16, 17, 28).

Lin et al. suggested that fingerprint and high wavenumber region Raman spectroscopy combined could have advantages for tissue characterization because of the complementary information. The diagnostic accuracy with integrated fingerprint and high wavenumber was found to be superior to either fingerprint (accuracy 86.1%) or high wavenumber (accuracy 84.2%) alone (17). For clinical use, this would require a significant step in probe development that can be avoided if the use of the high wavenumber region alone provides sufficient clinical information.

Considering the current limitations and published studies, future work may include the exploration of a different set-up that is optically designed to have an increased laser spot size (for instance 250 μm) and spectral acquisition time. This can improve the histopathological correlation and increase the signal-to-noise ratio, and thus the discriminative power of the classification model. Deep learning algorithms can be incorporated for a more accurate classification model. However, for these deep learning models, the training data set must be extended to a larger cohort of patients, to better capture the variation in spectra, within and between patients.

10.6 CONCLUSION

High wavenumber Raman spectroscopy can discriminate laryngeal cancer from non-cancerous tissue structures. Contrary to the findings for oral cavity cancer, water concentration is not a discriminating factor for laryngeal cancer. Despite the current limitations, this study contributes to important steps towards the development of a Raman spectroscopy probe for therapeutic surgery guidance.

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10.8 SUPPORTING MATERIAL

S1 – Raman experiments with a fiber-optic needle probe

10.8.1 Materials and Methods

10.8.1.1 Fiber-optic needle probe prototype

A prototype Raman instrument, developed by the Erasmus MC, RiverD International B.V. The Netherlands, and art photonics GmbH. (Germany), was used (Figure 1). The development was supported by the Dutch Cancer Foundation (project number: 8027 / KaWeFis Batch 5).

The instrument is comprised of a custom-designed high wave number Raman Module (RiverD), enabling signal collection in the spectral range of 2500-4000 cm^{-1}, a 671nm laser (Gem671, 50 mW–250 mW, LaserQuantum, UK), and a charge-coupled device (CCD) camera fitted with a back-illuminated deep depletion CCD-chip (Andor iVac, 316 LDC-DD, Andor Technology Ltd., UK).

The Raman module is connected to the fiber-optic needle probe through a cable (Figure 1.a) containing a fiber-optic patch-cord (NIR 100/110AL-300-FC/PC-MS44, art photonics GmbH., Germany) and electrical wiring.

The fiber-optic needle probe is disposable and consists of a fiber-optic needle (30G x 12 mm Omnican® insulin needle, B. Braun Holding GmbH & Co., Germany) that has a 100 μm core fiber-optic inside (NIR100/110AL, AP11396, art photonics GmbH., Germany) (Figure 1.b). It is attached to an actuator (via FC/PC) that drives the needle into the tissue to a maximum depth of 10 mm (SmartAct type SLC1730L Linear positioner, SmarAct GmbH., Germany). The actuator is located inside the probe's plastic housing, to which the patch cable is attached (Figure 1.b).

The spectral resolution of the fiber-optic needle probe prototype is >15 cm^{-1}. The depth sensitivity is approximately 100 μm. This was experimentally determined based on the full half-width maximum of the step response (measured in a non-scattering medium).

Figure 1. Fiber-optic needle probe prototype.
a. Complete system. b. Close-up of the fiber-optic needle probe.

10.8.1.2 Tissue sampling and data collection

A Raman section was retrieved as described in the materials and methods of the main article. Raman point measurements were performed with the fiber-optic needle probe prototype from the surface of the Raman section to a depth of ≤3mm with a step size of 0.5 mm (Figure S2, blue arrow). Per step, a Raman spectrum was collected with an exposure time of 0.1 s and an average laser power of ≈80 mW (at the fiber-optic needle probe tip). Each point measurement was 1mm distant from the next measurement (Figure S2, green arrow).

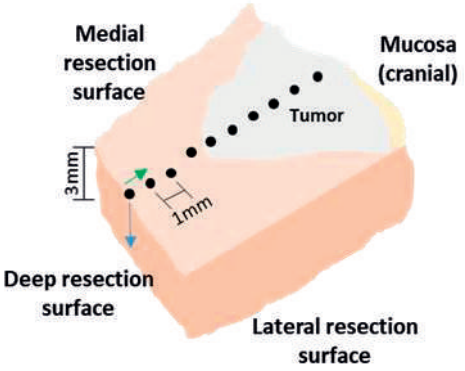

Figure 2. Raman section with point measurements from the resection surface (black dots).
Each point measurement is at 1 mm distance from the next (green arrow). Per point measurement, the fiber-optic needle probe collects Raman spectra from the surface to a depth of ≤3 mm and with a step size of 0.5 mm (blue arrow). Per step, one Raman spectrum is collected with an exposure time of 0.1s.

To allow the control of x and y positioning of the probe, a module for semi-automated scanning of the surface was used. This module contains a holder that grips on the probe housing (Figure 3).

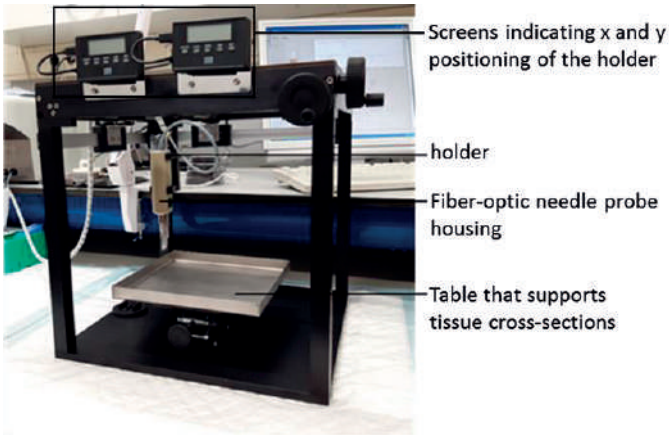

Figure 3. Module for semi-automated scanning of the surface of the Raman section.

After the experiment, the Raman section was added to the main specimen for fixation in formalin, for routine histopathologic evaluation.

10.8.1.3 Histopathology

Histopathologic evaluation of the Raman section was performed as described in the main article.

10.8.1.4 Calibration and pre-processing of spectra

All spectra were corrected for the instrument's wavelength-dependent detection efficiency and calibrated according to the instructions of the spectrometer supplier on the relative wavenumber axis (RiverD). Cosmic ray events were removed, and the signal background generated in the setup's optical path was subtracted (1). MATLAB (Mathworks, Natick, MA, USA) was used for data processing and data visualization. Saturated spectra and spectra with low signal intensity were discarded (less than 5% of the average spectra signal intensity). The intensity was measured within the range 2,700 cm^{-1} and 3,800 cm^{-1}. Low signal intensities were found at positions where the probe tip was not in full contact with the tissue. Fluorescence background signal was determined and subtracted using the multiple regression fitting method developed by *Barroso et al.* (2).

10.8.1.5 Spectral analysis

The analysis of the spectra was performed in MATLAB. Spectral analysis consisted of the extraction of Raman features that are strong discriminators of laryngeal squamous cell carcinoma (LSCC) and non-cancerous tissue.

10.8.1.5.1 Water content

The water content was extracted by calculating the ratio of the signal intensity between the bands at 3390 cm^{-1} and 2935 cm^{-1} for each calibrated and background corrected spectrum according to the method developed by *Caspers et al.* (2001) and described in detail in previous studies (3). A comparison between the frequency distribution of the water content for tumor spectra and for non-cancerous tissue spectra was performed.

10.8.2 Results

Forty Raman point measurements were performed on two Raman sections from 1 laryngectomy specimen (Figure 4). A pin was used to mark the first Raman point measurement, closest to the resection surface.

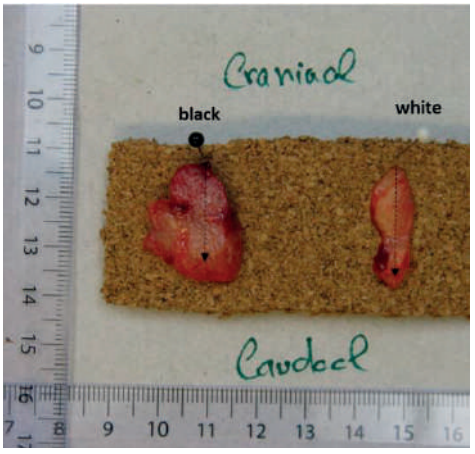

Figure 4. Raman sections: measurements with the needle probe were performed in a line, starting at the pin.

An example of a point measurement in non-cancerous tissue is shown in Figure 5 and an example for tumor is shown in Figure 6.

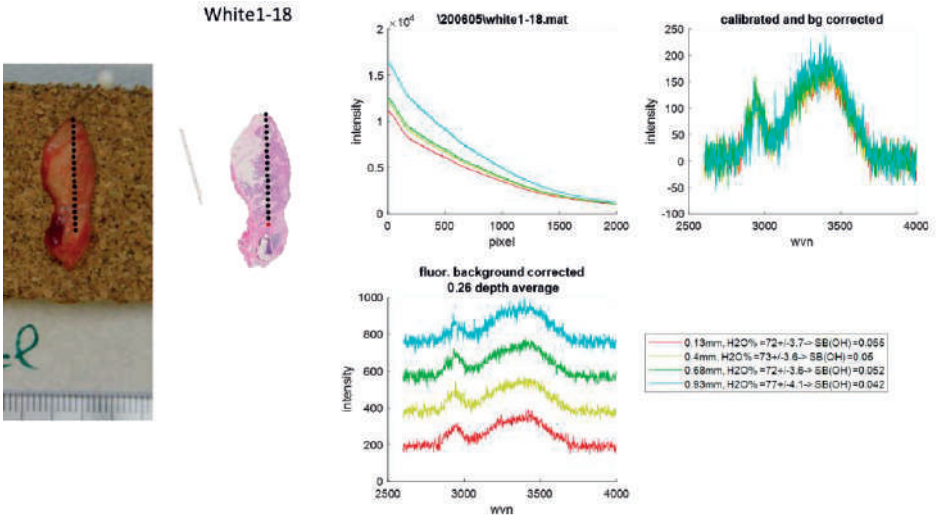

Figure 5. Example of a measurement on non-cancerous laryngeal tissue.

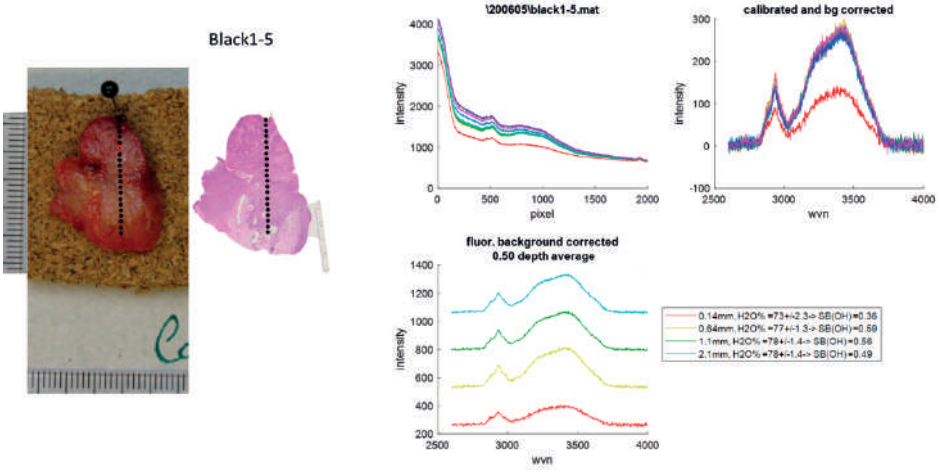

Figure 6. Example of a measurement on laryngeal squamous cell carcinoma.

The histogram of relative frequencies of water content distribution for non-cancerous tissue and tumor is shown in Figure 7. It shows that the water concentrations of non-cancerous tissue are high and highly overlapping with the water concentrations of tumor.

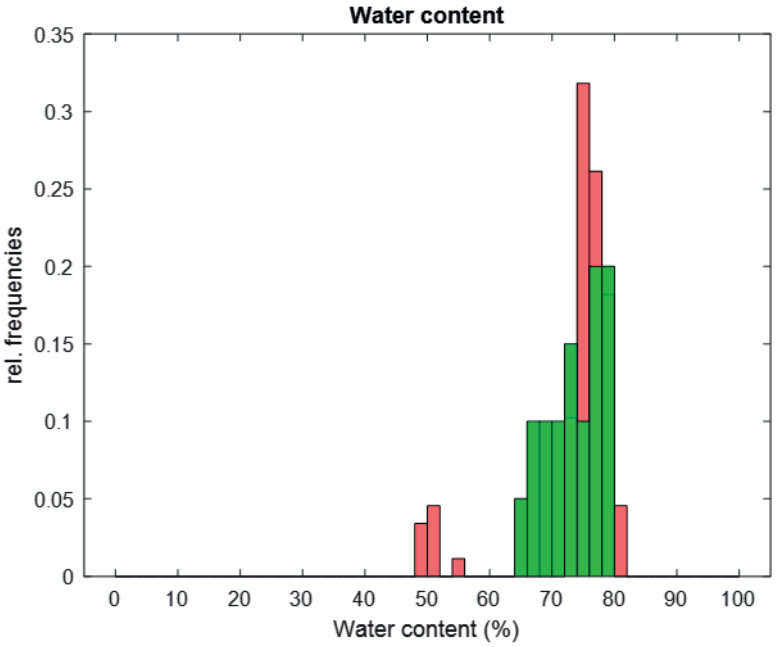

Figure 7. Water concentration distribution for laryngeal non-cancerous tissue (green) and laryngeal tumor (red).

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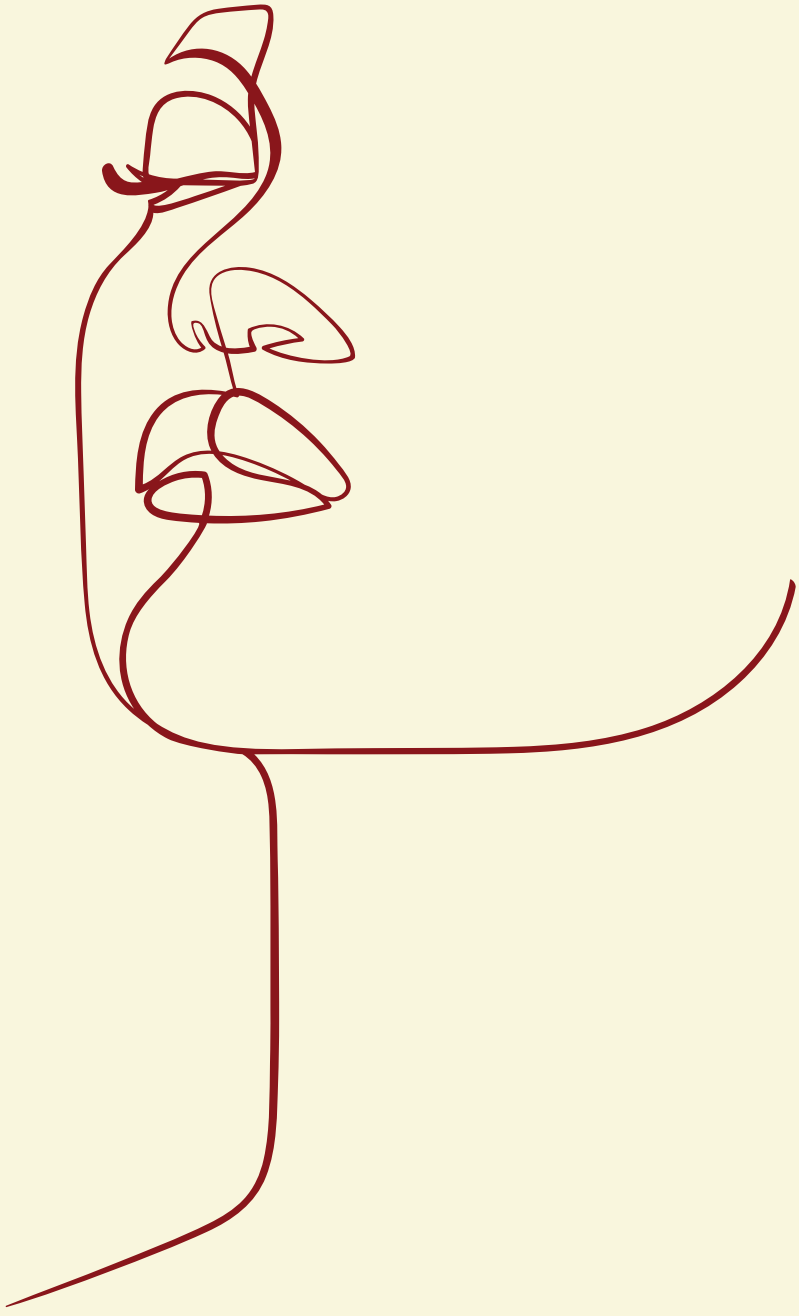

Chapter 11

Discussion - Optical techniques in head and neck cancer

In this thesis, we addressed a significant number of scientific questions and made improvements in various aspects of the surgicopathological workflow, with a particular focus on the intra-operative assessment of resection margins. However, as research progresses, new questions and challenges emerge. In this final chapter, we discuss our findings to enhance surgical treatment and compare them with current research, particularly in the field of optical techniques. We approached this step-by-step:

1. Pre-operative assessment with early and correct diagnosis and identifying prognostic factors.
2. Surgery and intra-operative assessment of resection margins and depth of invasion.
3. Post-operative assessment and treatment.

Finally, we explore the measures required to translate Raman spectroscopy into clinical practice.

11.1 PRE-OPERATIVE ASSESSMENT

Pre-operative assessment is crucial for diagnosis and selecting the appropriate treatment modality. Early and accurate diagnosis allows for timely intervention and offers the best chance of a cure. However, the current gold standard for diagnosis relies on histopathology obtained through tissue biopsy. This assessment is invasive, time-consuming, and prone to sampling errors. In this thesis, we have not explored the possibilities of enhancing this diagnostic assessment. Nevertheless, there is a clear need for an objective and rapid technique to enhance the diagnostic process.

11.1.1 Optical techniques for diagnosis

A comprehensive literature search shows a focus on real-time diagnosis through optical techniques, such as Raman spectroscopy, Elastic scattering spectroscopy, High-Resolution Micro Endoscopy, Narrow Band Imaging, Fluorescence, and Optical Coherence Tomography. Each technique has its own mechanism of action and different modes of data analysis, and all of them provide a real-time, non-invasive, and in situ optical signature using light of varying wavelengths to examine suspicious lesions. They can differentiate between normal and pathological lesions and aid in diagnosis (1).

Raman spectroscopy (RS), as extensively described in the introduction, is based on inelastic scattering of light by molecules in the tissue and provides detailed quantitative and qualitative information about its molecular composition. The technique can be directly applied to tissue since it is non-destructive, and there is no need for reagents or labeling. RS was first applied in the head and neck area by *Stone et al.* in 2000 to analyze laryngeal mucosa *ex vivo* using biopsy specimens. In this study, RS demonstrated a specificity of 90% and sensitivity of 92% for diagnosing invasive cancer (2). Subsequently, *Lau et al.* used a multivariate analysis to determine sensitivities of 89%, 69%, and 88%, and specificities of 86%, 94%, and 94% for normal tissue, carcinoma, and papilloma, respectively (3). Similar results were found in differentiating (pre)malignant oral lesions from normal tissue with a 100% sensitivity and 77% specificity (4). To evaluate RS in detecting premalignant conditions, *Singh et al.* obtained Raman spectra from premalignant, normal, and cancerous sites of the oral mucosa. They were able to differentiate the premalignant conditions based on the differences between the spectra (5). Despite many promising results, there are still hurdles to overcome for clinical adoption. Methods for data mining in RS research need improvement in efficiency to be used for clinical applications.

Elastic scattering spectroscopy (ESS) generates a wavelength spectrum that reflects the basic properties within tissues, such as the nucleus and subcellular organelles, and other components like proteins and lipids. It has been used in studies on premalignant and malignant *ex vivo* oral tissue (6, 7). A study assessing clinically suspicious oral lesions reported a sensitivity of 73% and specificity of 75% (7), while measurements of tumors in the mandible showed a sensitivity of 85% and specificity of 80% (8). For the assessment of tumor-positive lymph nodes, a sensitivity of 98% and specificity of 68% were reported, with false positives in 40% of cases (9). However, ESS has two main limitations. First, there is a scarcity of data on oral lesions, and there is a lack of correlation between optical and surgical methods. Second, formalin fixation can significantly impact the results as it may affect the obtained spectra (8). Consequently, more research needs to be conducted in this area.

High-Resolution Micro Endoscopy (HRME) is performed by placing a flexible fiber-optic probe in direct contact with suspicious mucosal lesions, which is preferentially stained with a fluorescent contrast agent. Illumination occurs using a light-emitting diode transmitted through the fiber-optic bundle. Simultaneously, the emitted light is collected by the probe, with each optical fiber serving as an individual pixel of the image. Hereby, HRME can provide microscopic images of

the cellular architecture of selected tissue in situ and in real-time, achieving a so-called "optical biopsy" (10, 11). HRME is cost-effective, non-invasive, and different sized scopes can be used for specific areas of interest. When compared with frozen and paraffin sections, HRME detects dysplastic mucosa with a sensitivity of 95% and specificity of 90% (12). With proper training, it can even achieve 98% sensitivity and 92% specificity (13). Several *ex vivo* studies have been published on the diagnostic value of HRME for identifying head and neck squamous cell carcinoma (SCC). *Vila et al.* conducted a study (14) that reported excellent sensitivity, specificity, and inter-rater reliability for HRME images (98%, 92%, and 84%, respectively). *Miles et al.* correlated HRME images obtained during surgery to differentiate between benign and malignant mucosal lesions. The mean accuracy, sensitivity, and specificity were 95.1%, 96%, and 95%, respectively (15). However, the HRME system has limitations, as it can only penetrate to a maximum depth of 50 μm, and keratinization can cause background artifacts (12, 16). Thus, HRME imaging is limited to the superficial mucosa and is unable to inspect submucosal tumors or tumor spread (10, 17). Additionally, the field-of-view of HRME is limited by the diameter of the bundle, which can cause sampling errors (10).

Narrow Band Imaging (NBI) is a high-resolution endoscopic technique that uses blue and green light to increase tissue contrast by identifying superficial capillaries and neoangiogenesis in abnormal tissue. There is no need for dyes, and it can target areas with suspicious vascular morphology for biopsy examination. The oral mucosa has clear branching vessels in the subepithelial layer, which appear cyan, and the capillaries in the epithelial layer appear brown. Abnormal vessels show brownish dots on dilation, and they weave and differ in shape, losing their regular capillary arrangement (18, 19). NBI seems to be a promising technique in the detection of laryngeal, pharyngeal, and oral malignancies. In 2013, *Li et al.* (20) performed a meta-analysis utilizing NBI in the evaluation of mucosal and sub-mucosal malignant lesions in the head and neck region. The overall sensitivity (90% vs. 62%), specificity (97% vs. 85%), and accuracy (98% vs. 89%) of NBI were superior to white-light imaging. Subgroup analysis based on the anatomical subsites also achieved similar results (21 - 23). A correlation was found between the T stage and changes in the intracapillary loops. If they showed angiogenesis and destruction, there was a greater likelihood of perineural and lymphovascular invasion. This confirmed that vascularity increases substantially as the disease progresses (24). Additionally, NBI can detect unknown primaries in patients with cervical lymphadenopathy. *Shinozaki et al.* found that 16 of 26 SCC lesions of the head and neck were detected using NBI (25). Some unknown primary tumors are difficult to detect because of the complex anatomy of the region. *Masaki et al.* used

NBI to locate these primary lesions and detected only 13.3% of primaries, whereas positron emission tomography (PET) detected up to 20% (26). The limitation of NBI is that it can detect only superficial lesions. The penetration depth of blue and green light is respectively 170 μm and 240 μm. This could limit the evaluation of some lesions in the oral cavity and oropharyngeal subsites, where the mean mucosal thickness measures up to 1300 μm (27). NBI is designed for recognizing neoangiogenesis patterns, so scenarios with modified micro vascularity, such as previous radiation or surgery, inflammation, and vascular lesions, can lead to false-positive results (28).

Fluorescence imaging (FI) is based on the illumination light from a filtered light source (low-intensity excitation) or laser (high-intensity excitation) that travels through tissue and is absorbed by targeted fluorophores, which can either be endogenous (i.e., autofluorescence) or exogenous (i.e., injected fluorescein). The re-emitted light can be detected and provide a real-time fluorescence color image (29). Autofluorescence can be detected due to endogenous fluorophores, such as structural proteins. The endogenous fluorophores appear green when excited by light (30, 31). Precancerous and malignant lesions appear as red-violet due to an altered metabolism in tumor cells and a loss of fluorescent signal resulting from epithelial thickening (30, 32). The diagnostic value of autofluorescence imaging (AFI) devices in oral SCC has been widely studied. A meta-analysis determined the accuracy for the diagnosis of oral SCC and/or dysplasia (33). The mean sensitivity and specificity were 72.4% and 63.79%, respectively. However, the values of sensitivity ranged from 20% to 100%, and specificity ranged from 15.3% to 100%, according to the included studies. *Huff et al.* investigated whether combining AFI with conventional oral examination could detect more oral abnormalities. The results suggested that the combination yielded more mucosal abnormalities than the conventional method alone (1.3% vs. 0.83%), with 83% of these being histopathology-confirmed premalignant disease (34). Similar findings were reported by *Truelove et al.,* adding AFI to conventional oral examination improved the detection of oral premalignancies, which were missed by conventional examination alone (35). The diagnostic value of AFI devices in laryngeal cancer has been widely investigated. A meta-analysis reported that the sensitivity (91% vs. 73%), specificity (84% vs. 79%), and accuracy (88% vs. 77%) of AFI were superior to white-light imaging alone (30). Even though the AFI modalities are regarded as practical, cost-effective, and non-invasive, they have the disadvantage of low specificity (31, 32, 36). The false positives are related to tissues with rich micro vascularity causing scattering and autofluorescence loss, seen in granulation tissue, inflammation, and edema. False

negatives are also observed in regions with overgrowth of bacteria (bacteria may produce extra fluorophores) or hyperkeratosis (keratin is strongly fluorescing).

Optical coherence tomography (OCT) uses infrared light and records reflections below the surface to produce an image of tissue, including the basement membrane, epithelial and subepithelial layers, and micro anatomical structures (37). In an *in vivo* study, there was close agreement between the histological findings and the images obtained, with sensitivity and specificity ranging between 80% and 98% (38). Another *in vivo* study reported sensitivity and specificity rates of 100% for the detection of oral SCC, compared to 93% and 69% for dysplastic lesions, respectively (39). A systematic review in 2018 found that OCT was able to distinguish between benign and (pre)malignant oral lesions with high accuracy (40). The status of the basal membrane was the most important parameter for differentiating SCC from dysplasia or benign lesions (41). The basal membrane is also an important parameter in laryngeal cancer. In a healthy larynx, OCT can identify the different layers. However, in laryngeal cancer, the boundary of the basal membrane is lost, leading to unrecognizable structures, which hampers identification and diagnosis (42). For *ex vivo* studies, the results are less promising. *Jerjes et al.* reported that changes in oral lesions could be identified, but OCT could not differentiate between the lesions (37). OCT is still far from implementation in everyday clinical practice. A limitation is that it cannot differentiate between malignancy and inflammation (43). Also, there is no classification to consistently diagnose lesions, and results still need to be validated (43). Finally, there can be considerable variation between observers (43, 44).

11.1.2 Assessment of prognostic factors

Treatment is based on the diagnosis in the pre-operative stage. Unfortunately, not many prognostic factors can be determined during this phase. In this thesis, we focused on two prognostic factors: resection margins and depth of invasion (DOI). Resection margins cannot be assessed pre-operatively, and therefore, often a second surgery and/ or (chemo) radiotherapy is needed. Also, DOI is usually determined days after the initial surgery based on final pathological evaluation. Hence, in some cases, a second surgery is needed for an extended neck dissection (END). While it is known that DOI can be estimated from pre-operative imaging, an analysis of measurement variations according to the imaging modality and the depth of the tumor itself is lacking. The accuracy of imaging-based estimation of DOI in relation to the tumor's histological DOI is being investigated. *Waech et al.* reported that CT and MRI measurements of DOI lead to an overestimation of the histological DOI, especially in tumors with DOI <5 mm, with upstaging by imag-

ing in over 50% of the cases (45). *Baba et al.* reported that DOI on MRI and CT strongly correlates with the pathological DOI. However, the radiological DOI is often 2-3 mm larger than the pathological DOI (46). Subsequently, they compared DOI determined on an MRI to DOI on a contrast-enhanced CT scan (CECT). DOI determined on a CECT correlates and is better approximated to the pathological DOI. The authors concluded that CECT could be useful for pre-operative staging of patients with tongue SCC (47). Most studies agree; DOI can be measured with a pre-operative MRI but is not accurate for tumors with DOI <5 mm (48 - 51).

11.2 SURGERY AND INTRA-OPERATIVE ASSESSMENT

Intra-operative assessment (IOA) can be of great value for patient outcome. The assessment can be used to measure resection margins and DOI during the initial surgery to achieve complete treatment. In this thesis, we focused on the IOA of resection margins. Our group recently published a review on the performance of IOA. The authors concluded that the sampling and interpretation error are higher for defect-driven IOA of resection margins when compared with specimen-driven IOA (52). They also showed that there is a low number of studies on the performance of IOA available. However, with the upcoming awareness of the need for IOA of resection margins, there will be enough evidence in the literature to perform a thorough systematic review/meta-analysis in the near future (52).

11.2.1 Implementation and evaluation of intra-operative assessment

Based on the findings from our research group, the pathologists at Erasmus MC have adopted an extended specimen-driven IOA of resection margins, including reporting on the exact margin and its extent (e.g., "resection margin posterior is 2.1 mm, over a distance of 2 cm"). To enable colleagues at other institutes to implement this specimen-driven IOA of resection margins, our research group published the protocol of this method in detail (Figure 1) (53). Additionally, we implemented a bi-weekly multidisciplinary meeting to discuss all patients postoperatively. Multiple studies have reported an association between multidisciplinary counseling and improved survival, as summarized in the systematic review by *Hong et al.* (54). *Nguyen et al.* (55) state that following the recommendations of a multidisciplinary tumor board provides optimal care for patients with locally advanced head and neck cancer. The postoperative multidisciplinary discussion and detailed pathological workup will provide the advantage of collecting data prospectively.

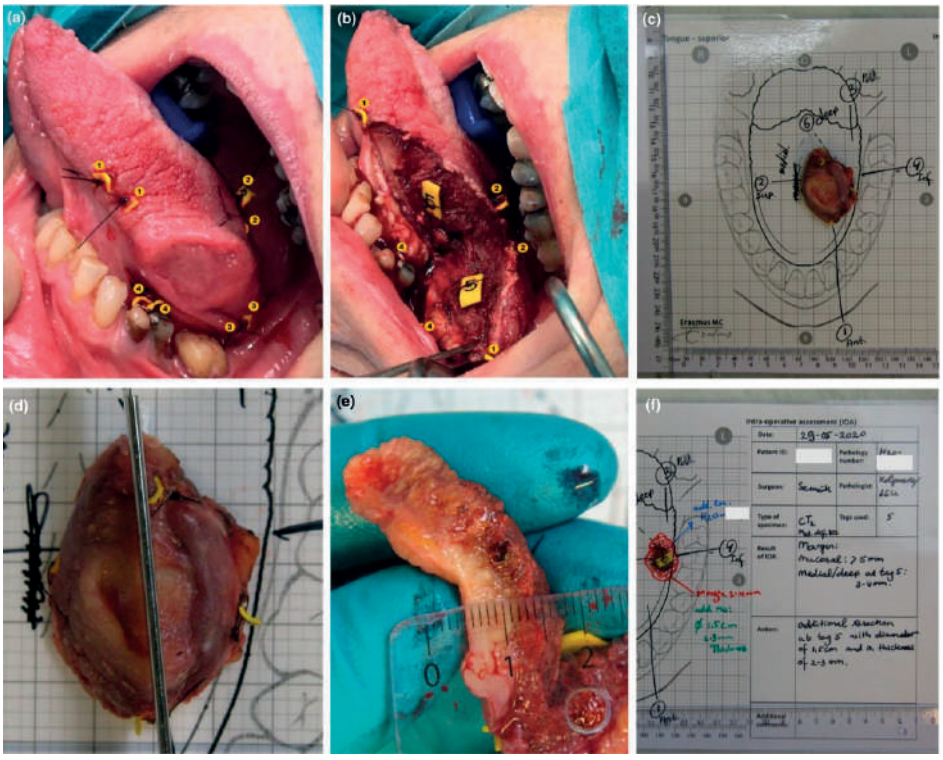

Figure 1. Illustration of the intra-operative assessment of resection margins including the relocation method.

(a) The surgeon attaches numbered tags in a pair-wise manner on both sides of the resection line, superficial and deep during the resection. (b) After the tumor resection has been completed, one numbered tag of each pair is attached to the specimen and the other tag remains in the wound bed. (c) Anatomical template of the tongue with the specimen, patient information, and the annotated tags. These templates have been designed to facilitate the preservation of anatomical orientation of the specimen during the IOARM. (d) The pathologist and surgeon inspect and palpate the specimen for suspicious areas (i.e., areas where margin might be less than ≤ 5 mm). If a suspicious area is found, the pathologist makes one or more parallel incisions perpendicular to the resection surface (with a mutual distance of 5–6 mm). This enables the visualization and measurement of the margin. (e) Measuring the margin with a ruler. If an inadequate margin is detected, its location is indicated based on the numbered tags. Advice is given for an additional resection in the indicated area, including the thickness. (f) Result of IOA of resection margins (e.g., *at the location of tag nr. 5, the margin is 3–4 mm*) is recorded at the template, together with the recommendation for additional resection (e.g., *area of tissue enclosing tag 5, with a diameter of 1.5 cm and the thickness 3–4 mm*)

Unfortunately, there are several possible sources of bias for specimen-driven IOA of resection margins apart from relocation. During surgery, it can become evident that achieving adequate resection margins is virtually impossible due to the close proximity of vital structures. Although pre-operative planning is of essential importance, it does not always reflect the intra-operative situation. In recent years, there has been much debate about adequate resection margins for OCSCC (56). There is evidence that >5 mm margins improve patient outcome (57 - 59). Yet, several authors suggest that margins between 2–3 mm could be sufficient without

negatively influencing patient outcome (60 - 62). *Nason et al., Binahmed et al.*, and *Pathak et al.* (60, 63, 64) state that survival improves with each additional millimeter of clear resection margin and propose a minimum margin of 3 mm to be considered an adequate resection margin. *Zanoni et al.* (61) showed that for tongue cancer, resection margins between 2.2 and 5 mm have no greater risk for local recurrence than margins of >5 mm. *Jang et al.* (62) reported no effect of margin status on local recurrence, except for small (<3 mm diameter) tumors, as did *Barry et al.* (65) for T1/T2 tumors. *Dik et al.* (66) concluded that a margin of 3 mm with ≤2 other adverse histological features is as safe as a margin of 5 mm in relation to local recurrence. Another recent study by *Buchakijan et al.* showed that only a margin of <1 mm was associated with an increased risk of local recurrence (67). *Brinkman et al.* found 3 mm for demarcation between close and clear, and yielded a good separation between survival curves of clear (≥3 mm), close (1-<3 mm), or involved (<1 mm) margins. They conclude that a 3 mm margin is significantly associated with survival in oral cavity SCC and may be useful for demarcation between close and clear (68). However, the evidence is still scarce and insufficient to alter the guidelines. A meta-analysis must be performed to reach a consensus on the definition of clear and close resection margins for oral cavity SCC.

In the larynx and hypopharynx, there seems to be consensus that resection margins of 1-5 mm can be accepted (69). The anatomy here is complex, and resection margins are limited by surrounding critical structures like skin, prevertebral space, and vital neurovascular structures. Although a margin of >5 mm (according to the RCP) is the goal, the question is whether this margin is always feasible or even necessary. An extensive literature search in Medline, Embase, and the Cochrane Collaboration showed a lack of studies regarding the clinical relevance of resection margins in the larynx and hypopharynx. However, the clinical relevance of resection margins for laser surgery in early glottis carcinoma is more extensively researched. Studies propose a margin of 2-5 mm as adequate for this subsite of the larynx (70 - 75).

11.2.2 Optical techniques for assessment of resection margins

Intra-operative assessment still has limitations, such as being time-consuming and having sampling errors. Optical techniques may offer a solution.

Hamdoon et al. tested OCT in the assessment of oral SCC resection margins (43). They identified tumor-positive margins by architectural changes and an increased epithelial thickness on OCT images. The sensitivity was 81.5%, and specificity was 87%. However, the technology is only useful for assessing peripheral margins.

Vicini et al. (76) evaluated the use of NBI in patients with head and neck SCC who underwent surgery. Frozen section analysis of surgical margins revealed a significantly lower rate of positive superficial margins in the NBI group compared with the white-light imaging group (12.1% vs. 42.1%). *Tirelli et al.* reported a sensitivity and specificity of NBI in assessing margins in oral cancer of 100% and 88.9%, respectively (77). *Sifrer et al.* evaluated IOA of margins by NBI (78). The rate of a complete resection in the NBI group and the control group was 88.9% and 70.9% ($p = 0.047$), whereas the ratio of histopathological negative margins was 95.9% and 88.4%, respectively ($p = 0.017$). A systematic review reported that, in oral and oropharyngeal cancer surgery, the use of NBI was associated with significantly fewer positive superficial margins than in the control group. However, the resection extent was sometimes mistakenly increased due to non-optimal specificity (79). A systematic review on the use of NBI in laser surgery of laryngeal carcinoma reported a significant reduction of positive superficial margins ($P < 0.05$) (80).

Plaat et al. examined patients who underwent laser surgery for early glottic cancer using white light with or without NBI. For patients treated by laser surgery on white light alone, 24% developed local recurrences, and only 2% in the NBI group ($P < 0.01$). Two-year recurrence-free survival was 82% in the white light group and 98% in the NBI group ($P < 0.05$) (81). Similar findings were reported by *Garofolo et al.* in early glottic carcinoma (82). However, this technique is only useful for mucosal margins (83).

Intra-operative *in vivo* FI of head and neck cancer is complex because most tumors cannot be evenly illuminated due to the difficult anatomical area in which they are located. Therefore, margin analysis is performed *ex vivo* on the excised specimen using camera systems that can be used in the operating room. The strategy for targeted FI can be combined with frozen section analysis of fluorescent spots identified on the resection specimen. Several targeted tracers show potential for intra-operative fluorescent margin assessment, although larger trials showing positive predictive values and better local control are currently lacking (84). AFI can be used to delineate surgical margins in oral lesions. *Poh et al.* found that surgical margins needed to be extended by up to 2.5 cm when compared with examination using white light alone (85). *Baek et al.* detected tumor-positive margins immediately after excision using fluorescence-guided intra-operative imaging (86). As the penetrating depth of AFI illumination is relatively shallow, AFI is best suited to evaluate only superficial margins.

One study was found on HRME, allowing real-time analysis of tissues, enabling informed decisions as to resection margins plus reduced thermal and orientation artifacts. However, there have been no studies investigating this capability, possibly because of the narrow field-of-view (12, 16).

The above-mentioned optical techniques might be promising for real-time assessment of resection margins. However, they are only suitable for superficial mucosal margins and cannot be applied to the submucosal layers of soft tissues. It is described that 87% of inadequate margins are found in the deeper (submucosal) layers (87).

Our research group focused on RS. In 2015, *Barroso et al.* could discriminate between oral cancer and healthy tissue based on water concentration (sensitivity 99%, specificity 92%) (88). In the third study of this thesis, we investigated how water concentration changes across the tumor border toward the healthy surrounding tissue. We found a transition from a high to a lower water concentration, from tumor (76% ± 8% of water) towards healthy surrounding tissue (54% ± 24% of water), over 4 to 6 mm across the tumor border. The water concentration distributions between the regions were significantly different ($P < 0.0001$). These results show a clear correlation between the tumor border and changes in water concentration (89).

In advanced oral cavity SCC, resections of the tumor can also comprise of a mandibulectomy. In contrast to soft tissue, for bone resection margins, no standard technique can be used intra-operatively for margin assessment (90). RS also shows promising results for bone assessment. In a study by *Barroso et al.,* RS was used to investigate the water concentration of tumor located in the bone. An average water concentration of 77% ± 6% was found. This is similar to the results found for soft tissue in their previous study. The average water concentration of healthy bone is 44%, which is lower than that of soft tissue (54%) (89). These results could improve the number of adequate resection margins and reduce the extent of adjuvant treatment. Equally important, having a method for IOA of bone resection margins might give the surgeons the confidence to resect the bone closer to the suspected tumor border. In this way, healthy bone can be spared, which will improve the reconstruction and reduce post-operative complications (90).

In head and neck oncological surgery, resection margins are also critical for laryngeal cancer. Yet, achieving adequate margins is difficult and sometimes not possible, due to the complex anatomy of the larynx. The ninth chapter of this thesis

investigated whether Raman spectroscopy could be used to discriminate laryngeal SCC from non-cancerous tissue. Both laryngeal cancer and all non-cancerous tissue structures showed high water concentrations of around 75%. Discriminative information was only found to be present in the CH-stretching region of the Raman spectra of the larynx (discriminative power of 0.87). Further research is needed to extend the dataset with a larger cohort of patients to better capture the variation in spectra, within and between patients (91).

11.2.3 Depth of invasion

Depth of invasion is regarded as the best predictor for (occult) lymph node metastasis (LNM) (92, 93). Unfortunately, in current practice, DOI is determined only days after initial surgery based on final pathological evaluation. This can lead to the need for a second surgery for an END in some cases. Many authors, including us, base their decision on END according to a 20% risk (NPV 80%) of occult LNM (94 - 99). This cut-off value of 20% is based on the publication by *Weiss et al.* in 1994 (100), which considered the side effects of surgery (END) and radiotherapy at that time. However, it is reasonable to assume that treatment modalities have substantially improved in the almost 30 years since that study. Therefore, we suggest considering a risk lower than 20% when deciding on END. A new study is needed to determine the morbidity of surgery versus radiotherapy. This consideration and decision can also be discussed with the patient, providing clear information on both the side effects of END and the risk of occult LNM (101).

To date, there is no reliable method for estimating DOI at the time of initial surgery (102). Intra-operative measurement of DOI for early-stage oral cavity SCC with a cN0 status could be of great value. This would enable the surgeon to perform an END at the initial surgery if DOI is ≥4 mm. Only three studies have analyzed the accuracy of frozen section analyses for measuring DOI intra-operatively, and all of them report a high correlation between the measurement on frozen section and H&E-stained section (103 - 105). A specimen-driven IOA of the resection margins, recommended as the standard of care by the AJCC 8th edition (93), could easily include DOI measurements. However, there are logistical downsides, such as reserved OR time for END that might not always be performed, and it might not be feasible in hospitals lacking the necessary facilities.

Another option to identify occult LNM at the initial surgery is sentinel lymph node biopsy (SLNB). According to the NCCN guidelines, SLNB, if available with technical expertise and experience, is the best predictor for LNM and should guide decision-making on END (92). SLNB has reported sentinel lymph node detection

rates of 95% and sensitivity of 0.93 with a NPV of 0.88-1 (102, 106 - 110). Studies suggest that SLNB-navigated neck dissection may replace END without a survival disadvantage and reduce postoperative neck disability (111). However, SLNB might not be suitable for certain cases, such as floor of the mouth tumors, or tumors at the upper gingiva and hard palate. Careful planning and perseverance are required for establishing SLNB-guided neck dissection (106, 112, 113).

Both IOA of DOI and SLNB frozen section have limitations in terms of logistics, time consumption, and planning. Recent studies have evaluated the value of molecular tumor biomarkers to optimize neck strategy selection criteria. The expression of cortactin has shown promise as an immunohistochemical tumor marker to identify low-risk patients who may not benefit from SLNB or END (114). Another study by *de Herdt et al.* investigated whether the receptor tyrosine kinase MET positivity is associated with LNM in early tongue SCC and found it to outperform DOI >4 mm in predicting LNM (115).

Raman spectroscopy shows promising results in differentiating tumor from healthy surrounding tissue in the oral cavity. Currently, measurements are being analyzed from healthy lymph nodes and lymph nodes containing tumor, with the aim of improving IOA of occult LNM and clinical decision-making on treatment of the neck.

11.3 POST-OPERATIVE ASSESSMENT AND ADJUVANT TREATMENT

The post-operative assessment involves the final pathological evaluation of the resection specimen. The histopathology report provides information on tumor characteristics, such as the type of tumor, differentiation grade, diameter, and invasion to surrounding tissues like bone invasion, perineural growth, and lymphovascular invasion. Additionally, the status of resection margins is mentioned. Unlike tumor characteristics, resection margins can be influenced by the surgeon and pathologist due to IOA.

Adjuvant treatment options include surgery, radiotherapy, or chemotherapy, and are based on resection margin status, DOI, or other tumor characteristics. In cases of inadequate margins in a primary closed wound bed, a second surgery in the form of re-excision may be performed. Intra-operative assessment of resection margins becomes valuable in such situations, as discussed above.

Post-operative radiotherapy (PORT) is recommended for advanced tumor stages (T3/T4), positive or close resection margins, extra-nodal extension (ENE), two or more pathologically positive lymph nodes, perineural invasion, and lymphovascular invasion (92). During the implantation of the relocation method, surgeons express the desire for tags that can be detected and identified by CT or MRI to aid in adjuvant radiotherapy. This would enable more accurate targeting of the desired area for radiation. Developing biocompatible or soluble and radio-opaque tags requires considering various factors, including their effects on the targeted volume, toxicity, and patient outcome, which calls for collaboration between surgeons, pathologists, and radiotherapists.

The recommendation for PORT in relation to margin status is currently debated. A positive margin (<1 mm) is an absolute indication for PORT. However, there is no consensus on when to indicate PORT in cases of a close margin. Literature reports different locoregional control and long-term survival rates after combined surgery and PORT versus surgery alone (116 - 119). The benefits of combined therapy should be weighed against the potential long-term side effects of PORT, such as xerostomia and osteoradionecrosis (119, 120). Some authors advocate a watch-and-wait approach after surgery before deciding on PORT (119, 121, 122). *Dik et al.* compared the impact of re-resection, PORT and watchful waiting. They found no evidence of benefit for any local adjuvant therapy in cases where the margin was 3 mm with only one or two more adverse histological features (66). *Welinder et al.* conducted a study to determine if PORT affected disease-free survival in patients with oral SCC and close surgical margins. They found no significant difference in disease-free survival between surgery-plus-RT vs. surgery-only ($p = 0.72$). Their findings support the trend toward a watch-and-wait approach (123). However, there is a need for prospective, randomized, controlled trials to determine whether adherence to current guidelines regarding PORT for close margins has only a minor impact on patient outcome. Until such trials are conducted, a watch-and-wait approach regarding PORT for patients with close margins can be discussed with the patient, surgeon, and radiotherapist.

Finally, concurrent chemotherapy with Cisplatin is recommended with PORT for patients with positive margins and extra nodal extension. This combination has shown to improve local control and disease-free survival compared to PORT alone based on landmark studies (124, 125). However, chemotherapy is a systemic therapy and cannot be influenced by the surgeon or pathologist.

Currently, imaging techniques, as described above, do not have a place in post-operative assessment or adjuvant treatment.

11.4 RAMAN SPECTROSCOPY – TRANSLATION TO THE CLINICS

This thesis emphasizes that improving resection margin status through intra-operative assessment is the key focus in head and neck oncological surgery.

Specimen-driven IOA of resection margins has shown promise, but it does add extra time to the surgical procedure. On average, it takes about 30 minutes, including transferring the specimen to the pathology department. In some cases, the surgical procedure must be put on hold until the IOA of resection margins results are known. Moreover, IOA of resection margins remains subjective and limited in the number of incisions that can be placed on the resected specimen without interfering with final histopathological evaluation.

It is important to be open to innovative modalities with the goals of further improving accuracy and enabling more widespread implementation. Raman spectroscopy is the most promising optical technique to fill this gap, enabling objective inspection of the entire resection surface (52). In recent years, major technological advances in Raman spectroscopic systems for clinical applications have been made in detector technology, fiber-optic probe design, combination with other techniques, and new laser opportunities for low-cost systems (126).

Currently, at the Erasmus MC Cancer Institute, RS is implemented in a prototype instrument employing a fiber-optic needle probe (Figure 2) (127). This fiber-optic needle is inserted into the specimen, from the resection surface toward the tumor. Based on the Raman spectra collected 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. By performing multiple insertions on the resection specimen, a complete assessment of all resection margins is possible. This prototype demonstrates the feasibility of an easy-to-use device. Data analysis can take place in real-time and within a short period of time, while the patient is still in the operating room. Moreover, the measurements could be carried out in, or close to, the operating room and would not necessarily be conducted by a pathologist (128). The mean absolute error in the margin length prediction by the prototype was <1 mm. This is within the estimated 1 mm uncertainty in the gold standard

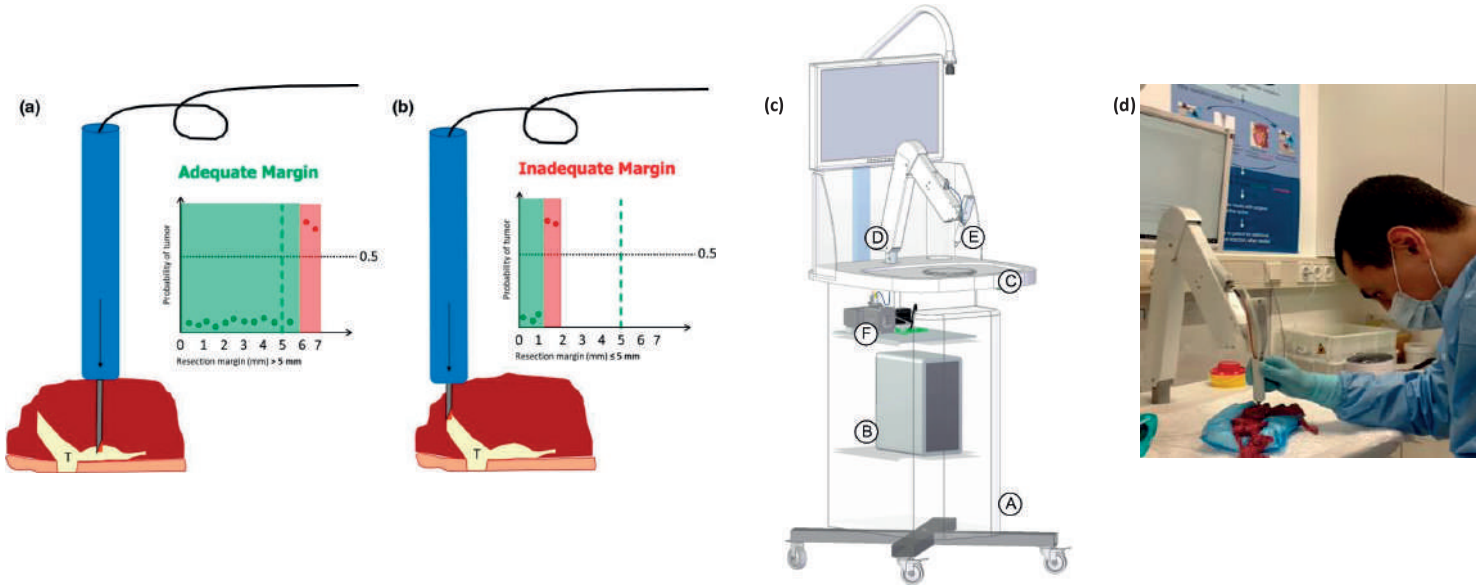

Figure 2. Illustration of specimen-driven intra-operative assessment of resection margins based on Raman spectroscopy.
The fiber-optic needle determines the resection margin as a distance between the resection surface and tumor border. The fiber-optic needle is driven into the specimen, from the resection surface toward the tumor border. Raman spectra are collected along the insertion path. In the graphs, each measurement is presented as a dot; the *x*-axis shows the measured resection margin, and the *y*-axis shows the probability of individual measurements to be classified as tumor or not. (a) Example of adequate margin (6 mm). (b) Example of inadequate margin (1.5 mm), an additional resection is needed. (c) Main components of the device. (d) Positioning of the fiber-optic needle probe on a specimen.

histological margin length assessment. Moreover, the prototype showed no positive or negative bias in margin length with respect to histology. Consequently, it may be expected that the prototype will indicate adequate and inadequate margins with high accuracy. This is confirmed by a test on 180 profiles of the margin length prediction validation dataset. Both the tissue classification model and the margin length prediction model, although validated on independent datasets, are still based on data of limited sizes. Therefore, it is expected that they can be further improved.

The next step towards the implementation of the technology is the development of a measurement protocol for systematic IOARM and result reporting. It appears feasible to determine the resection margins on a specimen at up to 100 locations within an acceptable time frame of 15 minutes, which is currently not achievable in any other way (128).

In the past, a disadvantage of RS was the low measurement speed. One way to overcome this is to complement RS with other optical techniques, such as autofluorescence imaging (129). For instance, *Kong et al.* and *McGregor et al.* have used AFI to quickly scan large areas of tissue for the selection of the measurement locations for RS, thereby reducing the time spent on redundant or non-relevant Raman measurements (130, 131). We also started a project for oral cavity surgery guidance based on these two optical techniques. The combination of these techniques is being developed to allow for a rapid and accurate specimen-driven IOA of all resection surfaces that will fit into the surgico-pathological workflow.

The number of scientific groups working towards the implementation of Raman spectroscopy devices in clinical procedures is growing. The major requirements for translating Raman equipment into clinical practice are a well-defined clinical need, proven patient benefits and/or cost-effectiveness, equipment changes that impact clinical practice, demonstrated safety, reproducibility, robustness, and reliability (e.g., through large clinical trials), and regulatory approval. However, most of the studies (89%) still take place at the same technology readiness level (TRL-4, or class B). In these studies, algorithms have been developed for the detection of (pre-) malignant tissue based on *in vivo* or *ex vivo* measurements, under conditions that approach the intended clinical environment. Validation of these algorithms based on large independent datasets obtained in the actual clinical workflow needs to take place (126).

There are still problems that need to be solved to successfully bring the technique to end-users in the hospital setting. Companies developing commercial Raman spectroscopy applications have indicated several hurdles when translating Raman systems into the clinics. One important issue can be ensuring that high-quality data can be acquired consistently and robustly under intra-operative conditions, with minimal disruption of the surgical workflow. It is also essential to ensure that real-time high-accuracy tissue classification is provided to surgeons in an intuitive way and using simple metrics (user-friendliness). The fact that Raman spectroscopy is still a relatively unknown technique for most clinicians was another hurdle mentioned. Approximately 30% of the reviewed studies are published in medical journals. To raise awareness and increase acceptance of Raman technology results, Raman studies should be presented at medical conferences and published in medical journals. It can also be beneficial to incorporate this technique, and other optical techniques, in the curriculum of clinicians or clinical researchers (126).

Better communication between clinicians and spectroscopists will facilitate understanding of the clinical requirements and challenges. The gap between technology developers and clinicians is narrowing due to the contribution of multidisciplinary networks like the International Society for Clinical Spectroscopy (ClirSpec), European Photonics Industry Consortium (EPIC), and Raman4Clinics. These networks target especially the translational aspects and actively pursue standardization of sample preparation, measurements, data analysis protocols, which will help form a basis for transferability and thereby accelerate development (126).

Although Raman spectroscopy is a proven technology, considerable improvements in instrumentation are still needed to enable further development towards clinical applications.

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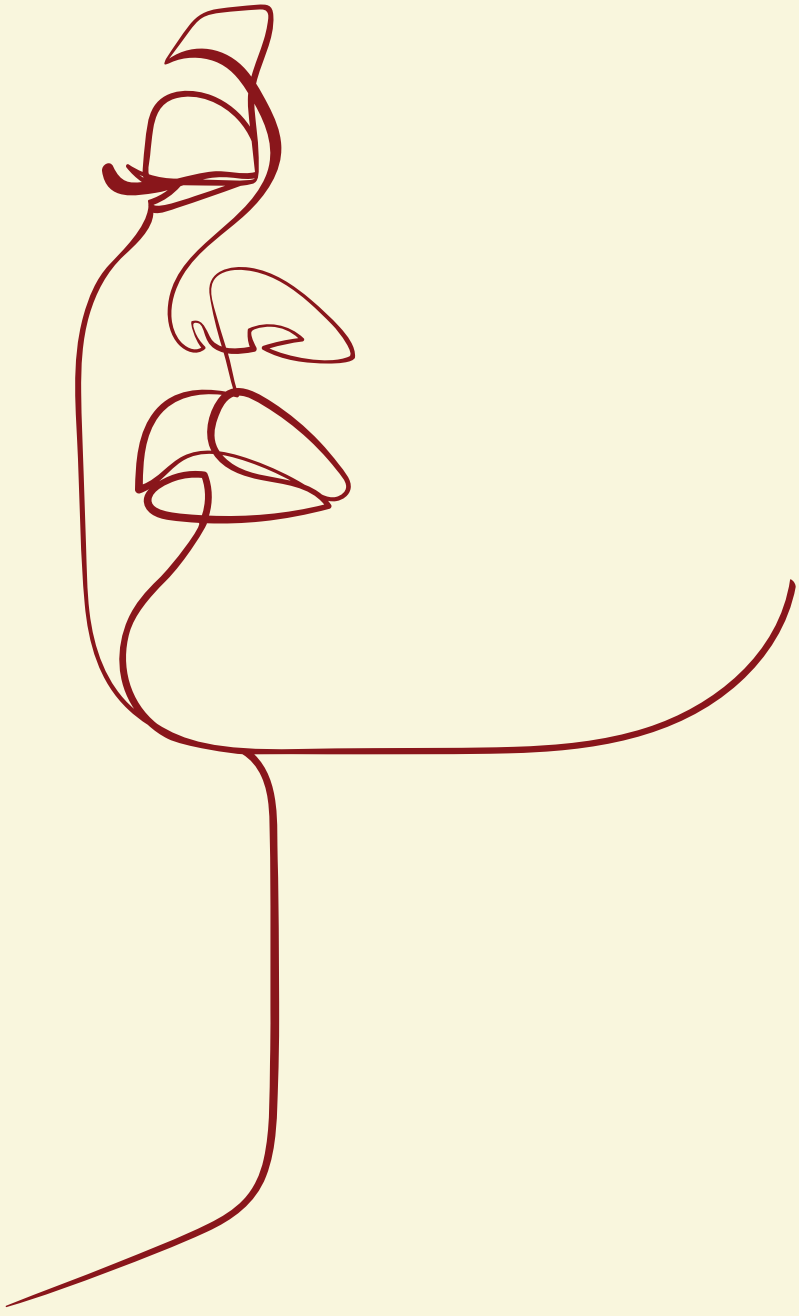


Chapter 12

Summary / samenvatting

SUMMARY

Head and neck cancer ranks the 6th most common malignancy worldwide, significantly affecting both life expectancy and quality of life. The outcome of these patients is influenced by various factors related to both the patient and the tumor itself, but only the radicality of the surgery can be influenced by the surgeon. The status of the resection margins can serve as a proxy for the radicality of surgery.

The Royal College of Pathologists (RCP) defines resection margins for head and neck cancer as: clear (>5 mm), close (1-5 mm), and positive (<1 mm). Achieving clear margins is crucial to reduce the risk of local recurrence. To assist surgeons in achieving adequate resections (i.e., clear margins), specimen-driven intraoperative assessment (IOA) is strongly recommended.

Another crucial histopathological factor is the depth of invasion (DOI), which is considered an independent predictor for lymph node metastasis (LNM) in early-stage oral cavity cancer. During histopathological assessment of the resected specimen, DOI is measured from the level of the basement membrane of the adjacent normal mucosa. For cases where DOI is ≥4 mm, an elective neck dissection (END) is recommended. If DOI can be determined pre- or intra-operatively, it may impact the decision of whether to perform an END in cN0 patients. Our hypothesis is that IOA, including resection margins and DOI, can lead to improved outcome in oral cancer surgery.

In a previous retrospective study, we showed that 85% of oral cavity cancer patients had inadequate (i.e., <5 mm) resection margins, a finding that aligns with similarly high numbers reported in the existing literature. In **Chapter 2**, we delved into the current practices IOA for oral squamous cell carcinoma (OCSCC) at the Erasmus MC. We conducted a comparative analysis of two patient cohorts: one treated between 2010 and 2012, when IOA was not yet the standard of care, and another treated between 2013 and 2017, where IOA had become the standard. We scrutinized the frequency, type (specimen-driven vs. defect-driven assessment), and outcomes of the IOA. We observed a significant increase in specimen-driven assessment (from 5% to 34%), a decrease in positive margins (from 43% to 16%), and a notable reduction in the occurrence of inadequate margins ($p < 0.001$) between the two time periods. Additionally, specimen-driven IOA demonstrated a significantly decreased recurrence rate and an improvement in disease-specific survival. These favorable results provide evidence for the adoption of this method in OCSCC surgery.

Various techniques, such as ultrasound, cytology, and optical methods, are currently being explored for intra-operative use in surgical oncology. Among these, advances in optical techniques have presented unique opportunities for objective, real-time, and rapid screening. Raman spectroscopy (RS) is an optical technique that is being investigated for IOA. RS can be effectively employed to assess both the mucosa and deep soft tissue layers. Notably, RS is nondestructive and does not require the use of reagents or labeling, making it easier to implement in clinical settings. RS is based on inelastic scattering of light and offers detailed information about the molecular composition of tissue, represented as a Raman spectrum - a specific optical fingerprint of the tissue sample. The Raman spectrum exhibits characteristic peaks corresponding to molecular vibration, allowing for discrimination between cancerous and healthy tissue based on changes in the tissue's molecular composition.

Earlier research conducted by our group demonstrated that OCSCC can be discriminated from the healthy surrounding tissue based on the higher water concentration present in tumor with RS. In **Chapter 3**, we further investigated how the water concentration changes across the tumor border towards the healthy surrounding tissue. For this study, we analyzed tissue sections from 20 patients who were undergoing surgery for OCSCC. Our findings revealed a distinct transition from a high water concentration in the tumor (76% ± 8%) to a lower concentration in the healthy surrounding tissue (54% ± 24%), occurring over a distance of 4 to 6 mm along the tumor border. The differences in water concentration distributions between these regions were found to be statistically significant ($P < 0.0001$). This discovery underlines the potential of RS as a valuable tool for objective IOA of resection margins.

Although specimen-driven IOA can be beneficial, it has a limitation in accurately relocating inadequate resection margins. This challenge is particularly evident in the head and neck region, where achieving an optimal additional resection is not always feasible. In **Chapter 4**, we introduced a method to relocate inadequate resection margins in the wound bed. During the resection of an oral cavity tumor, the surgeon places numbered tags on both sides of the resection line (superficial and deep) in a pair-wise manner. After the resection, one tag from each pair remains attached to the specimen, while the corresponding tag remains in the wound bed. If an inadequate margin is detected on the resected specimen during IOA, these paired tags are used to accurately relocate the margin in the wound bed. We implemented this paired tagging method in 80 resections and, in 31 of those

cases, detected inadequate margins. Additional resections could be performed accurately at inadequate margins.

Although we obtained promising results with the relocation method, we encountered some practical limitations. To overcome these, we initiated the development of a tagging instrument, in collaboration with the faculty of Industrial Design Engineering at Delft University of Technology. **Chapter 5** details the project, which followed the fundamental design process of Pahl and Beitz (analysis, conceptualization, embodiment, and evaluation). The project involved a proof-of-concept for the anchoring method of the tags. Once the design was finalized, we constructed a prototype from aluminum to showcase the applicator's size and mechanical principles. To assess its usability and effectiveness, the intended users tested the prototype on cadavers. The design was then evaluated against a set of requirements. Subsequently, we created a more sophisticated model using stainless steel, intended as a 1:1 scale prototype. However, we encountered difficulties in achieving this scale and ensuring its functionality due to the small dimensions involved. Based on the requirements evaluation and other findings from the design, prototyping, and user testing stages, the report concludes with a list of recommendations for future developers to continue the development and manufacturing process.

During the implementation of the relocation method, the surgeons expressed a preference for tags that possess specific qualities, such as biocompatibility, solubility, and radio-opacity. Tags with these characteristics can be easily detected and identified through CT, MRI, or Cyberknife imaging. These properties allow for utilization during post-operative radiotherapy (PORT), which in turn facilitates a more precise targeting of the treatment volume. In **Chapter 5**, we also present a new research protocol aimed at exploring the potential of relocation tags in assisting with the reduction of the PORT target volume, ultimately leading to a decrease in toxicity.

In **Chapter 6**, our focus was on investigating DOI and validating the cut-off value of 4 mm, as per the guideline of the National Comprehensive Cancer Network, to determine the necessity of performing an END. We conducted a retrospective study on patients who underwent surgical treatment for early-stage OCSCC. For patients with cN0, an END was carried out when the DOI measured ≥4 mm, while those with DOI <4 mm were placed under a watchful waiting protocol. Among the patients who underwent an END, 36% were found to have occult LNM (pN+). In the watchful waiting group, regional recurrence was observed in 5.2% for DOI <4 mm

and 24.1% for DOI ≥4 mm. Notably, for DOI ≥4 mm, patients who underwent an END had a higher regional recurrence-free survival rate compared to those under watchful waiting ($p = 0.002$). A Receiver-Operator-Curve analysis revealed that a DOI cut-off value of 4.0 mm is the optimal threshold for predicting occult LNM (sensitivity 95.1% and specificity 52.9%). Consequently, we established that a DOI ≥4 mm is a reliable and accurate cut-off value.

However, there exists differences in the frequency of regional metastasis and prognosis among the various subsites of the oral cavity. Notably, buccal squamous cell carcinoma (SCC) appears to display a more aggressive tumor behavior compared to other subsites within the oral cavity. **Chapter 7** focused on elucidating the risk of regional disease in buccal SCC. We conducted an international multicenter retrospective study, analyzing patients treated for T1-T2 buccal SCC. Out of 101 cases, 77 were staged as cN0, with 32 of them undergoing END. The study showed an occult nodal metastasis rate of 28%. Moreover, an END was associated with a reduction in regional recurrence when compared to the observation group (8.9% vs. 6.3%, $p = 0.67$). It was also noted that regional recurrence was linked to a DOI >5 mm. Furthermore, in the pT2cN0 subgroup, END led to an improvement in overall survival ($p < 0.01$). Based on our findings, we recommend an END for patients with cT2N0 buccal SCC, especially for tumors with DOI >5 mm.

Resection margins hold significant prognostic value for all subsites within the head and neck. While achieving >5 mm margins is generally attainable during tongue excisions, obtaining such margins poses a challenge for resections in the larynx and hypopharynx. The complex anatomy and proximity to critical structures limit the extent of resection margins. The research described in **Chapter 8** was dedicated to investigating the feasibility of obtaining margins >5 mm and whether an alternative guideline should be considered. A team of head and neck surgeons and a pathologist from EMC performed total laryngectomy (TL) and total laryngopharyngectomy (TLP) on a tumor-free cadaver. The resection surfaces were carefully identified, and the maximum feasible margins (MFM) were measured for each tumor location and subsite. The MFM were defined as resection margins limited to 1-5 mm, considering the anatomy and limited thickness of different tissue layers at the resection surface. The results indicated that, for a TL, resection margins >5 mm were not feasible on the ventral and dorsal resection surfaces. Similarly, for a TLP, resection margins >5 mm were not feasible on the ventral, dorsal, and lateral resection surfaces. These findings challenge the existing guidelines set by the RCP. Therefore, we propose a new guideline with MFM of >1 mm, instead of >5 mm, to enable an adequate resection in larynx and hypopharynx tumor surgery.

To substantiate our proposal with oncological outcome data, we delved into resection margins in laryngeal and hypopharyngeal surgery in **Chapter 9**. This entailed a retrospective cohort study involving patients who underwent TL or TLP. The resection margins were defined in accordance with the guidelines of the RCP. The study population was divided into four groups: primary laryngeal SCC (pLSCC), recurrent (rLSCC), primary hypopharyngeal SCC (pHSCC), and recurrent (rHSCC). Within each group, we examined the relationship between resection margin status and rates of recurrence and survival. The study included 107 cases of pLSCC, 100 cases of rLSCC, 45 cases of pHSCC, and 16 cases of rHSCC. Positive resection margins were found in 54% for pLSCC, 29% for rLSCC, 62% for pHSCC, and 44% for rHSCC. Regarding pLSCC and rLSCC, there was a linear association between the chance of recurrence and the narrowing margins. Furthermore, a multivariate survival analysis for pLSCC and rLSCC demonstrated significantly worse disease-free survival in cases with positive margins. The study revealed that close and clear margins had similar survival rates for pLSCC and rLSCC and pHSCC. This finding suggests that a margin >5 mm may not have significant clinical relevance in terms of survival. Consequently, accepting a margin of 1-5 mm in certain subsites could be appropriate.

While we propose that resection margins of 1-5 mm can be considered acceptable in laryngeal and hypopharyngeal cancer surgery, resection margins <1 mm are associated with significantly worse outcomes and should be avoided. In this context, the use of RS for IOA can be beneficial. **Chapter 10** focuses on investigating whether RS can effectively differentiate between LSCC and the surrounding non-cancerous tissue based on water concentration. The experiments involved analyzing resection specimens from patients who underwent surgical treatment for primary LSCC. Both cancerous and non-cancerous tissues in the larynx had high water content, around 75%. However, the CH-stretching region of the Raman spectra from the larynx provided h distinguishing information, with an accuracy of 0.87. Unlike oral cavity cancer, water concentration can't differentiate laryngeal cancer using RS. To achieve a more accurate classification model, deep learning algorithms can be incorporated. However, to accomplish this, the training data set must be expanded to include a larger cohort of patients, allowing for better capturing the variation in spectra both within and between patients.

In the final chapter (**Chapter 11**), we discuss the value of IOA and its impact on patient outcomes. To ensure complete treatment, IOA is crucial for measuring resection margins and DOI during the initial surgery. Building on the findings of our group, the pathologists at the Erasmus MC have adopted an extended

specimen-driven IOA approach for evaluating resection margins and reporting on them. With this method, the margins are assessed by inspection, palpation, and perpendicular incisions (grossing). The IOA is accompanied by the relocation method and supported by frozen section analysis. The report includes the exact margins and their extent (e.g., "resection margin posterior is 2.1 mm, over a distance of 2 cm"). To facilitate the implementation of this specimen-driven IOA in other institutions, we have published a detailed protocol of this method. Additionally, we have introduced a two-weekly multidisciplinary meeting where all postoperative patients are discussed. This collaborative approach allows for better management of patient cases and ensures a well-coordinated treatment plan.

However, we recognize that IOA still has limitations, including its time-consuming nature and potential for sampling errors. To address these challenges, optical techniques emerge as potential solutions. A comprehensive literature search reveals a focus on various optical techniques, such as Raman spectroscopy, Elastic scattering spectroscopy, High-Resolution Micro Endoscopy, Narrow Band Imaging, Fluorescence, and Optical Coherence Tomography. Each technique offers a unique mechanism of action and different modes of data analysis. They provide real-time, non-invasive, and in situ optical signatures by using light of varying wavelengths to examine suspicious lesions. These techniques can effectively differentiate between normal and pathological lesions, aiding in accurate diagnosis.

Among these optical techniques, RS stands out with promising results in differentiating tumor from healthy surrounding tissue. Currently, at the Erasmus MC, RS is implemented in a prototype instrument employing a fiber-optic needle probe. This probe allows for a comprehensive assessment of all resection margins. Although RS is a proven technology, there is still considerable scope for improvement in instrumentation, enabling further development towards clinical applications.

SAMENVATTING

Wereldwijd staat hoofd-hals kanker op de 6e plaats als meest voorkomende maligniteit. Deze diagnose heeft voor de patiënt aanzienlijke gevolgen voor zowel de levensverwachting als de kwaliteit van leven. De prognose voor deze patiënten wordt beïnvloed door verschillende factoren die verband houden met zowel de patiënt als de tumor zelf. Enkel de factor "radicaliteit van de operatie" kan worden beïnvloed door de chirurg. De status van de resectiemarges kan dienen als een indicatie voor de radicaliteit van de operatie. Het Koninklijk College van Pathologen (KCP) definieert resectiemarges voor hoofd-hals kanker als: vrij (>5 mm), krap (1-5 mm) en positief (<1 mm). Het verkrijgen van vrije marges is cruciaal om het risico op lokale recidief tumoren te verminderen. Om chirurgen te helpen bij het verkrijgen van een adequate resectie (i.e., vrije marges), wordt er sterk aanbevolen om tijdens de operatie een specimen-driven intra-operatieve assessment (IOA) uit te voeren.

Een andere cruciale histopathologische factor is de invasiediepte (ID), die wordt beschouwd als een onafhankelijke voorspeller voor lymfekliermetastasen (LKM) bij een vroeg stadium mondholte kanker. Tijdens de histopathologische beoordeling van het resectie preparaat wordt de ID gemeten vanaf het niveau van het basaalmembraan van het aangrenzende normale slijmvlies. Voor gevallen waarin de ID ≥4 mm is, wordt een electieve halsklierdissectie (EHKD) aanbevolen. Als de ID pre- of peroperatief bepaald kan worden, kan dit van invloed zijn op de beslissing om al dan niet een EHKD uit te voeren bij cN0-patiënten. Onze hypothese is dat IOA, inclusief resectiemarges en ID, kan leiden tot een verbeterde uitkomst bij operaties voor mondholte kanker.

In een eerdere retrospectieve studie toonden we aan dat 85% van de patiënten met mondholte kanker inadequate (i.e., <5 mm) resectiemarges had, een bevinding die overeenkomt met vergelijkbare hoge cijfers in de bestaande literatuur. In **Hoofdstuk 2** zijn we dieper ingegaan op de huidige praktijk van IOA voor plaveiselcelcarcinoom (PCC) van de mondholte bij het Erasmus MC. We hebben een vergelijkende analyse uitgevoerd van twee patiënten cohorten: één behandeld tussen 2010 en 2012, - toen IOA nog niet de standaard was -, en een andere behandeld tussen 2013 en 2017, waarin IOA tot de standaard behoorde. We onderzochten de frequentie, het type (specimen-driven versus defect-driven assessment) en de resultaten van de IOA. We observeerden een significante toename van specimen-driven assessment (van 5% naar 34%), een afname van positieve resectiemarges (van 43% naar 16%) en een opmerkelijke afname van inadequate resectie marges ($p < 0.001$)

tussen de twee tijdsperioden. Bovendien toonde specimen-driven IOA een significant verminderd recidief percentage en een verbetering van de ziekte-specifieke overleving. Deze gunstige resultaten vormen bewijs voor de invoering van deze methode bij mondholte kanker operaties.

Diverse technieken, zoals echografie, cytologie en optische instrumenten, worden momenteel onderzocht voor intra-operatief gebruik in oncologische chirurgie. Van deze technieken biedt de ontwikkeling in optische technologie unieke mogelijkheden voor een objectieve, real-time en snelle screening. Raman-spectroscopie (RS) is een optische techniek die wordt onderzocht voor IOA. RS kan effectief gebruikt worden om zowel het slijmvlies als diepere weefsellagen te beoordelen. RS is niet-destructief en vereist geen gebruik van reagentia of labels, wat implementatie in de kliniek makkelijker maakt. RS is gebaseerd op de inelastische verstrooiing van licht en biedt gedetailleerde informatie over de moleculaire samenstelling van weefsel, weergegeven als een Raman-spectrum; een specifieke optische vingerafdruk van het weefselmonster. Het Raman-spectrum vertoont karakteristieke pieken die overeenkomen met moleculaire vibratie, waardoor onderscheid kan worden gemaakt tussen tumor en gezond weefsel op basis van veranderingen in de moleculaire samenstelling van het weefsel.

Eerder onderzoek, uitgevoerd door onze groep, toonde aan dat mondholte PCC kan worden onderscheiden van het gezonde omringende weefsel op basis van de hogere waterconcentratie in het tumorweefsel met RS. In **Hoofdstuk 3** onderzochten we verder hoe de waterconcentratie verandert over de grens van de tumor naar het gezonde omringende weefsel. Voor deze studie analyseerden we weefselmonsters van 20 patiënten die een operatie ondergingen voor mondholte PCC. Onze bevindingen onthulden een duidelijke overgang van een hoge waterconcentratie in de tumor (76% ± 8%) naar een lagere concentratie in het gezonde omliggende weefsel (54% ± 24%), over een afstand van 4 tot 6 mm langs de tumorrand. De verschillen in waterconcentratiedistributies tussen deze gebieden bleken statistisch significant ($p < 0.0001$). Deze ontdekking benadrukt het potentieel van RS als waardevol hulpmiddel voor objectieve IOA van resectiemarges.

Hoewel specimen-driven IOA voordelig kan zijn, heeft het een beperking in het nauwkeurig lokaliseren van de inadequate resectiemarges. Deze uitdaging is met name duidelijk in het hoofd-halsgebied, waar het niet altijd haalbaar is om een optimale aanvullende resectie te verrichten. In **Hoofdstuk 4** introduceerden we een methode om inadequate resectiemarges in het wondgebied te lokaliseren. Tijdens de resectie van een tumor in de mondholte plaatst de chirurg genum-

merde tags aan beide zijden van de resectielijn (oppervlakkig en diep) op een gepaarde wijze. Na de resectie blijft één tag van elk paar aan het resectie preparaat bevestigd, terwijl de overeenkomstige tag in het wondgebied blijft. Als er tijdens IOA een inadequate marge wordt gedetecteerd op het resectie preparaat, worden deze gepaarde tags gebruikt om de marge nauwkeuring in het wondgebied te lokaliseren. We pasten deze "paired tagging" methode toe op 80 resecties en ontdekten bij 31 van die gevallen een inadequate marges. Aanvullende resecties konden nauwkeurig worden uitgevoerd in het geval van de inadequate marges.

Hoewel we veelbelovende resultaten behaalden met deze nieuwe relocatie methode, waren er enkele praktische beperkingen. Om deze beperkingen aan te gaan zijn we in samenwerking met de faculteit Industrieel Ontwerp van de Technische Universiteit Delft de ontwikkeling van een tagging instrument gestart. In **Hoofstuk 5** worden de details van dit project, dat het fundamentele ontwerpproces van Pahl en Beitz volgde (analyse, conceptualisering, belichaming en evaluatie). Het project omvatte een proof-of-concept voor de verankeringsmethode van de tags. Nadat het ontwerp was voltooid, construeerden we een prototype van aluminium om de grootte en mechanische principes van de applicator te demonstreren. Om de bruikbaarheid en effectiviteit ervan te beoordelen, testten de beoogde gebruikers het prototype op kadavers. Het ontwerp werd beoordeeld aan de hand van een reeks eisen. Vervolgens creëerden we een geavanceerder model met roestvrijstaal, bedoeld als een 1:1 schaalprototype. We ondervonden echter moeilijkheden bij het bereiken van deze schaal en het waarborgen van de functionaliteit vanwege de kleine afmetingen die ermee gepaard gaan. Op basis van de evaluatie van de eisen en andere bevindingen uit het ontwerp-, prototyping- en gebruikerstest stadia, sluit het rapport af met een lijst van aanbevelingen voor toekomstige ontwikkelaars om het ontwikkelings- en fabricageproces voort te zetten.

Tijdens de implementatie van de relocatie methode, gaven de chirurgen de voorkeur aan tags met specifieke eigenschappen zoals: bio compatibiliteit, oplosbaarheid en radio-opaciteit. Tags met deze kenmerken kunnen eenvoudig worden gedetecteerd en geïdentificeerd via CT, MRI of Cyberknife beeldvorming. Deze eigenschappen maken gebruik mogelijk tijdens postoperatieve radiotherapie (PORT), wat op zijn beurt een nauwkeurigere targeting van het volume van het doelgebied vergemakkelijkt. In **Hoofdstuk 5** presenteren we een nieuw onderzoeksprotocol gericht op het verkennen van het potentieel van de tags bij het helpen verminderen van het PORT-volume van het doelgebied, wat uiteindelijk leidt tot een afname van de toxiciteit.

In **Hoofdstuk 6** lag onze focus op het onderzoeken van ID en het valideren van de afkapwaarde van 4 mm, zoals voorgesteld in de richtlijn van het National Comprehensive Cancer Network, om de noodzaak van het uitvoeren van een EHKD te bepalen. We voerden een retrospectieve studie uit bij patiënten die een chirurgische behandeling ondergingen voor vroeg stadium mondholte PCC. Bij patiënten met cN0 werd een EHKD uitgevoerd wanneer de gemeten ID ≥4 mm was, terwijl diegenen met een gemeten ID <4 mm een expectatief protocol volgden. Van de patiënten die een EHKD ondergingen, bleek 36% occulte LKM te hebben (pN+). In de groep met een expectatief beleid werd er bij 5.2% een regionaal recidief waargenomen voor een ID <4 mm en 24.1% voor een ID ≥4 mm. Opmerkelijk was dat de patiënten met een ID ≥4 mm die een EHKD ondergingen een hoger overlevingspercentage zonder regionale recidieven hadden in vergelijking met de patiënten met een expectatief beleid ($p = 0.002$). Een receiver-operator-curve analyse toonde aan dat een ID afkapwaarde van 4,0 mm optimaal is voor het voorspellen van occulte LKM (sensitiviteit 95.1% en specificiteit 52.9%). Daarnaast hebben we vastgesteld dat een ID ≥4 mm een betrouwbare en nauwkeurige afkapwaarde is.

Er bestaan echter verschillen in de frequentie van regionale metastasen en de prognose tussen de verschillende locaties binnen de mondholte. Met name blijkt het PCC van de wang agressiever gedrag vertonen in vergelijking met andere locaties binnen de mondholte. **Hoofdstuk 7** richtte zich op het verduidelijken van het risico op regionale ziekte bij wang PCC. We voerden een internationale multicenter retrospectieve studie uit, waarbij patiënten werden geanalyseerd die werden behandeld voor een T1-T2 wang PCC. Van de 101 gevallen werden er 77 geclassificeerd als cN0, waarvan 32 een ID ondergingen. De studie toonde een percentage van 28% occulte LKM. Bovendien werd een EHKD geassocieerd met een vermindering van regionale recidieven in vergelijking met de observatiegroep (8.9% vs. 6.3%, $p = 0.67$). Er werd ook opgemerkt dat regionale recidieven werden gekoppeld aan een ID >5 mm. Bovendien leidde bij de pT2cN0-subgroep een EHKD tot een verbetering van de algehele overleving ($p < 0.01$). Op basis van onze bevindingen raden we een EHKD aan voor patiënten met cT2N0 wang PCC, vooral voor tumoren met een ID >5 mm.

De resectiemarges hebben een aanzienlijke prognostische waarde voor alle deelgebieden binnen het hoofd-halsgebied. Hoewel het over het algemeen haalbaar is om marges van >5 mm te bereiken tijdens resecties van een tumor van de tong, vormt het verkrijgen van dergelijke marges een uitdaging voor resecties in de larynx en hypofarynx. De complexe anatomie en de nabijheid van kritieke structuren beperken de omvang van de resectiemarges. Het onderzoek beschreven in **Hoofd-**

stuk 8 was gewijd aan het onderzoeken van de haalbaarheid van het verkrijgen van marges >5 mm en of een alternatieve richtlijn overwogen moet worden. Een team van hoofd-hals specialisten en een patholoog van het Erasmus MC voerde een totale laryngectomie (TL) en totale laryngopharyngectomie (TLP) uit op een tumorvrij kadaver. De resectieoppervlakken werden zorgvuldig geïdentificeerd en de maximaal haalbare marges (MHM) werden gemeten voor elke tumorlocatie en subsite. De MHM werden gedefinieerd als resectiemarges beperkt tot 1-5 mm, rekening houdend met de anatomie en de beperkte dikte van verschillende weefsellagen van het resectievlak. De resultaten toonden aan dat voor een TL resectiemarges >5 mm niet haalbaar waren op de ventrale en dorsale resectievlakken. Op dezelfde wijze waren voor een TLP resectie marges van >5 mm niet haalbaar op de ventrale, dorsale en laterale resectievlakken. Deze bevindingen dagen de bestaande richtlijnen van de KCP uit. Daarom stellen wij een nieuwe richtlijn voor met een MHM van >1 mm, in plaats van >5 mm, om een adequate resectie bij tumorchirurgie in de larynx en de hypofarynx mogelijk te maken.

Om onze voorgestelde richtlijn te onderbouwen met oncologische uitkomstgegevens zijn we dieper ingegaan op de resectiemarges bij larynx- en hypofarynxchirurgie in **Hoofdstuk 9**. Dit omvatte een retrospectieve cohortstudie bij patiënten die een TL of TLP ondergingen. De resectiemarges werden gedefinieerd volgens de richtlijnen van de KCP. De onderzoekspopulatie werd verdeeld in vier groepen: primair larynx PCC (pLPCC), recidief (rLPCC), primair hypofarynx PCC (pHPCC) en recidief (rHPCC). Binnen elke groep onderzochten we de relatie tussen de status van de resectiemarge en de percentages recidief en overleving. De studie omvatte 107 gevallen van pLPCC, 100 van rLPCC, 45 patiënten met een pHPCC en 16 patiënten met een rHPCC. Positieve resectiemarges werden gevonden bij respectievelijk 54% voor pLPCC, 29% voor rLPCC, 62% voor pHPCC en 44% voor rHPCC. Voor pLPCC en rLPCC was er een lineair verband tussen de kans op een recidief en het versmallen van de marges. Bovendien toonde een multivariate overlevingsanalyse voor pLPCC en rLPCC een significant slechtere ziektevrije overleving aan bij gevallen met positieve marges. De studie onthulde dat krappe en vrije marges verglijkbare overlevingspercentages hadden voor pLPCC, rLPCC en pHPCC. Deze bevinding suggereert dat een marge <5 mm mogelijk geen significante klinische relevantie heeft wat betreft de overleving. Daarom zou het accepteren van een marge van 1-5 mm op bepaalde locaties passend kunnen zijn.

Hoewel we voorstellen dat resectiemarges van 1-5 mm acceptabel kunnen worden beschouwd bij chirurgie van maligniteiten van de larynx- en hypofarynx, zijn resectiemarges <1 mm geassocieerd met aanzienlijk slechtere resultaten en moeten

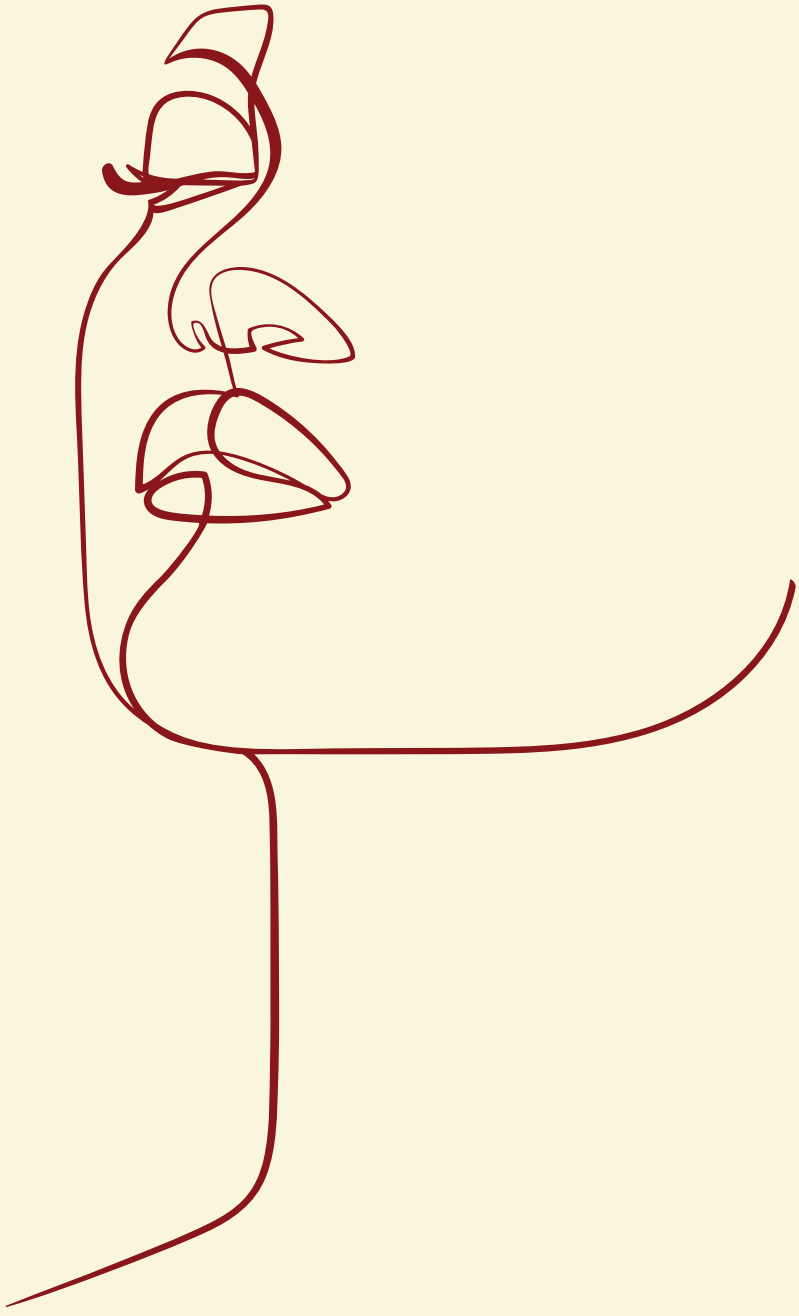
deze worden vermeden. In deze context kan het gebruik van RS voor IOA gunstig zijn. **Hoofdstuk 10** richt zich op het onderzoeken of RS effectief larynx PCC kan onderscheiden van het omliggende gezonde weefsel op basis van waterconcentratie. De experimenten omvatten het analyseren van resectiepreparaten van patiënten die een chirurgische behandeling ondergingen voor primair larynx PCC. Zowel tumor als gezond weefsel in de larynx hadden een hoge waterconcentratie, dit was ongeveer 75%. Echter, het CH-strechting gebied van de Raman-spectra van de larynx verschaft wel onderscheidende informatie, met een nauwkeurigheid van 0,87. In tegenstelling tot mondholte kanker kan er op basis van de waterconcentratie bij larynx PCC niet gedifferentieerd worden met RS. Om een nauwkeuriger classificatiemodel te bereiken, kunnen deep learning-algoritmen worden geïntegreerd. Om dit te bereiken, moet de trainingsdataset worden uitgebreid met een groetere groep patiënten, waardoor een betere vastlegging van de variatie in spectra zowel binnen, als tussen patiënten mogelijk is.

In het laatste hoofdstuk (**Hoofdstuk 11)** bespreken we de waarden van IOA en de impact ervan op de uitkomsten voor de patiënt. Om een volledige behandeling te waarborgen, is IOA cruciaal voor het meten van de resectiemarges en ID tijdens de initiële operatie. Voortbouwend op de bevindingen van onze groep hebben de pathologen van het Erasmus MC een uitgebreide specimen-driven IOA overgenomen voor het evalueren van resectiemarges en het rapporteren daarover. Met deze methode worden de marges beoordeeld door middel van inspectie, palpatie en loodrechte incisies (uitsnijden). De IOA wordt ondersteund door de relocation methode en ondersteund door analyse met vriescoupes. Het rapport bevat de exacte marges en hun omvang (bijvoorbeeld “resectiemarge posterieur is 2,1 mm, over een afstand van 2 cm”). Om de implementatie van deze specimen-driven IOA in andere instellingen te vergemakkelijken, hebben we een gedetailleerd protocol van deze methode gepubliceerd. Bovendien hebben we een tweewekelijkse multidisciplinaire vergadering geïntroduceerd waarin alle postoperatieve patiënten worden besproken. Deze samenwerking zorgt voor een beter management van de patiënt zorg en waarborgt een goed gecoördineerd behandelplan.

We erkennen echter dat IOA nog steeds beperkingen heeft, waaronder de tijdsintensieve aard en het potentieel voor sampling errors. Om deze uitdagingen aan te pakken, worden optische technieken naar voren gebracht als mogelijke oplossingen. Een uitgebreide literatuurstudie onthult een focus op verschillende optische technieken zoals: Raman-spectroscopie, Elastic scattering spectroscopie, High-Resolution Micro Endoscopie, Narrow Band Imaging, Fluorescentie en Optische Coherentie Tomografie. Elke techniek biedt een uniek werkingsmechanisme en

verschillende modi van gegevensanalyse. Ze leveren real-time, niet-invasieve en in situ optische “handtekeningen” door gebruik te maken van licht van variërende golflengtes om verdachte laesies te onderzoeken. Deze technieken kunnen effectief onderscheid maken tussen normale en pathologische laesies, wat bijdraagt aan een nauwkeurige diagnose.

Onder deze optische technieken springt RS eruit met veelbelovende resultaten bij het onderscheiden van tumoren van gezond omliggend weefsel. Momenteel wordt bij het Erasmus MC RS geïmplementeerd in een prototype-instrument met een fiber-optic needle probe. Deze probe maakt een uitgebreide beoordeling van alle resectie marges mogelijk. Hoewel RS een bewezen technologie is, is er nog aanzienlijke ruimte voor verbetering in instrumentatie, waardoor verdere ontwikkeling richting klinische toepassingen mogelijk is.



Chapter 13

Addendum

13.1 ABBREVIATIONS

ACE-27 - Adult Comorbidity Evaluation-27
AJCC - American Joint Committee on Cancer
AF - Autofluorescence
AFI - Auto-Fluorescence Imaging
ANOVA - Analysis of Variance
CCD - Charged-Coupled Device
CE - Contact Endoscopy
CECT - Contrast-Enhanced Computed Tomography
CI - Confidence Interval
cN0 - Clinically Node Negative
cN+ - Clinically Node Positive
ClirSpec - International Society for Clinical Spectroscopy
CRM - Confocal Raman Microscope
CRT - Chemo radiotherapy
CT - Computed Tomography
DFS - Disease-Free Survival
DSS - Disease-Specific Survival
DOI - Depth of Invasion
EMC - Erasmus MC Cancer Institute
EMSC - Extended Multiplicative Scatter Correction
END - Elective Neck Dissection
ECE - Extra-capsular Extension
EPIC - European Photonics Industry Consortium
ESS - Elastic Scattering Spectroscopy
FLI - Fluorescence Imaging
H&E - Hematoxylin and Eosin
HSCC - Hypopharyngeal Squamous Cell Carcinoma
HPV - Human Papillomavirus
HR - Hazard Ratio
HRME - High-Resolution Micro-Endoscopy
IOARM - Intra-Operative Assessment of Resection Margins
LDA - Linear Discriminant Analysis
LNM - Lymph Node Metastasis
LVI - Lymphovascular Invasion
LSCC - Laryngeal Squamous Cell Carcinoma
LR - Local Recurrence
MEC - Medical Ethics Committee

MFM - Maximum Feasible Margins
MRI - Magnetic Resonance Imaging
NBI - Narrow-band Imaging
ND - Neck Dissection
NCCN - National Comprehensive Cancer Network
NPV - Negative Predictive Value
n.a. - not applicable
OCSCC - Oral Cavity Squamous Cell Carcinoma
OR - Operating Room
OS - Overall Survival
OCT - Optical Coherence Tomography
PCA - Principal Component Analysis
PNI - Perineural Invasion
PORT - Post-operative Radiotherapy
PPV - Positive Predictive Value
RMH - Royal Melbourne Hospital
ROC - Receiver Operating Characteristic curve
RS - Raman Spectroscopy
RRFS - Regional Recurrence-Free Survival
RT - Radiotherapy
SCC - Squamous Cell Carcinoma
SD - Standard Deviation
SLNB - Sentinel Lymph Node Biopsy
TL - Total Laryngectomy
TLM - Transoral Laser Microsurgery
TLP - Total Laryngopharyngectomy
TNM - Tumor, Node, Metastasis classification
cTNM – clinical
pTNM - pathological
TRL - Technology Readiness Level
TR - Total Recurrence
TND - Therapeutic Neck Dissection

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13.4 PHD PORTFOLIO

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PhD training

General courses, seminars, and workshops

Basic Introduction Course on SPSS	2015
Systematic Literature research *Endnote, PubMed, Other Databases*	2015
3 Quarterly Progress Meeting – Raman group	2015 - 2019
Scientific Integrity	2016
Basic course on Regulations and organization for Clinical Investigators	2016
NVVO Basic course Oncology	2017
CLIRSPEC Summer School	2018
Otolaryngology residency program courses *BLS, Head and Neck anatomy, ENT radiology, ear surgery, nose surgery, basic FESS, children's airway*	2019 - 2023
Discipline transcending education *Health law, AIOS in Sync, Hospital management*	2019 - 2023

Oral presentations on (inter)national conferences

Research presentations Otolaryngology department	2015 - 2019
Otolaryngology Annual Research day, Rotterdam, The Netherlands	2016, 2019
9th AHNS, Seattle, United States of America	2016
52nd meeting of the ESSR, Amsterdam, The Netherlands	2017
Scientific meeting Dutch society for Otolaryngology, Nieuwegein, The Netherlands	2017, 2019
Patient presentations Otolaryngology residency program	2017 - 2024
AAV Erasmus MC researchers day	2018

Poster presentations on (inter)national conferences

6th THNO congress, Nice, France	2017
10th SPEC conference, Glasgow, Scotland	2018
6the IFHNOS, Buenos Aires, Argentine	2018

Teaching and supervision activities

Tutor first year medical students Erasmus MC	2015, 2016
Supervision graduation research MSc Student TU delft	2016 - 2017
ICK/ PKV education Otolaryngology medical students, Erasmus MC	2016 - 2024
Supervision research MSc Student Erasmus MC	2017 - 2018

Other activities

Organisation 7e Jonge Onderzoekersdag NWHHT	2016 - 2017
Organization 3 Quarterly Otolaryngology department meetings	2017, 2018
Basic Exam Surgery	2020
Member Otology board, Dutch society for Otolaryngology	2022 - 2024