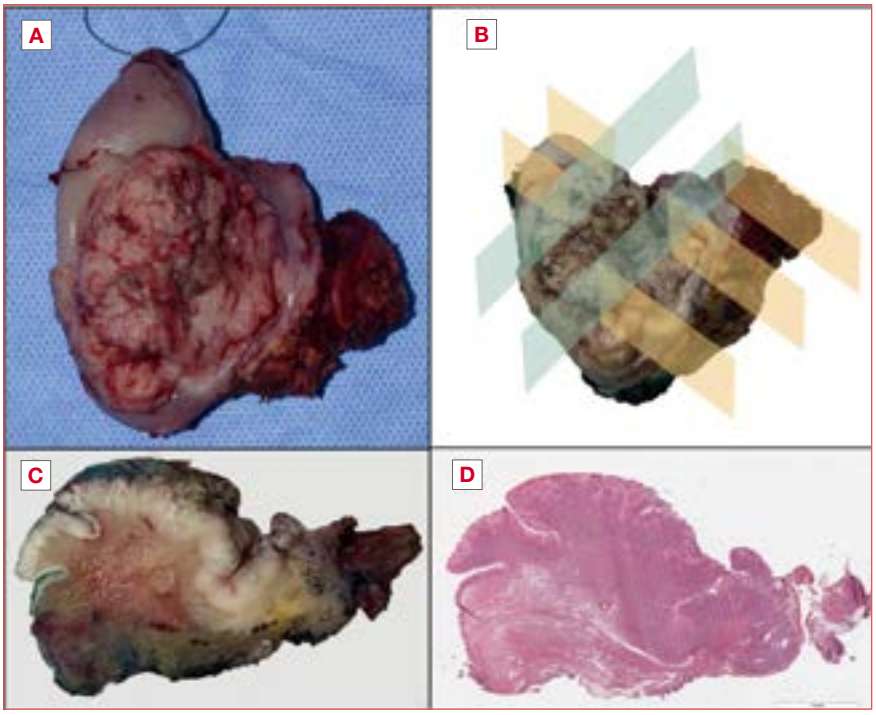
ACTA Otorhinolaryngologica Italica **2025**;45(Suppl. 1):S137-S157; doi: 10.14639/0392-100X-suppl.1-45-2025-N1141

The impact of histology and molecular biology in refining the prognostic value of surgical margins in head and neck oncology – A narrative review



Cover figure. **(A)** Surgical specimen of a right hemiglossopelvectomy. The tongue is oriented using a suture to indicate the tip; **(B)** Surgical specimen after formalin fixation, with resection margins marked in different colours of India ink to indicate the midline raphe, the margin on the floor of the mouth, the posterior and the anterior margin (the tip). The margins were assessed on the surgical specimen (sample-driven), which was sectioned using the "radial and perpendicular" method; **(C)** A macroscopic section of the lingual body obtained by radial sectioning. **(D)** The corresponding whole slide image stained with haematoxylin and eosin.

Patrizia Morbini[1], Giulia Querzoli[2,3], Andrea Ambrosini-Spaltro[4], Achille Tarsitano[5,6], Gabriele Molteni[3], Sara Bassani[7], Maria Berezina[6,8], Maria Pia Foschini[6,8]

[1] SC di Anatomia e Istologia Patologica, EO Ospedali Galliera, Genoa, Italy; [2] Pathology Unit, IRCCS Azienda Ospedaliero Universitaria di Bologna, Bologna, Italy; [3] Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy; [4] Pathology Unit, Morgagni-Pierantoni Hospital, Forlì, AUSL Romagna, Italy; [5] Oral and Maxillofacial Surgery Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy; [6] Department of Biomedical and Neuromotor Sciences (DIBINEM), Alma Mater Studiorum, University of Bologna, Bologna, Italy; [7] Otolaryngology-Head and Neck Surgery Department, University of Verona, Verona, Italy; [8] Unit of Anatomic Pathology at Bellaria Hospital, Bologna, Italy

Received: March 15, 2025
Accepted: March 21, 2025

Correspondence
Patrizia Morbini
E-mail: patrizia.morbini@galliera.it

How to cite this article: Morbini P, Querzoli G, Ambrosini-Spaltro A, et al. The impact of histology and molecular biology in refining the prognostic value of surgical margins in head and neck oncology – A narrative review. Acta Otorhinolaryngol Ital 2025;45(Suppl. 1):S137-S157. https://doi.org/10.14639/0392-100X-suppl.1-45-2025-N1141

© Società Italiana di Otorinolaringoiatria e Chirurgia Cervico-Facciale

OPEN ACCESS

This is an open access article distributed in accordance with the CC-BY-NC-ND (Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International) license. The article can be used by giving appropriate credit and mentioning the license, but only for non-commercial purposes and only in the original version. For further information: https://creativecommons.org/licenses/by-nc-nd/4.0/deed.en

Summary

The intraoperative evaluation of surgical margins (SM) using frozen section analysis is a widely adopted practice in surgery for head and neck cancer (HNC), offering real-time pathological assessment. However, its reliability is not without challenges, as inconsistencies in specimen processing, tissue shrinkage, and interobserver variability can impact its accuracy. This review explores key aspects of SM assessment in HNC, discussing different approaches, particularly dealing with intraoperative assessment and comparing sample-driven and tumour bed-driven approaches. It examines the accuracy of these techniques and their prognostic implications, especially in cases where a positive frozen section analysis leads to revision of margins. Another important factor discussed is the impact of tissue artefacts on margin interpretation, alongside practical recommendations for assessing bone margins. A crucial point emphasised throughout is the need for strong collaboration between surgeons and pathologists to enhance the reliability of intraoperative margin evaluation.

Beyond traditional histopathological methods, recent advancements in imaging techniques are also considered. Technologies such as narrow band imaging, Raman spectroscopy, optical coherence tomography, hyperspectral imaging, computer-assisted surgery, and confocal microscopy are gaining attention for their potential to refine margin assessment and improve surgical precision. Looking ahead, the focus should be on refining intraoperative SM assessment techniques, integrating artificial intelligence-driven imaging solutions, and developing standardised guidelines through large-scale meta-analyses. A multidisciplinary approach that brings together pathology, radiology, and surgical expertise will be crucial in optimising tumour resection strategies and help to reduce the risk of local recurrence.

Key words: frozen sections, surgical margin, head and neck cancer, accuracy assessment, prognostic impact, shrinkage

Introduction

Achieving an initially negative surgical margin predicts the optimal prognostic outcome for patients with head and neck squamous cell carcinoma (HNSCC), and surgical margins (SM) represent the only factor potentially controllable by the surgeon. Negative margins indicate that the tumour has been completely removed and serves as a guide for postoperative adjuvant treatments. However, the issue of margin adequacy, and the definition of "negative" margins, are objects of longstanding scientific debate. A previous American Head and Neck Society (AHNS) survey highlighted the spectrum of surgeons' opinions regarding margin adequacy, with nearly half defining a clear margin as > 5 mm [1]. In contrast, SM free from tumour but measuring < 5 mm are defined as "close".

Patient prognostic stratification according to conventional margin status is supported by robust and large literature data, as summarised by a recent meta-analysis on 8,435 patients from 26 studies, most of whom with oral squamous cell carcinomas (OSCC) (Cover figure and Figure 1). Hamman et al. reported a significant stepwise improvement in local recurrence (LR) and overall survival (OS) relative risks as margin status categorically improved from positive to close to clear[2]. Stratification by margin status defined as negative, close, and positive according to the cutoff of 5 mm has been introduced in the AJCC Staging Manual (8th edition) and in international guidelines. It is important to note that, while HNSCC included in the validation studies covered all head and neck subsites [3], albeit with a disproportionate prevalence of OSCC, the AJCC formally adopted the 5 mm cutoff for close tumours only for OSCC, and that most published literature exploring its strengths and limitations focuses on this tumour site [2].

Recent studies have challenged the 5 mm cut-off to reach a more precise definition of safe margin distance and negativity threshold. A recent review summarised the results of the most relevant studies exploring optimal cutoff values for microscopic surgical distance in OSCC with reference to different outcome measures (2-, 3-, or 5-year local control [LC], OS, LR, loco-regional recurrence [LRR], loco-regional control [LRC], disease-specific survival [DSS], or loco-regional recurrence free survival [LRRFS]) [4]. Significant cutoff values ranged between 1 and 7.6 mm, confirming that consensus on the extent of safe margins is far from reached. A meta-analysis in 2023 including 7 studies concluded that margins between 4 and 4.9 mm had a similar LR risk ratio compared with margins ≥ 5 mm, while margins < 4 mm had significantly higher risks [5]. No pooled data are available for other outcome parameters, including survival, due to the high heterogeneity of the available studies. Interestingly, recent studies have found that treating SM as continuous, rather than categorical, variables improves the correlation with survival [6,7], as well as with recurrence [8].

Controversies exist on how to treat positive (and possibly close) margins, be it by re-excision in case of intraoperative identification of positive margins, or adjuvant therapy, as originally proposed by the EORTC and RTOG studies [9,10]. This is mostly due to contradictory results obtained by works aimed at assessing the prognostic impact of such decisions. There are several technical reasons beyond intrinsic tumour behaviour that may explain the lack of robust evidence in this field; among them, the limited standardisation of sample handling by surgeons and pathologists and of tissue processing plays a crucial role: in particular, it is virtually impossible to quantify and standardise the amount of thermal damage and shrinkage suffered by the tissue in the pre-analytical phases. Moreover, the approach to the evaluation of SM is influenced by the practice of the surgical team and pathologists, with particular reference to the sampling of margins from the tumour bed or from the resection specimen and could benefit from the application of recently developed technologies, including artificial intelligence (AI).

We will cover the above topics in the following sections to provide an overview of the status of margin assessment in HNSCC.

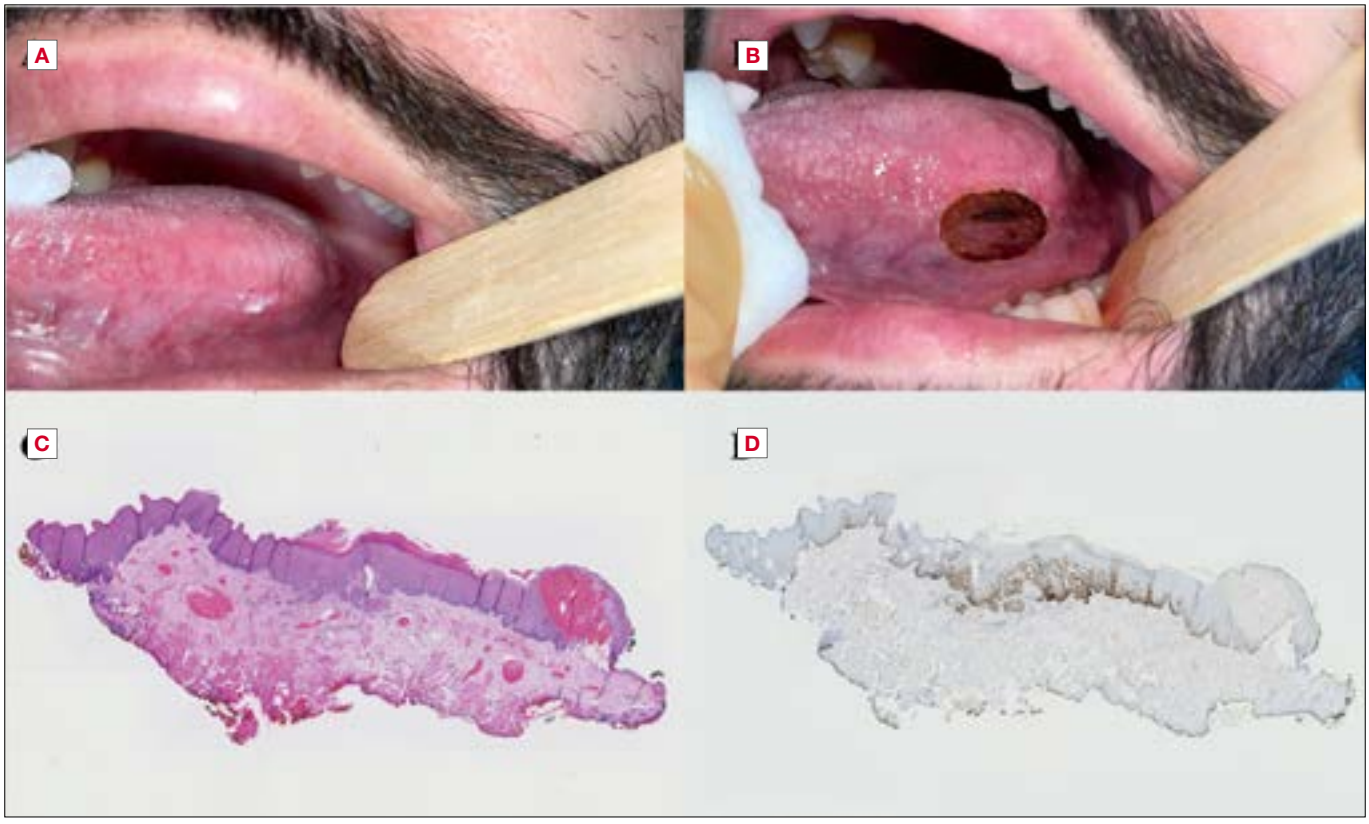


Figure 1. **(A)** A neoplastic lesion on the left lingual border in a male patient affected by graft-versus-host disease; **(B)** Tumour bed after lesion resection; **(C)** Whole slide image of the lesion stained with haematoxylin; **(D)** Immunohistochemical analysis for p53 shows a pattern of basal and suprabasal overexpression, while the resection margins are negative.

Dysplastic margins

The impact of dysplasia at margins of HNSCC resection is not well understood. Most respondents to the AHNS survey [1] considered carcinoma in situ as a positive margin, and 76% considered dysplasia at the margin as a negative SM. On the other hand, the College of American Pathologists guidelines recommend that moderate and severe dysplasia at SM should be considered as positive margins [11]. A recent large retrospective study on 565 resected OSCCs showed that dysplastic and close SM did not significantly affect LRR or OS in comparison with negative SM [12]; however, patients with severe dysplasia at margins had a higher recurrence rate than those with mild and moderate dysplasia, even if more than half received adjuvant radiation therapy [12]. Other studies reported that dysplastic SM was not a significant risk factor in OSCC and that the severity of dysplasia had no impact [13]. Senarath et al. [14] recently reviewed articles correlating outcomes with the presence of dysplastic margins and concluded that moderate to severe dysplasia at the margin required re-excision. Overall, due to the lack of definite evidence, surgeons tend to address SM with high-grade dysplasia on a case-by-case basis, highlighting the need for definite guidelines on this topic and the importance that pathologists precisely document the presence and grade of dysplasia on margins.

Margins at extra-oral sites

There is a lack of conclusive evidence regarding SM beyond the oral cavity. The anatomical differences among subsites in the head and neck region make it difficult to directly compare the clinical significance of a margin greater than 5 mm across these areas [15]. A key question remains: is achieving a margin greater than 5 mm feasible in the oropharynx, larynx, and hypopharynx? Several studies have indicated that the anatomical constraints of the head and neck region limit SM due to the thinness of various tissue layers [16]. In the literature, only a limited number of studies have specifically addressed SM in



Figure 2. **(A)** Total laryngectomy specimen. It is crucial to assess all resection margins. Intraoperatively, frozen sections must always be performed, especially on the superior margin at the level of the vallecula/base of the tongue and the inferior margin at the level of the piriform sinuses; **(B)** A whole slide image (WSI) of a cross-section obtained at the tumour site in the corresponding total laryngectomy specimen; **(C)** Open partial horizontal laryngectomy (OPHL) Type II specimen. Proper orientation of the specimen is essential. It is important to evaluate the margins and assess thyroid cartilage infiltration, as this can influence the indication for adjuvant treatment; **(D)** A WSI of a cross-section obtained at the tumour site in the corresponding OPHL Type II specimen; **(E)** Type VI cordectomy. Close collaboration with the surgeon is essential, particularly in orienting the specimen in the operating room. In this case, the margins to be evaluated include: the posterior vocal cord margin, the inferior subglottic margin, and the superior margin towards the ventricle. The anterior and lateral margins, adjacent to the perichondrium of the thyroid cartilage, may also be positive. However, in such cases, achieving negative margins is challenging. If the posterior, inferior, or superior margins are positive, a wider endoscopic resection may be performed. However, if the anterior or lateral margins are involved and further excision is necessary, an open surgical approach is required. In general, an indication for wider endoscopic resection is given only in cases of multiple positive superficial margins or a positive deep margin. However, depending on the surgeon's experience, an endoscopic follow-up approach may also be considered. This is because margins can appear negative intraoperatively, but due to shrinkage from formalin fixation or the effects of the carbon dioxide laser, may be positive in the final histological examination; **(F)** A WSI of a cross-section obtained from the cordectomy specimen.

total laryngectomy/laryngopharyngectomy procedures [17-23] (Fig. 2). Among them, only one adhered to the Royal College of Pathologists (RCP) guidelines [24]. Two studies classified close margins as being less than 5 mm and considered margins as positive when tumour cells were present at the resection surface [22-25]. The remaining studies described margin status using qualitative terms rather than precise numerical values, employing classifications such as 'positive', 'microscopically positive', 'tumour at the resection surface', 'negative', 'safe margins', or 'no invasive tumour at the resection surface'. Due to these inconsistencies, determining the clinical relevance of SM in the larynx and hypopharynx remains challenging. Achieving consensus on the prognostic significance of SM at these sites would require meta-analyses of studies that share standardised definitions, methodologies, and postoperative treatment strategies [26]. From a practical point of view, for glottic cancers a smaller margin of 1-2 mm is considered sufficient with particular regards to transoral laser resections [27,28]. As part of the Department of Veterans Affairs Laryngeal Cancer Study, one paper reported 50% survival outcomes for clear margins, 57% for close (less than 5 mm) margins, and 27% for involved margins in laryngeal SCC [29]. In transoral endoscopic and robotic approaches for oropharyngeal cancers, margins of 1.5-2 mm are considered acceptable[4]. In hypopharyngeal tumour, submucosal patterns of spread could predispose to inadequate margins [30]. A study has shown that the propensity for submucosal tumour extension was least in the superior direction (3-10 mm) and highest in the medial (2-37 mm), lateral (2-37 mm), and inferior direction (3-35 mm) [31]. The same group has also emphasised adequate radial clearance and suggested that a radial clearance of less than 1 mm is an independent prognostic factor for OS, DFS, and regional RFS [32]. Hence, greater margins up to 30 mm have been recommended in hypopharyngeal tumours. To integrate the minimal free margin proposed by the Royal College of Pathologists guidelines and establish it as a standard of care for laryngeal and hypopharyngeal cancer, further investigation into its clinical relevance is essential.

Intraoperative evaluation of tumour margins

The intraoperative assessment of SM by frozen section analysis is a widely accepted and commonly utilised approach in HNSCC surgery. Its reliability and clinical value have been well demonstrated. The 2024 NCCN guidelines report: 'Frozen section margin assessment is always at the discretion of the surgeon and should be considered when it will facilitate complete tumour removal. Achieving adequately wide margins may require resection of an adjacent structure in the oral cavity or laryngopharynx such as the base of the tongue and/or anterior tongue, mandible, lar-

ynx, or portions of the cervical oesophagus. This technique enables rapid pathological evaluation during surgery [33]. Ideally, margin assessment should follow a uniform, standardised approach for all cases. However, specific standards for head and neck procedures have not yet been established. Findings from the survey "Critical evaluation of frozen section margins in head and neck cancer resections" indicate that there is no universally standardised approach for frozen section margin analysis in head and neck surgeries. According to the responses, 32% of participants reported using multiple processes for intraoperative margin assessment, while 18% stated that more than one method was applied for final margin evaluation, although this step was determined by the pathologist. The approach often varies by institution and tends to be internally developed to fit specific institutional practices [34]. Additionally, many pathologists face uncertainty regarding the exact areas sampled for frozen section analysis and the margins that require further assessment. To address these challenges, various intraoperative techniques and strategies for SM evaluation have been developed [35] (Tab. I).

Defect-driven *vs* sample-driven

There is currently no evidence-based data confirming that intraoperative margin assessment improves local control. Conducting research is challenging due to the variability in margin sampling, evaluation, and reporting; however, it is clear that margins can be assessed from the specimen, referred to as specimen margins or sample-driven margins (SDM), or from the tumour bed, known as tumour bed margins (TBMs) or defect-driven margins [1,34,36]. In the SDM approach, the entire resected specimen is sent to the pathologist for margin assessment. Conversely, in the defect-driven approach, the surgeon samples the tumour bed, collecting margins from the wound, or cavity, after completing the resection. In the latter method, the selection of TBMs, including their size, location, and quantity, is not directly guided by the formal margin assessment of the resected specimen. The debate extends beyond the method of margin sampling to the reliability of peripheral margin interpretation. In fact, there is a weak correlation between margins taken from the surgical bed and those directly sampled from the resected specimen. Most studies on margin assessment overlook the distinction between these two approaches and fail to consider their relationship. Research suggests that prognostically significant margins are those derived from the actual resection specimen, while tumour bed sampling alone has been associated with poorer local control [37,38]. In a retrospective multicentre cohort study, Maxwell et al. found that among 95 patients relying solely on TBMs, the sensitivity was just 24.2% (95% CI: 16-34%), whereas the specificity reached 92% (95% CI: 85-97%) when SM were used as the reference standard [39]. Similarly, Buchakjian et al. reported that TBMs had a 35% sensitivity in detecting positive specimen margins [40]. Another study by Chang et al. found that TBMs failed to identify positive glossectomy margins in 67% of cases [37].

In evaluating the two types of margins, an economic aspect also emerged. The assessment of intraoperative margins has been associated with prolonged surgical times and increased costs [41]. Moreover, these additional expenses can extend beyond the operating room, as false negative results may necessitate further therapeutic escalation [42]. The current 2024 NCCN guidelines do not specify which type of margin between SDM and TBM is best to use intraoperatively with frozen sections [33].

TBM sampling remains widely used because of its efficiency and ease of execution, enabling surgeons to influence the pathology report. In a 2021 AHNS survey conducted among head and neck surgeons, 45% still favoured the tumour bed sampling method [43]. This preference may stem from its technical advantages, as it is a faster approach that allows surgeons greater control over the processing of pathological specimens. However, a survey

Table I. Intraoperative evaluation of tumour margins.

Key points	Description
Frozen section analysis	Widely used in head and neck cancer surgery for intraoperative margin assessment
NCCN guidelines	Frozen section margin assessment is at the surgeon's discretion; wide margins may require additional tissue resection
Proximal and distal nerve margins	Recommended for frozen section analysis to ensure complete tumour removal
Reliability of frozen sections	Demonstrated to be a valuable diagnostic tool for rapid intraoperative pathological evaluation
Lack of standardisation	There is no universally accepted standard for frozen section margin evaluation in head and neck surgery
Institutional variability	Different institutions apply varying approaches, often developed internally
Survey findings	32% of respondents use multiple intraoperative margin assessment methods; 18% apply more than one method for final evaluation

conducted among Canadian surgeons highlights that a slightly higher number of surgeons preferred a tumour-driven approach [44].

Over the years, efforts have been made to refine this technique by incorporating TBM orientation. Van Lanschot et al. explored the feasibility of a similar approach using paired markers placed on both the specimen and the surgical bed. The primary specimen underwent intraoperative analysis by both surgeon and pathologist. Based on these assessments, additional resections were carried out. As a result, margin status improved in 28 of 31 cases, and surgeons reported that identifying the positive SDM from the specimen to the wound bed became easier [45].

Villemure-Poliquin et al. evaluated the accuracy of an oriented TBM technique in comparison to an SDM technique [46]. Their findings indicated that the use of an oriented TBM approach did not negatively impact LC, LRC, or DFS when compared to SDM. Moreover, the oriented TBM method demonstrated higher sensitivity than the SDM technique, with rates of 50% and 30%, respectively. The effectiveness of this approach improved when performed by the surgeon who originally developed the technique, suggesting that its application may vary depending on the operator's experience and consistency. These findings are favourable compared to previous studies, which reported lower sensitivity values, ranging between 15% and 32% [39-47] (Tab. II).

Table II. Defect-driven *vs* sample-driven margin assessment.

Key points	Sample-driven margins (SDM)	Defect driven or tumour-bed margins (TBM)
Definition	Margins assessed on the resected specimen	Margins taken from the surgical bed after resection
Guidance	Pathologist-driven margin assessment	Surgeon-guided margin selection
Correlation	Stronger correlation with true SM	Weaker correlation with actual resection specimen margins
Local control	Better local control	Lower local control, higher false negative rate
Sensitivity	Higher sensitivity in detecting positive margins	Lower sensitivity (reported between 15% and 35%)
Surgical time	Potentially increases operative time for thorough assessment	Faster, as it avoids waiting for detailed pathological processing
Economic aspect	Higher costs due to extended surgery and pathology processing	Lower initial costs, but false negatives may lead to additional treatment expenses
Surgeon preference	Less commonly preferred due to logistical challenges	More widely used due to efficiency and ease of execution
Advancement	Standardised in most pathology departments	Some methods attempt to improve accuracy by orienting TBM samples

Accuracy assessment

The interpretation of a negative TBM remains unclear [48]. Unlike SDM, which can be categorised as positive, close, or negative, TBM is assessed in a binary manner, either positive or negative. Additionally, frozen sections taken from the tumour bed do not allow precise distance measurements as it would be on a resected specimen, and for this reason interpreting frozen margins remains challenging. If an SDM is negative and the corresponding TBM is also negative, the margin is unequivocally classified as negative. However, ambiguity arises in other situations. For instance, if a specimen has a close margin (ranging from 2 to 5 mm) but a negative TBM, should it still be considered a close margin? Similarly, if a TBM is initially positive but turns negative after multiple reassessments, should that margin be classified as negative or close in the final pathology report, provided that the latter scenario could result in escalated therapy? Currently, no standardised guidelines exist to categorise such cases, and adjuvant treatment decisions vary across institutions.

Prognostic impact of margin revision after positivity on frozen section analysis

A highly debated topic concerns the impact and management of SM, either SDM or TBM, that were initially positive at intraoperative frozen sections but later revised to negative. Although identifying positive margins intraoperatively and immediately resecting occult disease may seem logical, a definitive prognostic benefit and therapeutic significance of these revised margins continues to be a subject of discussion [49]. Therefore, although many surgeons attempt further tissue resection when encountering a positive intraoperative margin, it remains unclear whether this additional resection significantly affects prognosis [39,48,49]. Previous evidence suggests that survival rates and local tumour control among patients who undergo additional resections to achieve negative margins are comparable to those who achieve negative margins on the first attempt [50-52]. Zhang et al. found that immediate further

resection guided by frozen section analysis could enhance oncologic outcomes in patients with positive main specimen margins [51]. Additionally, they confirmed the poor sensitivity (17%) of TBMs in predicting the status of the margins of the main specimen [51]. However, the study included patients with diverse clinical characteristics, such as those who did or did not undergo adjuvant therapy and neck dissection, those at different tumour stages, and those with an uncertain SM status.

On the other hand, some researchers have suggested that re-resections provide limited benefit in reducing LRs and that tumours with an initial cut-through SM often exhibit more aggressive biological behaviour compared to those with an initially negative SM [53]. Patients with initially positive margins who undergo additional resections still tend to have a worse prognosis compared to those with negative margins from the outset. A meta-analysis of retrospective studies assessing LRFS in early-stage OSCC found that revising initially positive SM to negative margins using frozen section guidance does not yield the same outcome as having clear margins from the outset and does not significantly enhance LC [53]. Additionally, Bulbul et al. demonstrated that patients requiring additional resection were 2.9 times more likely to experience recurrence than those with initially negative margins, even if the final resection resulted in what was considered a negative margin [53]. Buchakjian et al. compared LC in patients with positive main specimen margins who underwent additional tissue resection and found that once a positive margin was encountered on the main specimen, additional tissue resection did not impact LC. The risk of LR was lowest when SM were initially negative, higher when positive margins were revised to negative, and highest when final margins remained positive [54]. Guillemaud et al. conducted a study on 65 patients, showing that the presence of tumour cut-through with positive frozen margins was associated with poorer LC, regardless of the final margin status after additional resection and revision [55]. Similarly, Patel et al. found that microscopic tumour with positive frozen margins remained a negative prognostic factor even when revised to negative margins [49]. Coutu et al. reported a cohort study indicating that an initial positive margin was an independent predictor of reduced survival and poorer LC, irrespective of the outcomes of additional resection [56]. Recently, Kim et al. investigated the oncologic impact of revised negative SM (R1-R0) in a cohort of 441 patients with early-stage (pT1-2/N0) OSCC who underwent surgery without adjuvant therapy. In their retrospective analysis, LR was significantly higher ($p = 0.045$) in the R1-R0 group (13.1%) than in the R0 group (5.5%), and LRFS was better in the R0 group; however, there were no significant differences in overall recurrence or OS between these two groups. In their multivariate analysis, the authors identified an initial positive margin as an independent risk factor for LR (HR = 2.2; 95% CI, 1-4.9; $p = 0.043$). A revised clear SM after an initial cut-through margin increased the risk of LR in early-stage OSCC, emphasizing the need for caution in managing these patients [57].

Overall, these studies reinforce the idea that the presence of a positive margin during the initial resection negatively affects prognosis and may not be fully corrected by further resection to achieve control rates comparable to those of patients with initially negative margins. Several hypotheses have been proposed to explain why additional tissue resection may not improve survival or LC. If a positive margin is not the result of a surgical error but rather due to diffuse tumour infiltration and the absence of a clearly defined tumour edge, this could indicate an underlying tumour biology that is less responsive to primary surgical treatment [56]. Moreover, studies have shown that the prognostic significance of positive margins during the initial resection is influenced by the presence of regional metastases. This further reinforces the idea that individual tumour biology plays a role in modifying the impact of a positive margin [49]. Secondly, identifying the precise location of a positive margin after tumour resection can be challenging. Given the low sensitivity of conventional tumour bed sampling techniques, it is difficult to ensure that an adequate revision has been achieved or that the re-resection has been performed in the appropriate location. The spatial relationship between the resected specimen and the revised margin plays a crucial role in ensuring proper margin revision. Studies have indicated that, when a frozen section is positive, surgeons often struggle to precisely identify the corresponding site for additional sampling [39,47-49]. Previous research has shown that accurately re-orienting the surgical specimen is difficult, often resulting in an error exceeding 10 mm [37,58,59]. Another challenge in margin assessment is tissue shrinkage, which varies depending on the resection site (e.g., dorsal tongue or hard palate *vs* floor of the mouth, buccal mucosa, or soft palate) [40,60] (Tab. III).

Are tissue artefacts on margins a real issue?

In 1997, Johnson et al. investigated the potential shrinkage of tumour-free margins caused by tissue fixation. By

simulating margins on the benign oral mucosa of dogs, they observed a reduction of 30-47% when comparing *in vivo*, post-fixation, and slide preparation measurements. However, the use of non-human subjects and non-malignant tissue raises concerns about the applicability of these findings to human OSCC and surrounding mucosa [54]. Mistry et al. examined margin shrinkage in the oral cavity 30 minutes after tumour resection, reporting an average reduction of 23%, but did not account for changes following formalin fixation [61]. Cheng et al. retrospectively analysed pre-excision margins in oral cavity specimens and compared them to the final histopathologic margins, finding an average reduction of 59%, and showed that this shrinkage occurs immediately post-resection and, to a lesser degree, following formalin fixation [62]. Several other factors may also influence the extent of tissue shrinkage, including the resection technique used, the time elapsed before transferring the specimen to a formalin container (cold ischaemia), and delays in tissue processing [63], but also intrinsic contractility of mucosal tissues. Umstattd et al. analysed shrinkage in OSCC along the pre-analytical phase, evaluating tumour and margin measurements at different stages: *in vivo*, post-resection, and post-formalin fixation. Their findings indicate that the most significant shrinkage occurs between pre- and post-excision measurements, while no significant changes were observed between post-excision and post-formalin fixation. Given that the primary alterations in measurement take place before fixation, it is suggested that tissue shrinkage is predominantly due to inherent tissue properties rather than the effects of formalin processing [64]. Additionally, it appears to vary across different regions within the same specimen and to affect less neoplastic than normal tissue; consequently, SM tend to move closer to the tumour mass [65]. This difference is likely due to the infiltrative nature of tumour tissue. The variation in shrinkage can be explained by the distinct structural properties of the tissues. The intra-neoplastic stroma is often characterised by dense collagenisation, which creates a compact and rigid framework that resists formalin-induced shrinkage more effectively than the more flexible and loosely structured normal tissues. Studies have shown that shrinkage is more pronounced in the tongue (average shrinkage of 23.5%) compared to buccal mucosa (21.2%) [61]. In a recent study, Michcik et al. introduced the term macroscopic resection margins (MRM) to eliminate clinical ambiguities in their research. MRM refers to the visibly unchanged tissue surrounding the resected OSCC, extending from the tumour's outer edge to the surgical cut line. Their findings suggest that variations in MRM size are influenced by the specific tumour location. Notably, tongue tumours exhibited the most significant reduction in margins, with a median shrinkage of 1.5 mm. In comparison, tumours located in the floor of the mouth and maxilla showed slightly less shrinkage, with median values of 0.8 and 0.9 mm, respectively [66]. Additionally, tumours at earlier stages (T1/T2) tend to exhibit greater shrinkage than those at more advanced stages (T3/T4) [61].

Following surgical excision and fixation, the specimen undergoes visual assessment during the grossing process. In HNSCC, the approach to sectioning remains a topic of debate due to the absence of standardised protocols, unlike in organs such as the colon or breast. Typically, specimens are sectioned using either the "radial or perpendicular" method, which involves cutting at a right angle to the margin (Cover figure), or the "parallel or en-face" (shave margin) technique. Notably, only the first method enables precise measurement of the microscopic distance between the (inked) margin and the tumour [67]. Although the grossing step may appear straightforward and systematic, it is essential to acknowledge the possibility of errors during the initial sampling stages. Additionally, individual pathologist interpretation can introduce a degree of subjectivity in the subsequent histological assessment [68]. Regardless of the grossing method used for tumour processing, each subsequent histological step, including dehydration, clearing and infiltration, microtomy, haematoxylin and eosin (H&E) staining, and slide preparation, has the potential to induce tissue alterations that may affect microscopic evaluation [69].

As a result of all of the above, pathological margins are often significantly smaller than in-situ margins, which can influence the assessment of marginal status.

This brings up an important question: Can specimen shrinkage be reliably predicted? Moreover, how can this knowledge be applied to improve margin evaluation? A noteworthy study by Burns et al. explores this issue in greater detail [70]. The study identified an average shrinkage of 26% following resection and processing, leading to the suggestion that margin measurements should be adjusted accordingly. Implementing this adjustment could reduce the need for overly extensive resections, particularly in cases where tumour location limits surgical options due to functional or aesthetic concerns. Additionally, it may help prevent unnecessary re-resections or adjuvant therapy in cases with close or positive margins. It is important to emphasise that this is currently just a proposal. As suggested by Sengupta et al., variations in tissue shrinkage across different institutions and laboratories should be thorough-

ly assessed before determining a universally optimal margin distance. Each institute and laboratory should measure and quantify tissue shrinkage while accounting for all possible influencing factors [71]. If a strong inter-institutional correlation in the data is identified, the recommended margin distance could be applied universally. However, if significant discrepancies arise, tissue shrinkage should be reassessed based on influencing factors to determine an appropriate margin distance for each institution and possibly anatomical area. Additionally, the authors recommend that pathology reports on margin assessment include the percentage of shrinkage, enabling surgeons to interpret the findings with both aspects in mind. These institutional measurements could contribute to improved prognosis for HNSCC. Therefore, future studies should focus on the correlation between tissue shrinkage and the optimal margin distance required for favourable prognostic outcomes [72]. Recently Michcik et al., using modern 3D imaging techniques, developed a protocol for creating virtual images of OSCC that can be utilised in various ways, including enhancing surgeon-pathologist communication, mapping and describing tumours, and presenting cases at multidisciplinary board meetings. The creation of virtual images provides nearly unlimited possibilities for conducting and repeating measurements at countless points. Compared to traditional methodologies described in the literature, the use of virtual OSCC images represents a significant breakthrough, unlocking new opportunities that were previously unavailable [66] (Tab. IV).

Suggestions on how to evaluate the bone margin using frozen sections

While frozen sections provide intraoperative histological assessment of soft tissue margins, microscopic evaluation of bone margins is generally not available within the surgical timeframe, provided that calcified bone prevents the preparation of frozen sections. As a result, the surgical planning for bone-infiltrating carcinomas relies primarily on preoperative imaging techniques, such as MRI and CT. Despite advancements in imaging technology, accurately defining the extent of bone involvement before surgery remains a challenge. Additionally, preoperative imaging data cannot be directly applied intraoperatively to distinguish malignant from healthy tissue in real time [3]. As a result, uncertainty persists regarding bone infiltration, which may lead to misjudgment of the extent of carcinoma involvement, potentially causing either over- or under-treatment. Multivariate analyses have demonstrated

Table III. Prognostic impact of margin revision after positivity on frozen section analysis.

Key points	Description
Negative margins	Clear margins improve prognosis in head and neck cancer
Surgical challenges	Obtaining negative margins in the first attempt can be difficult
Margin revision debate	Uncertainty exists on whether re-resection improves outcomes
Recurrence risk	Higher recurrence rates in patients with revised margins compared to initially negative ones
Tumour biology role	Patients with aggressive tumours may not benefit from additional resection
Re-resection challenges	Difficult to identify and accurately resect positive margins

Table IV. Are tissue artefacts on margins a real issue?

Key points	Description
Specimen processing	After excision, tissue is fixed and sectioned, but standardisation in head and neck cancers is lacking
Tissue shrinkage	Margins shrink due to processing, with reductions up to 59%, depending on tissue type and location
Impact on margin assessment	Shrinkage leads to margins appearing smaller, affecting classification of negative, close, or positive margins
Regional variability	Tongue tissue shrinks more (23.5%) than buccal mucosa (21.2%), impacting interpretation of margins
Predicting shrinkage	Studies suggest measurements of margins should be adjusted to compensate for shrinkage effects
Institutional differences	Margin shrinkage varies across institutions; standardised reporting could improve prognostic evaluation

that only medullary invasion has clinical significance, whereas cortical invasion alone does not [73-75]. According to the latest NCCN guidelines, segmental resection is advised in cases of medullary space invasion. Frozen section analysis of bone marrow can be utilised intraoperatively to assist in guiding resection decisions [27]. Given the preoperative challenges in assessing bone invasion and the intraoperative limitation of frozen sections to soft tissue evaluation, Nieberler et al. implemented intraoperative cytological assessment of bone (ICAB) SM as an alternative approach [3,76-78]. In their latest research, ICAB showed a sensitivity of 88% and a specificity of 78.6% in determining adequate resection [3]. However, its clinical imple-

mentation revealed technical and logistical challenges, including desiccation of bone margins, insufficient or excessive trabecular material, and interference from blood cells, leading to cytological samples with poor quality. To improve diagnostic accuracy, bone margins were rinsed to remove excess blood, bone dust, and floating carcinoma cells, which do not indicate true bone infiltration. It is also crucial to avoid contamination from instruments, gloves, or residual tumour tissue during transport to prevent false positive results. The quality of ICAB relies heavily on expert cytological assessment, as prior radiotherapy may alter cells morphology, and immature haematopoietic precursor cells can be misidentified as malignant. Additionally, complex margins, infiltrative carcinoma growth, and prior radiotherapy pose challenges to both frozen sections and ICAB.

Collaboration with the surgical team

At the institutional level**,** several measures can enhance the accuracy of SM assessment, such as dedicated head and neck pathologists, standardised synoptic reporting, uniform laboratory processing protocols, and external quality control through routine audits. To improve specimen orientation and facilitate interpretation, the Canadian surgeons survey reported employing 3D specimen orientation techniques, using various marking methods to indicate the closest representative margin [44]. Another promising area of research is the integration of 3D modelling to improve communication between surgeons and pathologists. Saturno et al. [79] and Sharif et al. [80] have demonstrated the feasibility of scanning surgically resected specimens to generate virtual 3D models, which can be annotated by pathologists for real-time intra- and postoperative consultations. While this technology remains in the experimental phase and was not directly examined in this survey, it holds potential as a valuable tool for enhancing SM assessment in the future.

The use of tissue biomarkers in the evaluation of tumour margins

The rationales supporting the search for biomarkers that could indicate either absence or presence of neoplastic transformation on SM are: (1) the observation that tumour tissue and non-neoplastic mucosa bear inherent molecular differences; (2) the theory of field cancerisation in the upper aerodigestive tract mucosa, and (3) the possibility that the number of tumour cells left beyond resection margins is too small to be detected by routine histopathology (minimal residual cancer, MRC) [81]. Several findings have shown that microscopically normal mucosa surrounding SCC may host molecular abnormalities shared with tumour tissue, suggesting preneoplastic transformation [82]. They also supported the hypothesis that the presence of these abnormalities on SM can negatively affect prognosis and possibly explain the limited association between conventional margin stratification and patient outcomes discussed in the above sections. Recurrence rates at the primary tumour site after R0 resection range between 12% and 30% [83,84]. For these reasons, morphological margin examination may be supported using ancillary techniques. These are roughly distinguished in two main groups, although significant overlap exists: molecular and immunohistochemical analyses. Both may integrate morphological examinations and contribute to clarifying SM status. Molecular techniques provide a more comprehensive description of tissues as well as of their genomic status. However, they are expensive and time-consuming, requiring many days to be performed. Immunohistochemical analyses, instead, are less expensive and require less time, representing a valid support for routine practice (Fig. 1). More recently, protein biomarkers have also been proposed for fluorescence-guided surgical identification of negative mucosal margins [85,86].

Not surprisingly, the number of biomarkers that are expressed differentially in HNSCC and normal mucosa, and that may signal the presence of tumour cells along SM, is very high, including p53, microsatellite instability, DNA ploidy, CCDN1, p16, Chk2, laminin-5, glycosylated oncofetal fibronectin, galectin-9, hypermethylated gene promoters [87-89], and overall genomic imbalance [90]. A relevant number of studies have aimed to identify if the presence of any of these biomarkers on SM could predict LR (Tab. V) [76,79,82,86,88,92-122]. The list of those that have been proven to significantly predict LR in small case-control series is relatively limited. Among these, the status of p53 has been most widely studied with increasingly accurate methods over time. Other promising biomarkers include overexpression of eIF4E, loss of PERP and cornulin, overall loss of heterozygosity (LOH), p16, cyclin D1, beta-catenin, MMP9 and PTHLH RNA levels, chromosome instability, and profile of genes with promoter methylation.

It is worth noting that the bulk of studies on so-called "molecular margins" was published in the first 2 decades of this century; moreover, there are no prospective studies validating the prognostic value of biomarker expression analysis on traditionally sampled HNSCC margins, and

biomarkers have not yet been applied as a support to surgical or therapeutic decisions. In reviewing the literature and discussing the multitude of molecular markers that have been evaluated for use, one factor that limits their application is the low coverage of these markers within the SM, and the relatively low specificity for tumour. For the widely studied p53, the myriad of genetic variants makes it difficult to develop simple assays that can be integrated into the clinical setting and limits the utility of mutant p53 assessment. Other examples, which include the early genetic event involving LOH at 9p21 to 22, observed in approximately 70% of HNSCC cases, dysregulation of p16, either via homozygous deletion (67%) or increased promoter methylation (21%), or microsatellite instability (50-60%), illustrate that no marker is expressed homogeneously (Tab. V). Definite evidence of practical benefits in treatment decisions stemming from molecular evaluation of SM is still lacking. Possibly the most significant concern that has so far prevented the use of molecular alterations to assess the SM in clinical practice is determining a particular biomarker's predictive value for malignant disease.

Moreover, most of the methods reported so far are not compatible with the rapid response required for margin status by clinicians. However, Goldenberg and colleagues used quantitative methylation-specific PCR (QMSP) to determine the hypermethylation pattern of p16 and MGMT promoters in the SM. Although the number of patients in this study was small, the investigators showed that QMSP analysis could be performed within a period compatible with intraoperative assessment [122]. One further limitation is related to the complexity of complete tissue sampling over a large surface of marginal tissue. The concept of margin imprinting presented by Hayashi et al. [123] is interesting. Their strategy may ease the difficulty of collecting relevant SM samples that represent the entire area of irregular surgical resection.

More recently, whole genome sequences of HNSCCs available in public databases offer the opportunity for a different approach to molecular profiling of cancer, and possibly to overcome the limitations of single biomarker analysis. One recent study [124] provided a gene signature associated with positive SM in tongue SCC, possibly predicting tumour resectability and informed surgical decisions. Furthermore, a pioneering microRNA signature in peripheral blood has recently been associated with disease recurrence in OSCC [125], paving the way for the application of liquid biopsy in the future.

Non-histopathological approaches to the evaluation of tumour margins: back to the surgical bed

Evaluating tumour margins intraoperatively is crucial to ensure complete tumour resection and minimise recurrence. In recent years, significant efforts have been directed toward developing non-invasive optical imaging techniques to support clinical diagnosis and treatment. These imaging methods provide a way to analyse the structural and compositional characteristics of tissues and cells in vivo, by detecting variations in their optical properties, resulting in highly detailed images of organs and biological structures [126]. Various optical imaging modalities integrating surgery have been documented in the literature, including narrow band imaging (NBI), Raman spectroscopy (RS), optical coherence tomography (OCT), hyperspectral imaging (HSI), computer-assisted surgery (CAS), and fluorescence imaging. These techniques leverage anatomical and metabolic alterations, as well as the chemical composition of tissues [127]. Due to its ability to minimise patient exposure to harmful radiation while offering valuable insights into the properties of soft tissue, optical imaging has become an essential tool in clinical practice. It is widely used to identify abnormal lesions, enabling early disease detection, providing real-time visualisation of SM, assisting in tumour removal, and tracking treatment response.

NBI is a fast, noninvasive, and safe endoscopic technique that employs two specific wavelength filters, blue and green, to improve the visualisation of mucosal structures and superficial blood vessels, thus allowing the distinction of patterns of distribution between tumour and normal mucosa. With central wavelengths of 415 nm for blue light and 540 nm for green light, NBI takes advantage of haemoglobin's strong absorption properties, causing blood vessels to appear in darker shades, such as brown or cyan, in contrast to the surrounding mucosa [128]. As a result, surgeons utilise NBI to assess characteristics of intra-epithelial papillary capillary loops for early lesion detection and differentiation between benign and malignant conditions. Key features examined include the extent of vessel dilation, curvature, variations in vessel calibre and morphology, as well as colour alterations in surrounding mucosal tissue [129]. Research indicates that defining the incisional margin using NBI can lower the likelihood of positive tumour margins, enhance patient survival, and decrease recurrence rates compared to standard white-light endoscopy. In a study by Tirelli et al., NBI demonstrated a

Table V. Summary of studies evaluating the prognostic impact of biomarker expression on surgical margins.

Biomarker	Method	Site	Tumour-associated defect	Cases/controls	
TP53 gene	Sequencing Southern blot	Head neck	Mutation	5/20	
EIF4E protein	IICH	Head neck	Overexpression	22/43	
EIF4E protein P53 protein	IICH IICH	Larynx	Overexpression Overexpression	25/29	
TP53 gene P53 protein	Phage assay IICH	Oral cavity	Mutation Overexpression	5/6	
Microsatellites	PCR, Gel electrophoresis	Multiple	Instability	8/17	
P53 protein MMP9 protein 4E protein	IICH IICH IICH	Head neck	Overexpression	14/38	
TP53 gene	Plaque assay	Multiple	Mutation	15/61	
Microsatellites	PCR, Gel electrophoresis	Multiple	Instability	5/21	
DNA	DNA-image cytometry	Oral cavity	Aneuploidy	20/20	
TP53 gene	PCR, Plaque assay	ND	Mutation	13/12	
CDKN2A (p16), *CCNA1, DCC* genes	QMSP	Multiple	Promoter methylation	5/22	
BIRC5 protein CD44v6 protein	IICH IICH	Larynx	Overexpression Overexpression	41/71	
DNA	Interphase-FISH	Oral cavity	Polyploidy	4/15	
P53 protein	IICH	Oral cavity	Overexpression	4/15	
Cytokeratin 4 protein Cornulin protein	IICH IICH	Oral cavity and oropharynx	Hypoexpression Hypoexpression	23/23	
CDKN2A gene	QMSP	Tongue	Promoter methylation	6/24	
TP53 gene	LigAmp	Head neck	Mutations	15/80	
Chromosome 3p Chromosome 9p Chromosome 17p P53 protein	Sequence Sequence Sequence IICH	Oral cavity and oropharynx	LOH LOH LOH Overexpression > 5%	16/19	
TP53 gene Ly-6D mRNA	LigAmp qRT-PCR	Oral cavity	Mutation Expression	43/59	
PTHLH mRNA EPCAM mRNA MMP9 mRNA	qRT-PCR qRT-PCR qRT-PCR	Multiple	Overexpression 5x Overexpression 10x Overexpression 10x	4/51	
DCC, EDNRB, p16, *KIF1A* genes	QMSP	Head neck	Promoter methylation profile	6/11	
DSPP protein BSP protein OPN protein MMP-2 protein MMP-3 protein MMP-9 protein	IICH IICH IICH IICH IICH IICH	Oral cavity	Expression	9/11	
P53 protein Cyclin D1 protein	IICH IICH	Multiple	Overexpression Overexpression	35/81	
DCC, EDNRB, HOXA9, KIF1A, NID2, NR2B genes	QMSP	Head neck	Promoter methylation profile	Total 65	
PAX5 gene	QMSP; ddQMSP	Head neck	Promoter methylation	35/25	

	Prevalence	Specificity (%)	Sensitivity (%)	Analysis	Significance	Notes	Ref.
	13/25	5/5 (100)	12/20 (60)	KM/log-rank	Yes		82
	36/65	20/22 (91)	27/43 (63)	KM/log-rank	Yes		92
	32/54 6/54	21/25 (84) 6/23 (26)	18/29 (82) 31/31 (100)	KM/log-rank Multivariate KM/log-rank Multivariate	Yes Yes Yes No		93
	6/11	4/5 (80)	4/6 (67)	ND			94
	11/25	7/8 (88)	13/17 (76)	KM/log-rank Multivariate	Yes Yes		95
	24/52 28/52 27/52	9/14 (64) 11/14 (78) 12/14 (86)	23/38 (60) 21/38 (55) 22/38 (58)	KM/Cox proportional hazard/ multivariate	No No Yes/Yes		96
	50/76	14/15 (93)	25/61 (41)	KM/log-rank	Yes		76
	7/26	5/5 (100)	19/21 (90)	KM/log-rank	Yes		97
	16/40	14/20 (70)	18/20 (90)	Fisher exact test	Yes		98
	16/25	11/13 (85)	7/12 (58)	KM/log-rank	Yes	Deep margins also evaluated	99
	11/27	5/5 (100)	16/22 (73)	KM/log-rank	Yes	At least 1 marker positive	100
	44/112 35/112	26/41 (63) 20/41 (49)	53/71 (75) 56/71 (79)	Cox proportional hazard/ multivariate	Yes/Yes Yes/Yes		101
	8/19	4/4 (100)	11/15 (73)	Fisher exact test	Yes		102
	8/19	3/4 (75)	10/15 (67)	Fisher exact test	No		103
	23/46 32/46	17/23 (74) 16/23 (70)	17/23 (74) 16/23 (70)	KM/log-rank	Yes Yes		104
	13/30	5/6 (67)	16/24 (67)	KM/log-rank	Yes		105
	35/95	9/15 (60)	54/80 (67)	Log-rank	No		106
	ND	ND ND (62) ND ND (62)	ND ND (74) ND ND (84)	KM/Cox proportional hazard	No Yes No Yes		107
	51/102 ND	ND ND	ND ND	KM/log-rank/multivariate	Yes/Yes No	Combined markers significant	108
	5/55 6/55 13/55	0/4 (0) 0/4 (0) 4/4 (100)	46/51 (90) 45/51 (88) 42/51 (82)	KM/log-rank Cox proportional hazard	No No Yes		79
	8/17	5/6 (83)	8/11 (72)	N/A	NA	Tissue imprints	109
	11/20 13/20 11/20 12/20 13/20 7/20	8/9 (89) 6/9 (67) 7/9 (78) 5/9 (55) 7/9 (78) 6/9 (67)	6/11 (54) 4/11 (36) 7/11 ((64) 4/11 (36) 5/11 (45) 10/11 (91)	KM/Cox proportional hazard	Yes No No No No Yes		110
	34/116 46/116	ND	ND	KM/Cox proportional hazard/ multivariate	No Yes/No	No association with OS	111
	ND	ND	ND	Cox proportional hazard	Significant combined markers; no single	Significant on margin imprint, negative histological	112
	16/60	ND	ND	Cox proportional hazard	Yes	Margin imprint and tissue	86

Table V. *continues.*

Biomarker	Method	Site	Tumour-associated defect	Cases/controls	
Microsatellite	Sequence	Oral cavity	Instability LOH	24/58	
P53 protein eIF4E Protein	IICH IICH	Oral cavity and oropharynx	Overexpression Overexpression	7/17	
Chromosome 9p Chromosome 17p P53 protein P14 protein P15 protein P16 protein	PCR/gel electrophoresis IICH IICH IICH	Oral cavity	LOH LOH Overexpression Overexpression Overexpression Overexpression	15/56	
Chromosome 1 & 7 P53	FISH IICH	Oral cavity	Copy number variation Overexpression	11/24 11/31	
βcatenin protein βcatenin protein βcatenin mRNA	Western blot IICH qRT-PCR	Oral cavity	Overexpression Nuclear expression Overexpression	26/54	
Microsatellite	Sequence	Oral cavity	Instability	26/29	
P53 protein	IICH	Oral cavity	Overexpression	16/20	
PERP protein	IICH	Multiple	Loss of expression	NA	
Conulin protein KI67/MIB1 protein ISG15 protein	IICH IICH IICH	Oral cavity	IRS-score (hig/low) % positive (hig/low) IRS-score (hig/low)	15/19	
TP53 gene P53 protein	RT-PCR IICH	Oral cavity	Amplification Overexpression	21/19	
TP53 gene Circulating miRNA	NGS + ddPCR	Multiple	VAFs Level quantification	47 49 (28 both tests)	

KM: Kaplan-Meier; IICH: immunohistochemistry; ND: not detailed; PCR: polymerase chain reaction; QMSP: quantitative methylation-specific PCR; FISH: fluorescent in situ hybridisation; LOH: loss o time PCR; NGS: next generation sequencing; ddPCR: digital droplet polymerase chain reaction.

sensitivity of 100% and a specificity of 88.9% in evaluating incisional margins [130].

NBI has notable limitations when evaluating deep incisional margins, as it is unable to detect submucosal tumour extension. Consequently, NBI is most effective for assessing the margins of superficial and early-stage tumours. For locally-advanced tumours, where precise evaluation of deep margins is required, fluorescence molecular imaging offers a more accurate depiction of tumour boundaries [131].

RS is a light scattering technique based on the Raman effect and is capable of generating unique spectral fingerprints that reveal the molecular composition and biochemical characteristics of tumour tissue. By reflecting histopathological changes, RS provides valuable insights for clinical applications, including early cancer detection, tumour margin assessment, identification of metastatic lymph nodes, and identification of recurrence. The method is fast, non-invasive, requires minimal sample material, does not rely on fluorescence properties, and eliminates the need for special labelling, making it a promising tool in cancer diagnostics [132]. RS has certain limitations. One challenge is the difficulty in fully distinguishing tumour from normal tissue. Due to biochemical similarities between SCC and the squamous epithelium, along with changes in nucleic acid and protein levels in inflamed connective tissue, there is a risk of misdiagnosing normal epithelium or inflammatory tissue as SCC [133,134]. However, studies suggest that false positive results do not impact surgical outcomes as long as a 5-10 mm margin around the tumour is excised [134]. Another limitation is the lack of a standardised diagnostic threshold to differentiate cancerous from non-cancerous tissue using spectral analysis. Yan et al. in their research introduced a novel approach combining Raman imaging technology with an AI generative model to enhance intraoperative margin assessment. In their research, Raman images were converted into H&E-stained histopathological representations using an AI generative model. This approach facilitated the transformation of RS images into visual histopathological images, eliminating the need for traditional tissue

	Prevalence	Specificity (%)	Sensitivity (%)	Analysis	Significance	Notes	Ref.
	40/82 76/82	ND ND	ND ND	KM/log-rank	Yes No		113
	11/24 16/24	4/7 (57) 1/7 (14)	10/17 (59) 15/17 (88)	KM/log-rank	No No		114
	14/65 17/67 22/71 39/71 30/71 30/71	6/13 (46) 6/15 (40) 10/15 (67) 11/15 (73) 9/15 (60) 15/15 (100)	44/52 (85) 41/52 (79) 44/56 (78) 28/56 (50) 35/56 (62) 38/56 (68)	KM/Cox proportional hazard/ multivariate Cox regression	Yes/Yes Yes/No Yes/No No/No Yes/No Yes/No	TP16+1 9p microsatellite significant in multivariate analysis	115
	18/35 12/42	9/11 (82) 4/11 (36)	15/24 (62) 16/31 (52)	KM/log rank test/multivariate Cox regression	Yes/Yes No		116
	47/80	21/26 (81)	28/54 (52)	KM/log-rank	Yes	Pooled results	117
	41/55	22/26 (85)	10/29 (34)	Logistic regression	Yes	Pre-selected MSI+ tumour	118
	32/72	14/16 (87)	38/40 (95)	KM/Cox proportional hazard	Yes	Dysplastic margins	119
	19/46	NA	NA	KM/log-rank	Yes		120
	10/34	7/15 (47) 2/15 (13) 3/15 (20)	16/19 (84) 18/19 (95) 18/19 (95)	Pearson Chi-square/binary logistic regression	Yes No No		121
	38/40	16/21 (76) 19/21 (90)	14/19 (74) 17/19 (89)	KM/Cox proportional hazard/ Multivariate COX regression	No No	Significant association with OS	122
	30/47 26/49	27/42 (64) 25/47 (53)	2/5 (40) 0	KM KM	Yes Yes	Significant biomarker combination	88

f heterozygosity; qRT-PCR: quantitative retro-transcribed polymerase chain reaction; NA: not assessed; OS: overall survival; MSI: microsatellite instability; IRS: immune response score; RT-PCR: real

section preparation and staining processes. The AI-generated H&E images provided a clear visualisation of the pathological characteristics of the tissue and demonstrated superior performance compared to conventional clinical practice, particularly in cases of HNSCC with metastatic lymph nodes or moderate differentiation [135].

HSI is a remote optical imaging technique that captures a single image across potentially hundreds of distinct wavelengths. This method provides detailed information about tissue composition, assisting surgeons in identifying tumour margins more precisely. Fabelo et al. highlighted the importance and effectiveness of HSI in detecting brain cancer in vivo and designed a visualisation system that leverages a machine learning-based classification algorithm for near-real-time guidance [136]. Additionally, research on deep learning approaches applied to the same dataset revealed superior performance compared to traditional machine learning methods. Boehm et al. conducted a proof-of-concept study to evaluate whether HSI, combined with AI through a Convolutional Neural Network (CNN) and a Graph Neural Network (GNN), can improve the differentiation between healthy and malignant tissue in *ex vivo* head and neck tumour specimens. The goal was to determine its potential for effective margin assessment. Their study included 32 patients diagnosed with HNSCC. Following surgical resection, their specimens underwent *ex vivo* HSI imaging, with annotated regions used to train the CNN and GNN models. Imaging parameters were carefully optimised to enhance efficiency, allowing for a more objective and rapid evaluation of SM. Their findings suggest that integrating HSI with neural networks provides additional benefits in distinguishing cancerous from healthy tissue. Despite the relatively small dataset (32 patients), the AI models demonstrated high accuracy [137].

CAS plays a crucial role in improving margin assessment during HNC surgery. By integrating imaging techniques such as CT, MRI, and PET, CAS enables precise preoperative visualisation and delineation of tumour margins, reducing the risk of leaving residual cancerous tissue [138,139]. In OSCC with mandibular involvement, CAS has shown

Table VI. Non-histopathological approaches to the evaluation of tumour margins.

Key points	Description
Importance of margin assessment	Intraoperative tumour margin assessment is crucial for complete tumour resection and reduction of recurrence risk
Optical imaging techniques	NBI, RS, OCT, HSI, CAS, and fluorescence imaging improve tumour margin visualisation
Narrow band imaging (NBI)	Enhances superficial tumour margin assessment but is ineffective for deep margins
Raman spectroscopy (RS)	Provides biochemical insights but has limitations in differentiating SCC from normal tissue
HyperSpectral Imaging (HSI)	Effective with AI assistance but limited by long acquisition times
Computer-sssisted surgery (CAS)	Improves preoperative planning but lacks conclusive oncological margin control evidence
Fluorescence imaging	Several markers (5-ALA, cetuximab-IRDye800CW, etc.) show promise for intraoperative tumour delineation
Ex vivo microscopy	Studies demonstrate high sensitivity and specificity in SCC margin assessment

effectiveness in controlling SM, with outcomes comparable to those of traditional surgical techniques. Some studies suggest that CAS enhances both surgical precision and postoperative functional and aesthetic results due to its accuracy in planning and executing resections [140]. However, there is no conclusive evidence that cutting guides significantly improve oncological margin control in mandibular SCC resections. Research into mixed reality systems, which integrate real-time 3D imaging with CAS, is exploring ways to further improve intraoperative margin assessment, though these technologies remain in experimental phases [141,142].

In recent years, there has been growing interest in the use of fluorescent markers to enhance tumour detection and delineation during surgery, providing real-time intraoperative guidance. These markers aim to provide a clear distinction between malignant and healthy tissues, ensuring precise excision margins. An ideal fluorescent marker should selectively bind to cancer cells, accumulate sufficiently to produce fluorescence, and effectively differentiate malignant from healthy tissue. Several fluorescent markers have been studied for their potential use in HNSCC detection, including toluidine blue, ICG, 5-ALA, and IRDye800CW-conjugated anti-EGFR antibodies. Toluidine blue has seen limited recent research [143] due to concerns over specificity. While ICG is FDA- and EMA-approved [144], its effectiveness in HNSCC remains uncertain [145], although newer ICG-based markers like ONM-100 may offer better tumour visualisation. 5-ALA, already used in glioma surgery [146], has shown promise for HNSCC in recent studies [147]. Meanwhile, fluorescently labeled cetuximab and panitumumab are gaining attention, with multiple ongoing clinical trials. Panitumumab-IRDye800CW has demonstrated potential for improving surgical decision-making [148]. Srinivasan et al. conducted a systematic review to identify and compare fluorescent markers used for the detection and delineation of HNSCC. Among the most promising candidates, they identified 5-ALA, cetuximab-IRDye800CW, panitumumab-IRDye800CW, and targeted ICG derivatives such as ONM-100 [149]. Recent studies have explored *ex vivo* microscopy for evaluating excision margins in HNSCC. Shavlokhova et al. [150] reported a sensitivity and specificity of 99% and 95% using *ex vivo* imaging of oral cavity lesions. A systematic review of 6 confocal laser endomicroscopy studies [151] showed 95% sensitivity and 93% specificity, while Zanoni et al. [152] demonstrated 90% sensitivity and 98.3% specificity for *in vivo* reflectance confocal microscopy (Tab. VI).

Conclusions

In HNC surgery, evaluation of margin status represents an important part of tumour staging and prognostic assessment. This may be obtained in frozen and definitive sections, sometimes with the use of ancillary techniques. It should be noted, however, that the risk of LR is affected not only by the surgical excision margin. Alongside this, T category, degree of differentiation, pN, lympho-vascular, perineural, depth, and pattern of invasion are other independent risk factors for LR.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

All authors contribuited equally to the work.

Ethical consideration

Not applicable.

References

1 Meier JD, Oliver DA, Varvares MA. Surgical margin determination in head and neck oncology: current clinical practice. The results of an international American Head and Neck Society member survey. Head Neck 2005;27:952-958. https://doi.org/10.1002/hed.20269

2 Hamman J, Howe CL, Borgstrom M, et al. Impact of close margins in head and neck mucosal squamous cell carcinoma: a systematic review. Laryngoscope 2022;132:307-321. https://doi.org/10.1002/lary.29690

3 Nieberler M, Stimmer H, Rasthofer D, et al. Defining secure surgical bone margins in head and neck squamous cell carcinomas: the diagnostic impact of intraoperative cytological assessment of bone surgical margins compared with preoperative imaging. Oral Oncol 2020;102:104579. https://doi.org/10.1016/j.oraloncology.2020.104579

4 Chen Y, Zhong NN, Cao LM, et al. Surgical margins in head and neck squamous cell carcinoma: a narrative review. Int J Surg 2024;110:3680-3700. https://doi.org/10.1097/JS9.0000000000001306

5 Young K, Bulosan H, Kida CC, et al. Stratification of surgical margins distances by the millimeter on LR in oral cavity cancer: a systematic review and meta-analysis. Head Neck 2023;45:1305-1314. https://https://doi.org/10.1002/hed.27339

6 Singh A, Mishra A, Singhvi H, et al. Optimum surgical margin in squamous cell carcinoma of the oral tongue: is the current definition adequate? Oral Oncol 2020;111:104938. https://doi.org/10.1016/j.oraloncology.2020.104938

7 Nason RW, Binahmed A, Pathak KA, et al. What is the adequate margin of surgical resection in oral cancer? Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:625-629. https://doi.org/10.1016/j.tripleo.2008.11.013

8 Dudkiewicz D, Yosefof E, Shpitzer T, et al. Rethinking surgical margins: a new approach to predict outcomes in oral squamous cell carcinoma. Laryngoscope 2025;135:161-167. https://doi.org/10.1002/lary.31744

9 Cooper JS, Zhang Q, Pajak TF, et al. Long-term follow-up of the RTOG 9501/intergroup phase III trial: postoperative concurrent radiation therapy and chemotherapy in high-risk squamous cell carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 2012;84:1198-1205. https://doi.org/10.1016/j.ijrobp.2012.05.008

10 Bernier J, Cooper JS, Pajak TF, et al. Defining risk levels in locally advanced head and neck cancers: a comparative analysis of concurrent postoperative radiation plus chemotherapy trials of the EORTC (#22931) and RTOG (# 9501). Head Neck 2005;27:843-850. https://doi.org/10.1002/hed.20279

11 www.cap.org/cancerprotocols2011

12 Kim Y, Lee C, Heo Y, et al. Impact of dysplasia at surgical margins on oncologic outcome after curative resection of oral tongue squamous cell carcinoma: significance of high-grade dysplastic surgical margins. Eur Arch Otorhinolaryngol 2024;281:441-449. https://doi.org/10.1007/s00405-023-08233-0

13 Chen TC, Chang HL, Yang TL, et al. Impact of dysplastic surgical margins for patients with oral squamous cell carcinoma. Oral Oncol 2019;97:1-6. https://doi.org/10.1016/j.oraloncology.2019.07.015

14 Senarath N, Jayasooriya P, Siriwardena B, et al. Epithelial dysplasia at excision margins of oral squamous cell carcinoma: a review on relationship to clinicopathological parameters and prognosis. Asian Pac J Cancer Prev 2021;22:2313-2321. https://doi.org/10.31557/APJCP.2021.22.8.2313

15 Bernard SE, van Lanschot CGF, Hardillo JA, et al. A new proposal for adequate surgical margins in larynx and hypopharynx tumor surgery-are the rcp guidelines feasible? Cancers 2024;16:2058. https://doi.org/10.3390/cancers16112058

16 Hinni ML, Zarka MA, Hoxworth JM. Margin mapping in transoral surgery for head and neck cancer. Laryngoscope 2013;123:1190-1198. https://doi.org/10.1002/lary.23900

17 Kutter J, Lang F, Monnier P, et al. Transoral laser surgery for pharyngeal and pharyngolaryngeal carcinomas. Arch Otolaryngol Head Neck Surg 2007;133:139-144. https://doi.org/10.1001/archotol.133.2.139

18 Bernard SE, van Lanschot CGF, Sewnaik A, et al. Clinical relevance of surgical margins in patients with total laryngectomy or laryngopharyngectomy. Cancers 2024;16:2038. https://doi.org/10.3390/cancers16112038

19 Bich TA, Vuong NL, Cam Tu NCHTN, et al. Long-term survival of patients after total pharyngolaryngoesophagectomy with gastric pull-up reconstruction for hypopharyngeal or laryngeal cancer invading cervical esophagus. Ann Otol Rhinol Laryngol 2023;132:511-518. https://doi.org/10.1177/00034894221098802

20 Mazerolle P, Philouze P, Garrel R, et al. Oncological and functional outcomes of trans-oral robotic surgery for pyriform sinus carcinoma: a French GETTEC group study. Oral Oncol 2018;86:165-170. https://doi.org/10.1016/j.oraloncology.2018.09.014

21 Omura G, Ando M, Saito Y, et al. Disease control and clinicopathological prognostic factors of total pharyngolaryngectomy for hypopharyngeal cancer: a single-center study. Int J Clin Oncol 2015;20:290-297. https://doi.org/10.1007/s10147-014-0709-z

22 Bova R, Goh R, Poulson M, et al. Total pharyngolaryngectomy for squamous cell carcinoma of the hypopharynx: a review. Laryngoscope 2005;115:864-869. https://doi.org/10.1097/01.MLG.0000158348.38763.5D

23 Grasl S, Frommlet F, Faisal M, et al. A new nomogram to predict oncological outcome in laryngeal and hypopharyngeal carcinoma patients after laryngopharyngectomy. Eur Arch Otorhinolaryngol 2023;280:1381-1390. https://doi.org/10.1007/s00405-022-07668-1

24 Wulff NB, Andersen E, Kristensen CA, et al. Prognostic factors for survival after salvage total laryngectomy following radiotherapy or chemoradiation failure: a 10-year retrospective longitudinal study in eastern Denmark. Clin Otolaryngol 2017;42:336-346. https://doi.org/10.1111/coa.12726

25 Spector JG, Sessions DG, Lenox J, et al. Management of T3N1 glottic carcinoma: therapeutic outcomes. Laryngoscope 2006;116:106-110. https://doi.org/10.1097/01.mlg.0000184767.62682.3d

26 Saraniti C, Speciale R, Gallina S, et al. Prognostic role of surgical margins in open oncologic laryngeal surgery: survival analysis of a cohort of 139 patients affected by squamous cell carcinoma. Braz J Otorhinolaryngol 2019;85:603-610. https://doi.org/10.1016/j.bjorl.2018.04.012

27 Nakayama M, Holsinger C, Okamoto M, et al. Clinicopathological analyses of fifty supracricoid laryngectomized specimens: evidence base supporting minimal margins. ORL J Otorhinolaryngol Relat Spec 2009;71:305-311. https://doi.org/10.1159/000261836

28 Fiz I, Mazzola F, Fiz F, et al. Impact of close and positive margins in transoral laser microsurgery for Tis-T2 glottic cancer. Front Oncol 2017;7:245. https://doi.org/10.3389/fonc.2017.00245

29 Bradford CR, Wolf GT, Fisher SG, et al. Prognostic importance of surgical margins in advanced laryngeal squamous carcinoma. Head Neck 1996;18:11-16. https://doi.org/10.1002/(SICI)1097-0347(199601/02)18:1<11::AID-HED2>3.0CO;2-1

30 Ho CM, Ng WF, Lam KH, et al. Submucosal tumor extension in hypopharyngeal cancer. Arch Otolaryngol Head Neck Surg 1997;123:959-965. https://doi.org/10.1001/archotol.1997.01900090073010

[31] Wei WI. The dilemma of treating hypopharyngeal carcinoma: more or less. Hayes Martin Lecture. Arch Otolaryngol Head Neck Surg 2002;128:229-232. https://doi.org/10.1001/archotol.128.3.229

[32] Ho CM, Ng WF, Lam KH, et al. Radial clearance in resection of hypopharyngeal cancer: an independent prognostic factor. Head Neck 2002;24:181-190. https://doi.org/10.1002/hed.10002

[33] NCCN Guidelines Version 4.2024 Head and Neck Cancers.

[34] Black C, Marotti J, Zarovnaya E, et al. Critical evaluation of frozen section margins in head and neck cancer resections. Cancer 2006;107:2792-2800. https://doi.org/10.1002/cncr.22347

[35] Shapiro M, Salama A. Margin analysis: squamous cell carcinoma of the oral cavity. Oral Maxillofac Surg Clin North Am 2017;29:259-267. https://doi.org/10.1016/j.coms.2017.03.003

[36] Hinni ML, Ferlito A, Brandwein-Gensler MS, et al. SM in head and neck cancer: a contemporary review. Head Neck 2013;35:1362-1370. https://doi.org/10.1002/hed.23110

[37] Chang AMV, Kim SW, Duvvuri U, et al. Early squamous cell carcinoma of the oral tongue: comparing margins obtained from the glossectomy specimen to margins from the tumor bed. Oral Oncol 2013;49:1077-1082. https://doi.org/10.1016/j.oraloncology.2013.07.013

[38] Baddour HM, Magliocca KR, Chen AY. The importance of margins in head and neck cancer. J Surg Oncol 2016;113:248-255. https://doi.org/10.1002/jso.24134

[39] Maxwell JH, Thompson LDR, Brandwein-Gensler MS, et al. Early oral tongue squamous cell carcinoma: sampling of margins from tumor bed and worse local control. JAMA Otolaryngol Head Neck Surg 2015;141:1104-1110. https://doi.org/10.1001/jamaoto.2015.1351

[40] Buchakjian MR, Tasche KK, Robinson RA, et al. Association of main specimen and tumor bed margin status with local recurrence and survival in oral cancer surgery. JAMA Otolaryngol Head Neck Surg 2016;142:1191-1198. https:doi.org/10.1001/jamaoto.2016.2329

[41] DiNardo LJ, Lin J, Karageorge LS, et al. Accuracy, utility, and cost of frozen section margins in head and neck cancer surgery. Laryngoscope 2000;110:1773-1776. https://doi.org/10.1097/00005537-200010000-00039

[42] Horwich P, MacKay C, Bullock M, et al. Specimen oriented intraoperative margin assessment in oral cavity and oropharyngeal squamous cell carcinoma. J Otolaryngol Head Neck Surg 2021;50:37. https://doi.org/10.1186/s40463-021-00501-5

[43] Bulbul MG, Zenga J, Tarabichi O, et al. Margin practices in oral cavity cancer resections: survey of American Head and Neck Society members. Laryngoscope 2021;131:782-787. https://doi.org/10.1002/lary.28976

[44] Daniel RC, Yan B, Chandarana S, et al. SM definition and assessment in head and neck oncology: a cross-sectional survey of Canadian Head and Neck Surgeons. J Otolaryngol Head Neck Surg 2024;53:19160216241296121. https://doi.org/10.1177/19160216241296121

[45] van Lanschot CGF, Mast H, Hardillo JA, et al. Relocation of inadequate surgical margins in the wound bed during oral cavity oncological surgery: a feasibility study. Head Neck 2019;41:2159-2166. https://doi.org/10.1002/hed.25690

[46] Villemure-Poliquin N, Roy ÈM, Nguyen S, et al. Tumor bed margins versus specimen margins in oral cavity cancer: too close to call? J Otolaryngol Head Neck Surg 2024;53:19160216241278653. https://doi.org/10.1177/19160216241278653

[47] Prabhu AV, Sturgis CD, Lai C, et al. Improving margin revision: characterization of tumor bed margins in early oral tongue cancer. Oral Oncol 2017;75:184-188. https://doi.org/10.1016/j.oraloncology.2017/10.013

[48] Kubik MW, Sridharan S, Varvares MA, et al. Intraoperative margin assessment in head and neck cancer: a case of misuse and abuse? Head Neck Pathol 2020;14:291-302. https://doi.org/10.1007/s12105-019-01121-2

[49] Patel RS, Goldstein DP, Guillemaud J, et al. Impact of positive frozen section microscopic tumor cut-through revised to negative on oral carcinoma control and survival rates. Head Neck 2010;32:1444-1451. https://doi.org/10.1002/hed.21334

[50] Kwok P, Gleich O, Hübner G, et al. Prognostic importance of "clear versus revised margins" in oral and pharyngeal cancer. Head Neck 2010;32:1479-1484. https://doi.org/10.1002/hed.21349

[51] Zhang L, Judd RT, Zhao S, et al. Immediate resection of positive margins improves local control in oral tongue cancer. Oral Oncol 2023;141:106402. https://doi.org/10.1016/j.oraloncology.2023.106402

[52] Patel RS, Clark JR, Dirven R, et al. Prognostic factors in the surgical treatment of patients with oral carcinoma. ANZ J Surg 2009;79:19-22. https://doi.org/10.1111/j.1445-2197.2008.04791.x

[53] Bulbul MG, Tarabichi O, Sethi RK, et al. Does clearance of positive margins improve local control in oral cavity cancer? A meta-analysis. Otolaryngol Head Neck Surg 2019;161:235-244. https://doi.org/10.1177/0194599819839006

[54] Buchakjian MR, Ginader T, Tasche KK, et al. Independent predictors of prognosis based on oral cavity squamous cell carcinoma surgical margins. Otolaryngol Head Neck Surg 2018;159:675-682. https://doi.org/10.1177/0194599818773070

[55] Guillemaud JP, Patel RS, Goldstein DP, et al. Prognostic impact of intraoperative microscopic cut-through on frozen section in oral cavity squamous cell carcinoma. J Otolaryngol Head Neck Surg 2010;39:370-377.

[56] Coutu B, Ryan E, Christensen D, et al. Positive margins matter regardless of subsequent resection findings. Oral Oncol 2022;128:105850. https://doi.org/10.1016/j.oraloncology.2022.105850

[57] Kim JY, Kim Y, Kim EH, et al. Initial negative surgical margins versus revised negative surgical margins in patients who underwent surgery without adjuvant therapy for early-stage oral tongue squamous cell carcinoma. Oral Oncol 2024;159:107046. https://doi.org/10.1016/j.oraloncology.2024.107046

[58] Kerawala CJ, Ong TK. Relocating the site of frozen sections--is there room for improvement? Head Neck 2001;23:230-232. https://doi.org/10.1002/1097-0347

[59] Scholl P, Byers RM, Batsakis JG, et al. Microscopic cut-through of cancer in the surgical treatment of squamous carcinoma of the tongue. Prognostic and therapeutic implications. Am J Surg 1986;152:354-360. https://doi.org/10.1016/0002-9610

[60] Johnson RE, Sigman JD, Funk GF, et al. Quantification of surgical margin shrinkage in the oral cavity. Head Neck 1997;19:281-286. https://doi.org/10.1002/(sici)1097-0347(199707)19:4<281::aid-hed6>3.0.co;2-x

[61] Mistry RC, Qureshi SS, Kumaran C. Post-resection mucosal margin shrinkage in oral cancer: quantification and significance. J Surg Oncol 2005;91:131-133. https://doi.org/10.1002/jso.20285

[62] Cheng A, Cox D, Schmidt BL. Oral squamous cell carcinoma margin discrepancy after resection and pathologic processing. J Oral Maxillofac Surg 2008;66:523-529. https://doi.org/10.1016/j.joms.2007.08.040

[63] Pangare TB, Waknis PP, Bawane SS, et al. Effect of formalin fixation on surgical margins in patients with oral squamous cell carcinoma. J Oral Maxillofac Surg 2017;75:1293-1298. https://doi.org/10.1016/j.joms.2016.11.024

[64] Umstattd LA, Mills JC, Critchlow WA, et al. Shrinkage in oral squamous cell carcinoma: an analysis of tumor and margin measurements

in vivo, post-resection, and post-formalin fixation. Am J Otolaryngol 2017;38:660-662. https://doi.org/10.1016/j.amjoto.2017.08.011

65 Weinstock YE, Alava I, Dierks EJ. Pitfalls in determining head and neck surgical margins. Oral Maxillofac Surg Clin North Am 2014;26:151-162. https://doi.org/10.1016/j.coms.2014.01.003

66 Michcik A, Jopek M, Pęksa R, et al. Oral squamous cell carcinoma and what we lose during formalin fixation: an evaluation of changes in macroscopic surgical margins utilizing virtual three-dimensional imaging techniques with analysis based on 947 measurements. Biomedicines 2024;12:2805. https://doi.org/10.3390/biomedicines12122805

67 Shah AK. Postoperative pathologic assessment of surgical margins in oral cancer: a contemporary review. J Oral Maxillofac Pathol 2018;22:78-85. https://doi.org/10.4103/jomfp.JOMFP_185_16

68 Abbey LM, Kaugars GE, Gunsolley JC, et al. Intraexaminer and interexaminer reliability in the diagnosis of oral epithelial dysplasia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995;80:188-191. https://doi.org/10.1016/s1079-2104(05)80201-x

69 Tirelli G, Zacchigna S, Boscolo Nata F, et al. Will the mininvasive approach challenge the old paradigms in oral cancer surgery? Eur Arch Otorhinolaryngol 2017;274:1279-1289. https://doi.org/10.1007/s00405-016-4221-0

70 Burns C, Gorina Faz M. An analysis of tumor margin shrinkage in the surgical resection of squamous cell carcinoma of the oral cavity. Cureus 2021;13:E15329. https://doi.org/10.7759/cureus.15329

71 Sarode SC, Sarode GS. A novel "microscopic method" of shrinkage calculation in the pursuance of shrinkage based histopathological guidelines for interpretation of surgical margins. Oral Oncol 2012;48:E15-E16. https://doi.org/10.1016/j.oraloncology.2012.01.011

72 Sengupta N, Sarode GS, Sarode SC, et al. Optimum surgical margins in squamous cell carcinoma of the oral tongue: does shrinkage matters? Oral Oncol 2021;113:105052. https://doi.org/10.1016/j.oraloncology.2020.105052

73 Ebrahimi A, Murali R, Gao K, et al. The prognostic and staging implications of bone invasion in oral squamous cell carcinoma. Cancer 2011;117:4460-4467. https://doi.org/10.1002/cncr.26032

74 Li C, Lin J, Men Y, et al. Does medullary versus cortical invasion of the mandible affect prognosis in patients with oral squamous cell carcinoma? J Oral Maxillofac Surg 2017;75:403-415. https://doi.org/10.1016/j.joms.2016.08.005

75 Fried D, Mullins B, Weissler M, et al. Prognostic significance of bone invasion for oral cavity squamous cell carcinoma considered T1/T2 by American joint committee on cancer size criteria: significance of bone invasion. Head Neck 2014;36:776-781. https://doi.org/10.1002/hed.23367

76 Nieberler M, Häusler P, Drecoll E, et al. Evaluation of intraoperative cytological assessment of bone SM in patients with oral squamous cell carcinoma. Cancer Cytopathol 2014;122:646-656. https://doi.org/10.1002/cncy.21428

77 Nieberler M, Häußler P, Kesting MR, et al. Intraoperative cell isolation for a cytological assessment of bone SM in patients with head and neck cancer. Br J Oral Maxillofac Surg 2017;55:510-516. https://doi.org/10.1016/j.bjoms.2017.02.006

78 Nieberler M, Häußler P, Kesting MR, et al. Clinical impact of intraoperative cytological assessment of bone surgical margins in patients with head and neck carcinoma. Ann Surg Oncol 2016;23:3579-3586. https://doi.org/10.1245/s10434-016-5208-1

79 Saturno MP, Brandwein-Weber M, Greenberg L, et al. Utilizing 3D head and neck specimen scanning for intraoperative margin discussions: proof of concept of our novel approach. Head Neck 2023;45:10-21. https://doi.org/10.1002/hed.27171

80 Sharif KF, Lewis JS, Ely KA, et al. The computer-aided design margin: ex vivo 3D specimen mapping to improve communication between surgeons and pathologists. Head Neck 2023;45:22-31. https://doi.org/10.1002/hed.27201

81 Van Houten VMM, Leemans CR, Kummer JA, et al. Molecular diagnosis of surgical margins and local recurrence in head and neck cancer patients. Clin Cancer Res 2004;10:3614-3620. https://doi.org/10.1158/1078-0432.CCR-03-0631

82 Westra WH, Sidransky D. Phenotypic and genotypic disparity in premalignant lesions: of calm water and crocodiles. J Natl Cancer Inst 1998;90:1500-1501. https://doi.org/10.1093/jnci/90.20.1500

83 Leemans CR, Tiwari R, Nauta JJP, et al. Recurrence at the primary site in head and neck cancer and the significance of neck lymph node metastases as a prognostic factor. Cancer 1994;73:187-190. https://doi.org/10.1002/1097-0142

84 De Carvalho AC, Kowalski LP, Campos AHJFM, et al. Clinical significance of molecular alterations in histologically negative surgical margins of head and neck cancer patients. Oral Oncol 2012;48:240-248. https://doi.org/10.1016/j.oraloncology.2011.10.018

85 Van Schaik JE, Van Der Vegt B, Slagter-Menkema L, et al. Identification of new head and neck squamous cell carcinoma molecular imaging targets. Oral Oncol 2024;151:106736. https://doi.org/10.1016/j.oraloncology.2024.106736

86 Lauwerends LJ, Zweedijk BE, Galema HA, et al. Tumor marker expression in head and neck malignancies to identify potential targets for intraoperative molecular near-infrared imaging. Mol Diagn Ther 2024;28:811-820. https://doi.org/10.1007/s40291-024-00742-w

87 Brennan JA, Mao L, Hruban RH, et al. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med 1995;332:429-435. https://doi.org/10.1056/NEJM199502163320704

88 Babji D, Nayak R, Bhat K, et al. Comparative evaluation of immunohistochemical expression of p16 with p16 microsatellite marker by PCR in surgical margins of oral squamous cell carcinoma. Indian J Otolaryngol Head Neck Surg 2019;71:716-723. https://doi.org/10.1007/s12070-018-1517-y

89 Braakhuis BJM, Bloemena E, Leemans CR, et al. Molecular analysis of surgical margins in head and neck cancer: more than a marginal issue. Oral Oncol 2010;46:485-491. https://doi.org/10.1016/j.oraloncology.2010.01.019

90 Baldan F, Gnan C, Lazarevic M, et al. Somatic genomic imbalances in 'tumor-free' surgical margins of oral cancer. Int J Oral Maxillofac Surg 2023;52:831-838. https://doi.org/10.1016/j.ijom.2022.12.008

91 Nathan CO, Franklin S, Abreo FW, et al. Analysis of surgical margins with the molecular marker eIF4E: a prognostic factor in patients with head and neck cancer. J Clin Oncol 1999;17:2909-2914. https://doi.org/ 10.1200/JCO.1999.17.9.2909

92 Nathan CO, Sanders K, Abreo FW, et al. Correlation of p53 and the proto-oncogene eIF4E in larynx cancers: prognostic implications. Cancer Res 2000;60:3599-3604.

93 Partridge M, Li SR, Pateromichelakis S, et al. Detection of minimal residual cancer to investigate why oral tumors recur despite seemingly adequate treatment. Clin Cancer Res 2000;6:2718-2725.

94 Sardi I, Franchi A, Ferriero G, et al. Prediction of recurrence by microsatellite analysis in head and neck cancer. Genes Chromosomes Cancer 2000;29:201-206. https://doi.org/10.1002/1098-2264(2000)9999:9999<::aid-gcc1031>3.0.co;2-x

95 Nathan CO, Amirghahri N, Rice C, et al. Molecular analysis of surgical margins in head and neck squamous cell carci-

noma patients. Laryngoscope 2002;112:2129-2140. https://doi.org/10.1097/00005537-200212000-00003

[96] Temam S, Casiraghi O, Lahaye JB, et al. Tetranucleotide microsatellite instability in surgical margins for prediction of local recurrence of head and neck squamous cell carcinoma. Clin Cancer Res 2004;10:4022-4028. https://doi.org/10.1158/1078-0432.CCR-04-0199

[97] Handschel J, Oz D, Pomjanski N, et al. Additional use of DNA-image cytometry improves the assessment of resection margins. J Oral Pathol Med 2007;36:472-475. https://doi.org/10.1111/j.1600-0714.2007.00564.x

[98] Huang X, Pateromichelakis S, Hills A, et al. p53 mutations in deep tissues are more strongly associated with recurrence than mutation-positive mucosal margins. Clin Cancer Res 2007;13:6099-6106. https://doi.org/10.1158/1078-0432-CCR-07-1369

[99] Tan HK, Saulnier P, Auperin A, et al. Quantitative methylation analyses of resection margins predict local recurrences and disease-specific deaths in patients with head and neck squamous cell carcinomas. Br J Cancer 2008;99:357-363. https://doi.org/10.1038/sj.bjc.6604478

[100] Zhao H, Ren J, Zhuo X, et al. Prognostic significance of Survivin and CD44v6 in laryngeal cancer surgical margins. J Cancer Res Clin Oncol 2008;134:1051-1058. https://doi.org/10.1007/s00432-008-0391-5

[101] Bergshoeff VE, Hopman AH, Zwijnenberg IR, et al. Chromosome instability in resection margins predicts recurrence of oral squamous cell carcinoma. J Pathol 2008;215:347-348. https://doi.org/10.1002/path.2349

[102] Simon RM, Paik S, Hayes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. J Natl Cancer Inst 2009;101:1446-1452. https://doi.org/10.1093/jnci/djp335

[103] Schaaij-Visser TB, Graveland AP, Gauci S, et al. Differential proteomics identifies protein biomarkers that predict local relapse of head and neck squamous cell carcinomas. Clin Cancer Res 2009;15:7666-7675. https://doi.org/10.1158/1078-0432.CCR-09-2134

[104] Sinha P, Bahadur S, Thakar A, et al. Significance of promoter hypermethylation of p16 gene for margin assessment in carcinoma tongue. Head Neck 2009;31:1423-1430. https://doi.org/10.1002/hed.21122

[105] Poeta ML, Manola J, Goldenberg D, et al. The ligamp TP53 assay for detection of minimal residual disease in head and neck squamous cell carcinoma surgical margins. Clin Cancer Res 2009;15:7658-7665. https://doi.org/10.1158/1078-0432.CCR-09-1433

[106] Graveland AP, Golusinski PJ, Buijze M, et al. Loss of heterozygosity at 9p and p53 immunopositivity in surgical margins predict local relapse in head and neck squamous cell carcinoma. Int J Cancer 2011;128:1852-1859. https://doi.org/10.1002/ijc.25523

[107] Pena Murillo C, Huang X, Hills A, et al. The utility of molecular diagnostics to predict recurrence of head and neck carcinoma. Br J Cancer 2012;107:1138-1143. https://doi.org/10.1038/bjc.2012.213

[108] Roh JL, Westra WH, Califano JA, et al. Tissue imprint for molecular mapping of deep surgical margins in patients with head and neck squamous cell carcinoma. Head Neck 2012;34:1529-1536. https://doi.org/10.1002/hed.21982

[109] Ogbureke KU, Weinberger PM, Looney SW, et al. Expressions of matrix metalloproteinase-9 (MMP-9), dentin sialophosphoprotein (DSPP), and osteopontin (OPN) at histologically negative surgical margins may predict recurrence of oral squamous cell carcinoma. Oncotarget 2012;3:286-298. https://doi.org/10.18632/oncotarget.373

[110] Sakashita T, Homma A, Suzuki S, et al. Prognostic value of cyclin D1 expression in tumor-free surgical margins in head and neck squamous cell carcinomas. Acta Otolaryngol 2013;133:984-991. https://doi.org/10.3109/00016489.2013.795287

[111] Hayashi M, Wu G, Roh JL, et al. Correlation of gene methylation in surgical margin imprints with locoregional recurrence in head and neck squamous cell carcinoma. Cancer 2015;121:1957-1965. https://doi.org/10.1002/cncr.29303

[112] Lin JC, Wang CC, Jiang RS, et al. Impact of microsatellite alteration in surgical margins on local recurrence in oral cavity cancer patients. Eur Arch Otorhinolaryngol 2017;274:431-439. https://doi.org/10.1007/s00405-016-4215-y

[113] Singh J, Jayaraj R, Baxi S, et al. Immunohistochemical expression levels of p53 and eIF4E markers in histologically negative surgical margins, and their association with the clinical outcome of patients with head and neck squamous cell carcinoma. Mol Clin Oncol 2016;4:166-172. https://doi.org/10.3892/mco.2015.689

[114] Wang X, Chen S, Chen X, et al. Tumor-related markers in histologically normal margins correlate with locally recurrent oral squamous cell carcinoma: a retrospective study. J Oral Pathol Med 2016;45:83-88. https://doi.org/10.1111/jop.12334

[115] Pierssens DDCG, Borgemeester MC, van der Heijden SJH, et al. Chromosome instability in tumor resection margins of primary OSCC is a predictor of local recurrence. Oral Oncol 2017;66:14-21. https://doi.org/10.1016/j.oraloncology.2016.12.029

[116] Roy S, Kar M, Roy S, et al. Role of β-catenin in cisplatin resistance, relapse and prognosis of head and neck squamous cell carcinoma. Cell Oncol 2018;41:185-200. https://doi.org/10.1007/s13402-017-0365-1

[117] Liu SA, Wang CC, Jiang RS, et al. Genetic analysis of surgical margins in oral cavity cancer. Br J Surg 2018;105:E142-E149. https://doi.org/10.1002/bjs.10693

[118] Yang XH, Ding L, Fu Y, et al. p53-positive expression in dysplastic surgical margins is a predictor of tumor recurrence in patients with early oral squamous cell carcinoma. Cancer Manag Res 201911:1465-1472. https://doi.org/10.2147/CMAR.S192500

[119] Holmes BJ, von Eyben R, Attardi LD, et al. Pilot study of loss of the p53/p63 target gene PERP at the surgical margin as a potential predictor of local relapse in head and neck squamous cell carcinoma. Head Neck 2020;42:3188-3196. https://doi.org/10.1002/hed.26358

[120] Govindaraj PK, Kallarakkal TG, Mohd Zain R, et al. Expression of Ki-67, Cornulin and ISG15 in non-involved mucosal surgical margins as predictive markers for relapse in oral squamous cell carcinoma (OSCC). PLoS One 2021;16:E0261575. https://doi.org/10.1371/journal.pone.0261575

[121] Kamat MS, Puranik RS, Das Rai AB, et al. Assessing immuno-expression of p53 protein and TP 53 gene amplification in histologically negative surgical margins of oral squamous cell carcinoma patients and normal oral mucosa. Clin Oral Investig 2022;26:6235-6243. https://doi.org/10.1007/s00784-022-04574-y

[122] Goldenberg D, Harden S, Masayesva BG, et al. Intraoperative molecular margin analysis in head and neck cancer. Arch Otolaryngol Head Neck Surg 2004;130:39-44. https://doi.org/10.1001/archotol.130.1.39

[123] Hayashi M, Guerrero-Preston R, Sidransky D, et al. Paired box 5 methylation detection by droplet digital PCR for ultra-sensitive deep surgical margin analysis of head and neck squamous cell carcinoma. Cancer Prev Res 2015;8:1017-1026. https://doi.org/10.1158/1940-6207.CAPR-15-0180

[124] Saidak Z, Pascual C, Bouaoud J, et al. A three-gene expression signature associated with positive SM in tongue squamous cell carcinomas: predicting surgical resectability from tumor biology? Oral Oncol 2019;94:115-120. https://doi.org/10.1016/j.oraloncology.2019.05.020

[125] Ganci F, Allegretti M, Frascolla C, et al. Combined TP53 status in tumor-free surgical margins and circulating microRNA profiling predicts the risk of locoregional recurrence in head and neck cancer. Biomark Res 2024;12:32. https://doi.org/10.1186/s40364-024-00576-y

[126] Xu Y, Deng X, Sun Y, et al. Optical imaging in the diagnosis of OPMDs malignant transformation. J Dent Res 2022;101:749-758. https://doi.org/10.1177/00220345211072477

[127] Wu C, Gleysteen J, Teraphongphom NT, et al. In-vivo optical imaging in head and neck oncology: basic principles, clinical applications and future directions. Int J Oral Sci 2018;10:10. https://doi.org/10.1038/s41368-018-0011-4

[128] Wong Kee Song LM, Adler DG, Conway JD, et al. Narrow band imaging and multiband imaging. Gastrointest Endosc 2008;67:581-589. https://doi.org/10.1016/j.gie.2008.01.013

[129] Yagishita A, Fujii S, Yano T, et al. Endoscopic findings using narrow-band imaging to distinguish between basal cell hyperplasia and carcinoma of the pharynx. Cancer Sci 2014;105:857-861. https://doi.org/10.1111/cas.12440

[130] Tirelli G, Piovesana M, Gatto A, et al. Narrow band imaging in the intra-operative definition of surgical margins in oral cavity and oropharyngeal cancer. Oral Oncol 2015;51:908-913. https://doi.org/10.1016/j.oraloncology.2015.07.005

[131] De Wit JG, Van Schaik JE, Voskuil FJ, et al. Comparison of narrow band and fluorescence molecular imaging to improve intraoperative tumor margin assessment in oral cancer surgery. Oral Oncol 2022;134:106099. https://doi.org/10.1016/j.oraloncology.2022.106099

[132] Han R, Lin N, Huang J, et al. Diagnostic accuracy of Raman spectroscopy in oral squamous cell carcinoma. Front Oncol 2022;12:925032. https://doi.org/10.3389/fonc.2022.925032

[133] Ibrahim O, Toner M, Flint S, et al. The potential of Raman spectroscopy in the diagnosis of dysplastic and malignant oral lesions. Cancers 2021;13:619. https://doi.org/10.3390/cancers13040619

[134] Cals FLJ, Koljenović S, Hardillo JA, et al. Development and validation of Raman spectroscopic classification models to discriminate tongue squamous cell carcinoma from non-tumorous tissue. Oral Oncol 2016;60:41-47. https://doi.org/10.1016/j.oraloncology.2016.06.012

[135] Yan B, Wen Z, Xue L, et al. GenAI synthesis of histopathological images from Raman imaging for intraoperative tongue squamous cell carcinoma assessment. Int J Oral Sci 2025;17:12. https://doi.org/10.1038/s41368-025-00346-y

[136] Fabelo H, Halicek M, Ortega S, et al. Surgical aid visualization system for glioblastoma tumor identification based on deep learning and in-vivo hyperspectral images of human patients. In: Fei B, Linte CA, editors. Medical Imaging 2019: image-guided procedures, robotic interventions, and modeling. SPIE 2019. p. 35. https://doi.org/10.1117/12.2512569

[137] Boehm F, Alperovich A, Schwamborn C, et al. Enhancing surgical precision in squamous cell carcinoma of the head and neck: hyperspectral imaging and artificial intelligence for improved margin assessment in an ex vivo setting. Spectrochim Acta A Mol Biomol Spectrosc 2025;332:125817. https://doi.org/10.1016/j.saa.2025.125817

[138] Karnatz N, Möllmann HL, Wilkat M, et al. Advances and innovations in ablative head and neck oncologic surgery using mixed reality technologies in personalized medicine. J Clin Med 2022;11:4767. https://doi.org/10.3390/jcm11164767

[139] Catanzaro S, Copelli C, Manfuso A, et al. Intraoperative navigation in complex head and neck resections: indications and limits. Int J Comput Assist Radiol Surg 2017;12:881-887. https://doi.org/10.1007/s11548-016-1486-0

[140] Al-Sabahi ME, Jamali OM, Shindy MI, et al. Aesthetic reconstruction of onco-surgical mandibular defects using free fibular flap with and without CAD/CAM customized osteotomy guide: a randomized controlled clinical trial. BMC Cancer 2022;22:1252. https://doi.org/10.1186/s12885-022-10322-y

[141] Nyirjesy SC, Heller M, von Windheim N, et al. The role of computer aided design/computer assisted manufacturing (CAD/CAM) and 3-dimensional printing in head and neck oncologic surgery: a review and future directions. Oral Oncol 2022;132:105976. https://doi.org/10.1016/j.oraloncology.2022.105976

[142] Ceccariglia F, Cercenelli L, Badiali G, et al. Application of augmented reality to maxillary resections: a three-dimensional approach to maxillofacial oncologic surgery. JPM 2022;12:2047. https://doi.org/10.3390/jpm12122047

[143] Allegra E, Bianco MR, Mignogna C, et al. Early glottic cancer treated by transoral laser surgery using toluidine blue for the definition of the surgical margins: a pilot study. Medicina 2020;56:334. https://doi.org/10.3390/medicina56070334

[144] Zhou J, Yang F, Jiang G, et al. Applications of indocyanine green based near-infrared fluorescence imaging in thoracic surgery. J Thorac Dis 2016;8:S738-S743. https://doi.org/10.21037/jtd.2016.09.49

[145] Scott-Wittenborn N, Jackson RS. Intraoperative imaging during minimally invasive transoral robotic surgery using near-infrared light. Am J Otolaryngol 2018;39:220-222. https://doi.org/10.1016/j.amjoto.2017.09.001

[146] Hadjipanayis CG, Stummer W. 5-ALA and FDA approval for glioma surgery. J Neurooncol 2019;141:479-486. https://doi.org/10.1007/s11060-019-03098-y

[147] Filip P, Lerner DK, Kominsky E, et al. 5-Aminolevulinic acid fluorescence-guided surgery in head and neck squamous cell carcinoma. Laryngoscope 2024;134:741-748. https://doi.org/10.1002/lary.30910

[148] van Keulen S, Nishio N, Fakurnejad S, et al. The clinical application of fluorescence-guided surgery in head and neck cancer. J Nucl Med 2019;60:758-763. https://doi.org/10.2967/jnumed.118.222810

[149] Srinivasan A, Kaminskaite V, Winter SC. The use of fluorescent markers to detect and delineate head and neck cancer: a scoping review. Clin Otolaryngol 2025;50:220-240. https://doi.org/10.1111/coa.14263

[150] Shavlokhova V, Flechtenmacher C, Sandhu S, et al. Detection of oral squamous cell carcinoma with ex vivo fluorescence confocal microscopy: sensitivity and specificity compared to histopathology. J Biophotonics 2020;13:E202000100. https://doi.org/10.1002/jbio.202000100

[151] Sethi S, Ju X, Logan RM, et al. Diagnostic accuracy of confocal laser endomicroscopy for the diagnosis of oral squamous cell carcinoma: a systematic review and meta-analysis. Int J Environ Res Public Health 2021;18:12390. https://doi.org/10.3390/ijerph182312390

[152] Zanoni DK, Demétrio De Souza França P, Valero C, et al. A prospective double-blinded comparison of reflectance confocal microscopy with conventional histopathology for in vivo assessment in oral cancer. Clin Cancer Res 2024;30:2486-2496. https://doi.org/10.1158/1078-0432.CCR-23-1361