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Received: 12 October 2023 | Revised: 9 December 2023 | Accepted: 11 December 2023

DOI: 10.1002/cncy.22800

ORIGINAL ARTICLE

Prediction of lymph node status in patients with surgically treated head and neck squamous cell carcinoma via neck lavage cytology: A pilot study

Hugo Rimbach[1] | Maximilian Linxweiler MD[1] | Sandrina Körner PhD[1] | Sigrun Smola MD[2] | Barbara Linxweiler[3] | Stefanie Speicher[1] | Johanna Helfrich[1] | Erich-Franz Solomayer MD[3] | Mathias Wagner MD[4] | Bernhard Schick MD[1] | Jan Philipp Kühn MD[1]

[1]Department of Otorhinolaryngology, Saarland University, Homburg/Saar, Germany

[2]Institute of Virology, Saarland University, Homburg/Saar, Germany

[3]Department of Gynecology, Saarland University, Homburg/Saar, Germany

[4]Department of Pathology, Saarland University, Homburg/Saar, Germany

Correspondence
Jan Philipp Kühn, Department of Otorhinolaryngology, Saarland University, Kirrbergerstraße 100, 66421 Homburg/Saar, Germany.
Email: jan.kuehn@uks.eu

Funding information
Homburger Forschungsförderungsprogramm

Abstract

Background: Neck dissection is a standardized surgical procedure for patients with head and neck squamous cell carcinoma (HNSCC) and plays a critical role in the choice of adjuvant treatment based on histopathological findings. Saline irrigation is routinely performed at the end of surgery. However, this irrigant is not used for diagnostic purposes.

Methods: Intraoperative irrigation of the neck dissection wound was performed in 56 patients with HNSCC ($N = 93$ neck dissections), and the cytological suspension obtained was processed via the liquid-based cytology (LBC) technique, Papanicolaou staining, and immunocytochemical staining. Microscopic preparations were screened for the presence of tumor cells and classified as positive, borderline, or negative. These results were correlated with the histopathological and clinical data.

Results: Neck lavage LBC demonstrated high diagnostic value in detecting lymph node metastases (N+) with extracapsular spread (ECS), with a specificity, sensitivity, negative predictive value, and positive predictive value of 93.1%, 100%, 100%, and 80%, respectively. Tumor cells were detected in 4.8% of N− cases, 20% of N+ cases without ECS, and 100% of N+ cases with ECS. Receiver operating characteristic curve analysis showed an area under the curve of 0.8429 for the prediction of N+ ($p < .0001$) and 0.9658 for the prediction of N+ with ECS ($p < .0001$).

Conclusions: Differential lavage cytology can provide valid and rapid information on the lymph node status in patients with HNSCC and showed an excellent correlation with histopathology. Thus, neck lavage LBC may facilitate faster and more reasonable planning of adjuvant treatment and help improve the therapeutic management of patients with HNSCC.

The first two authors contributed equally to this article.

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KEYWORDS
cytology, head and neck squamous cell carcinoma, lymph node metastasis, neck dissection, neck lavage cytology

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) accounts for approximately 90% of all malignancies in the head and neck region.[1] It is the eighth most prevalent tumor worldwide, and causes approximately 878,000 new cases and 444,000 deaths annually. Patients' prognosis is influenced by several clinical, histopathological, and molecular factors, including comorbidities, tumor localization, human papillomavirus (HPV) status,[2] and International Union Against Cancer (UICC) tumor stage.[3–5] Of critical importance, lymph node metastases are frequently detected at the initial diagnosis, and node-positive disease considerably worsens patient outcomes. Extracapsular spread (ECS) of lymph node metastases, defined as tumor extension beyond the lymph node capsule into the surrounding tissue, significantly worsens patients' overall survival, particularly in HPV-negative HNSCC cases, compared to lymph node–positive disease without ECS. In addition, ECS is associated with higher rates of locoregional recurrence and distant metastasis.[6,7] Consequently, current European[8] and US[9] guidelines for the treatment of HNSCCs recommend incorporating platinum-based chemotherapy with neck irradiation in cases of N+, ECS+ disease on the basis of evidence from phase 3 clinical trials RTOG 9501[7] and EORTC 22931.[10] Currently, the diagnosis of ECS can only be confirmed via histopathological examination of the resected lymph nodes after surgery, which usually takes several days to 1 week before adjuvant chemoradiation can be planned in cases of ECS-positive neck disease. Neck dissection is a highly standardized surgical procedure that represents an essential part of treatment for the majority of patients with HNSCC and is indicated in any clinically positive nodal disease and locally advanced tumor (T3–T4).[11] At the end of each neck dissection, the wound cavity is routinely irrigated with an antiseptic solution and isotonic saline before drainage and wound closure.[11] This irrigation fluid is commonly discarded and not used for diagnostic purposes. By contrast, other disciplines, including gynecology, abdominal surgery, and pneumology, routinely use irrigation fluids from predefined or surgically created body cavities for diagnostic purposes. For instance, bronchoalveolar lavage is used in pneumology and has a relevant role in detecting tuberculosis or other bacterial and fungal infections, as well as neoplastic cells of malignant lesions.[12–14] Wash cytology is widely used in gynecology. This technique was first described in the 1950s[15,16] and has become an established and standardized part of gyneco-oncological surgical procedures. Several clinical studies have not only proven its applicability for diagnostics but also shown a significant correlation between lavage cytology results and patient outcomes in malignancies of the ovary, endometrium, and uterine cervix.[17] Additionally, in abdominal surgery, studies have demonstrated that positive peritoneal lavage cytology is linked to poor prognosis, especially in conditions such as gastric[18] and pancreatic cancer.[19] However, no studies have been conducted on the potential use of lavage cytology in HNSCC, particularly for neck dissection. Liquid-based lavage cytology has the potential to provide relevant pathological information in a shorter time, especially for large surgical specimens, because cytopathological results are typically available much faster than histopathological reports. This feature is of particular importance because the risk of local recurrence increases with the prolonged time between surgical treatment and the start of adjuvant radiotherapy or chemoradiation in HNSCC. Hence, adjuvant therapy should be initiated as soon as possible, with a maximum interval of 6 weeks.[20]

Against this background, our study aimed to investigate the potential application of intraoperative liquid-based lavage cytology in patients with surgically treated HNSCC undergoing neck dissection and correlate cytological findings with histopathological neck status and clinical features, including patient outcome.

MATERIALS AND METHODS

Patients and clinical data

Fifty-six patients with HNSCC, with 93 neck dissections, were included in this pilot study. The patient cohort comprised 51 male (91%) and five female patients (9%) with a mean age of 66 years. All patients were histopathologically diagnosed with squamous cell carcinomas located in the oropharynx (n = 29), larynx (n = 17), hypopharynx (n = 8), or oral cavity (n = 2). Clinical characteristics are summarized in Table 1.

All patients were treated at the Department of Otorhinolaryngology at Saarland University (Homburg/Saar, Germany), with a median follow-up of 26 months. All patients provided informed consent to participate in the study. The study was conducted in accordance with the Declaration of Helsinki and relevant national and international regulations. This study was approved by the Saarland Medical Association Ethics Review Committee (index number 218-10).

Surgical procedures

Two types of neck dissection were performed in this study (Table 1). Modified radical neck dissection is a surgical procedure wherein all macroscopically visible cervical lymph nodes within predefined anatomical levels are removed but with additional resection of at least one nonlymphatic structure, such as the accessory nerve, internal

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TABLE 1 Clinical data of the patient cohort.

		Total
Patients with HNSCC, No.		56
Neck dissections, No.		93
Sex, No. (%)	Male	51 (91.1)
	Female	5 (8.9)
Age, median, years		66
Tumor localization, No. (%)	Oral cavity	2 (3.6)
	Oropharynx	29 (51.8)
	Hypopharynx	8 (14.3)
	Larynx	17 (30.3)
T stage, No. (%)	T1	23 (41.1)
	T2	17 (30.3)
	T3	6 (10.7)
	T4	10 (17.9)
N stage, No. (%)	N0	31 (55.4)
	N1	10 (17.8)
	N2	12 (21.4)
	N3	3 (5.4)
ECS, No. (%)	Negative	73 (78.5)
	Microscopically positive	9 (9.7)
	Macroscopically positive	11 (11.8)
M stage, No. (%)	M0	56 (100)
	M1	0 (0)
Grading, No. (%)	G1	0 (0)
	G2	27 (48.2)
	G3	29 (51.8)
HPV, No. (%)	Positive	15 (26.8)
	Negative	41 (73.2)
Therapy, No. (%)	Unilateral neck dissection	19 (33.9)
	Bilateral neck dissection	37 (66.1)
	Selective neck dissection	74 (79.6)
	Modified radical neck dissection	19 (20.4)
	Surgery only	20 (35.7)
	Surgery + RT	16 (28.6)
	Surgery + CRT	15 (26.8)
	Primary CRT + salvage surgery	5 (8.9)

Abbreviations: CRT, chemoradiotherapy; CT, chemotherapy; ECS, extracapsular spread; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; RT, radiotherapy.

jugular vein, or sternocleidomastoid muscle. In our study, no radical neck dissection was performed, which typically involves the resection of all three of the aforementioned structures. Selective neck dissection involves the resection of macroscopically visible cervical lymph nodes at predefined neck levels but without any additional resection of nonlymphatic tissue. The lymph node levels to be removed were determined preoperatively via sonographic findings and expected metastatic patterns based on the primary site of the disease.[21]

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Liquid-based lavage cytology

Saline irrigation was routinely performed at the end of a neck dissection, after careful bipolar coagulation to achieve a surgically blood-dry field. Therefore, maximum care was taken to prevent contact of the saline solution with the patient's skin and avoid contamination of the irrigation fluid with squamous epithelial cells of the skin. The neck dissection wound was irrigated with 50 mL of sterile 0.9% saline solution (B. Braun, Melsungen, Germany), which was then immediately collected in a 50-mL Perfusor syringe (B. Braun). The collected cellular material (10–30 mL) was transferred to a vial containing 10 mL of PreservCyt solution (Hologic, Marlborough, Massachusetts) after centrifugation of the irrigation fluid at 2000 rpm for 10 min, and the supernatant was discarded. Following the manufacturer's specifications, the cells were then transferred onto microscope glass slides with the ThinPrep system (Hologic). This technique allowed for the preparation of two to five microscope slides per neck dissection, which depended on the density of cells and blood contamination in the lavage suspensions.

Papanicolaou staining and cytomorphological analysis of liquid-based cytology preparations

For cytopathological analysis, Papanicolaou (Pap) staining of the liquid-based cytology (LBC) preparations was performed via a standard protocol. Additionally, immunocytochemistry (ICC) targeting cytokeratin (CK) AE1/3 was performed on all LBC samples. Two technical assistants with extensive experience in evaluating cytological samples of the uterine cervix and one board-certified cytopathologist independently classified the samples on the basis of the Pap and ICC slides as cytomorphologically positive (presence of AE1/3-positive squamous cell carcinoma cells), borderline (presence of AE1/3-positive severely dysplastic squamous cells without clear criteria for malignancy), or negative (no AE1/3-positive severely dysplastic squamous cells or AE1/3-positive squamous cell carcinoma cells).

ICC

After the LBC microscope slides were prepared as described above, they were fixed for 15 min in formaldehyde (4%, volume/volume) and washed twice with phosphate buffer solution (PBS) (pH 7.2). The slides were then placed in retrieval buffer (Tris/EDTA buffer solution, pH 9.0; Roche, Basel, Switzerland) for 25 min at 95°C to undergo heat-induced epitope retrieval. Thereafter, incubation in 3% (weight/volume) bovine serum albumin (BSA)/PBS (Sigma-Aldrich Chemie, Taufkirchen, Germany) was performed for 30 min at room temperature to block nonspecific protein-binding sites. After being washed with PBS (pH 7.2), the slides were coated with the primary antibody anti-CK AE1/3 (mouse anti-CK AE1/3, clone MAB3412; Chemicon, Temecula, California) at a 1:230 dilution in 1% (volume/volume) BSA/PBS for 60 min at room temperature. After three further washing steps with PBS (pH 7.2), antibody–antigen reactions were visualized with a fast red chromogen solution with the REAL Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (K5005; Dako, Santa Clara, California) according to the manufacturer's instructions. Subsequently, the slides were rinsed with PBS (pH 7.2) and counterstained with hematoxylin (alcohol-free; Sigma-Aldrich Chemie) for 5–6 min. Finally, the slides were dehydrated in an ascending alcohol series up to 100% xylene and syringed with tap water for 5 min. The slides were covered with Entellan (Sigma-Aldrich Chemie). Positive and negative controls were included for each staining series with the positive control slides provided with the Dako REAL Detection System Kit, which omitted the primary antibody incubation step.

HPV status

HPV DNA-specific polymerase chain reaction (PCR) was performed on all patients with formalin-fixed paraffin-embedded (FFPE) tissue samples extracted from their respective primary tumors. DNA was extracted from the FFPE tissue samples with the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. HPV PCR was performed with a LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany) and GP5+/6+ primers, as described by de Roda Husman et al.,[22] which can detect at least 27 mucosotropic HPV types, including HPV6, 11, 16, 18, 31, and 33. SYBR Green staining and gel electrophoresis were performed. Initial denaturation at 95°C for 15 min was followed by 45 PCR cycles with denaturation at 95°C for 10 s, annealing at 45°C for 5 s, and extension at 72°C for 18 s. After amplification, a melting curve was generated at temperatures between 45°C and 95°C, with the temperature increasing at a rate of 0.2°C s^{-1}. The melting temperatures for the HPV16- and HPV18-positive controls were 79°C and 82°C, respectively. Glyceraldehyde-3-phosphate dehydrogenase PCR was performed in parallel for each sample, which served as a positive control, as described by Ruprecht et al.[23] Regarding the histopathological grading of HNSCC cases at our institution, the respective information on tumor differentiation (G1/2/3) based on histomorphology is provided with the initial histopathology report before HPV DNA PCR is performed for those cases, as described above. Therefore, information on tumor grading is provided for HPV-positive HNSCCs (Table 1), although grading is not recommended for HPV-associated HNSCC in the European or College of American Pathologists guidelines.

Statistical analysis

Prism version 9 software (GraphPad Software, Boston, Massachusetts) was used for statistical analysis. To check whether the distributions of categorical variables (suspicious vs. nonsuspicious neck lavage cytology) differed between the predefined groups, the Fisher exact test was performed. Receiver operating characteristic (ROC)

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curves were generated, and area under the curve (AUC) analyses were conducted with Prism version 9, with 95% confidence intervals (CIs) and *p* values calculated with the Wilson–Brown method. For survival analyses, the Kaplan–Meier method and log-rank test were used. Statistical significance was set at $p < .05$ ($\alpha = .05$).

RESULTS

Cellular patterns and cytomorphological characteristics of neck lavage LBC preparations

First, three independent examiners morphologically analyzed the Pap-stained lavage LBC preparations from all neck dissections included in this study. All the LBC specimens predominantly showed single-cell layers and were eligible for further analysis. As shown in Figure 1, different cell types were detected in LBC microscopic preparations. Although immune cells, red blood cells, and fibroblasts clearly dominated the cellular pattern in all samples, cytomorphologically altered cells suspicious for squamous cell carcinoma were found in a subset of patients. These cells showed characteristic cytomorphological signs of malignancy, such as an increased nuclear/cytoplasmic ratio, anisocytosis and anisokaryosis, abnormalities in nuclear membrane structure, nuclear hyperchromasia, abnormal distribution of nuclear chromatin, prominent and large nucleoli, and discohesive cells. Further cellular abnormalities included enhanced mitotic activity, abnormal mitoses, cellular and nuclear pleomorphism, and tumor diathesis.[24] In a few cases, cellular overlap impaired the microscopic evaluation of cytological preparations with suspicious cells but without clear morphological characteristics of malignancy; therefore, those cases were classified as borderline (Figure S1).

For all neck dissection preparations, ICC staining targeting pan-CK (AE1/3) was performed to better identify squamous epithelial cells. We observed positive pan-CK staining in all cytomorphologically altered cells, which was suggestive of squamous cell carcinoma (Figure 2). No pan-CK–positive nondysplastic squamous cells were found in the ICC preparations, which indicated no contamination of the neck lavage preparations with skin squamous cells. Figure 2 illustrates hematoxylin and eosin–stained microscopic preparations of the resected lymph nodes (Figure 2A,D,G) and Pap-stained (Figure 2B,E,H) and ICC-stained (Figure 2C,F,I) cytological slides of the respective neck lavage preparations for three representative cases.

Correlation of neck lavage cytological pattern with histopathological lymph node status

On the basis of Pap- and ICC-stained cytological preparations, all neck lavage samples were classified as either cytomorphologically positive (presence of AE1/3-positive squamous cell carcinoma cells), borderline (presence of AE1/3-positive severely dysplastic squamous cells without clear malignancy criteria), or negative (no AE1/3-positive severely dysplastic squamous cells or AE1/3-positive squamous cell carcinoma cells) by all three examiners. Therefore, we found perfect interobserver agreement (3 of 3; 100%) in 93.5% of the cases (87 of 93). In three cases (3 of 93; 3.2%), two examiners diagnosed the LBC neck lavage preparations as positive, whereas the third examiner found only borderline squamous cells. In two further cases (2 of 93; 2.2%), two examiners classified cytological preparations as negative, whereas the third examiner reported a borderline finding. In one case (1 of 93; 1.1%), two examiners diagnosed the slides as borderline, whereas one examiner classified the case as negative. In these cases, the final diagnosis was adapted to the consensus of the two examiners, which outvoted the diagnosis of the single examiner. As shown in Figure 3A, 73.3% (22 of 30) of neck dissections with a histological diagnosis of squamous cell carcinoma lymph node metastasis (N+) were also detected in the lavage cytology preparations. In cases of histopathologically proven ECS of lymph node metastases, 100% (20 of 20) of neck lavage cytological samples were classified as positive. In 5% (3 of 60) of the cases with node-negative histology, neck lavage cytology revealed suspicious cells and were therefore classified as borderline. Notably, patients with lymph nodes that showed macroscopic ECS, as stated by either the pathologist or the surgeon, had a significantly higher percentage of suspicious cytology results than patients with only microscopic ECS (11 of 11 [100%] vs. 6 of 9 [67%]; $p = .0378$, χ^2 test; Table S1). Additionally, we tested the potential correlation between cytological diagnosis and lymph node size; however, the results were not significant (data not shown).

To calculate the diagnostic validity of neck lavage cytology, borderline and positive classifications were considered suspicious, whereas negative classifications were considered nonsuspicious. In our cohort of 56 patients with 93 neck dissections, neck lavage cytology had a sensitivity of 73.3% and a specificity of 95.2% for predicting squamous cell carcinoma metastases (N+ status), with a positive predictive value (PPV) of 88% and a negative predictive value (NPV) of 88.2%. Regarding the prediction of lymph node metastases with ECS, lavage LBC showed an even better diagnostic performance, with a specificity of 93.1%, sensitivity of 100%, NPV of 100%, and PPV of 80% (Figure 3B). ROC curve analysis (Figure 3C) revealed an AUC of 0.8429 for predicting positive lymph node status ($p < .0001$; 95% CI, 0.7423–0.9434) and 0.9658 for predicting positive lymph node status with ECS ($p < .0001$; 95% CI, 0.9305–1).

Notably, LBC results were received significantly faster than histopathology reports, with a median time span between cytological and histological diagnoses of 11 days ($p < .0001$; Figure S2).

Correlation of neck lavage cytology with further histopathological characteristics and clinical data

Next, we correlated neck lavage cytology findings with clinical and histopathological features, including HPV status, nodal ratio, number of metastatic lymph nodes per neck dissection, surgical neck

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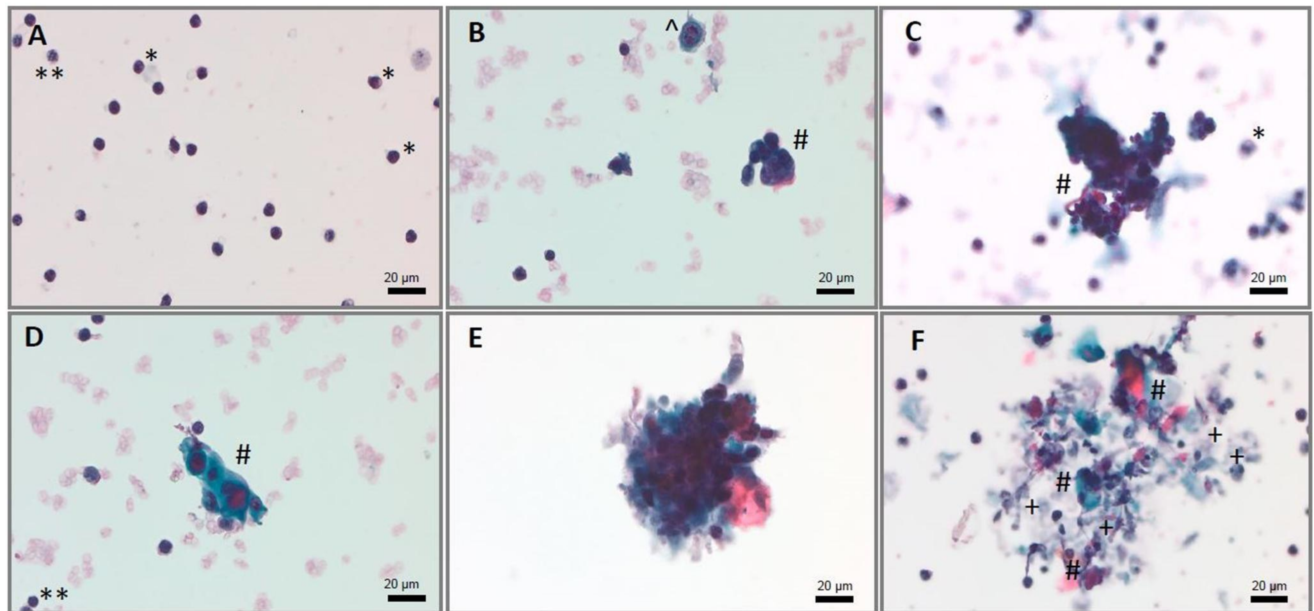

FIGURE 1 Cytomorphological characteristics of neck lavage liquid-based cytology preparations. (A) Inconspicuous cell pattern with macrophages (*) and degenerated leukocytes (**). (B) Conglomerate mass consisting of poorly differentiated tumor cells (#) and abnormal mitosis (^). (C) Large, very dense, crowded groups of tumor cells (#) and macrophages (*). (D) Crowded groups of moderately differentiated tumor cells with heterochromia, cellular cannibalism (#), and leukocytes (**). (E) Dense tumor cell cluster with prominent hyperchromasia and anisocytosis, as well as anisokaryosis and keratinization signs. (F) Crowded groups of poorly differentiated tumor cells (#) with tumor diathesis (+) and signs of keratinization.

dissection technique, unilateral/bilateral neck dissection, and tumor localization, as shown in Figure 4. Herein, we found a significantly higher percentage of suspicious neck lavage cytologies in HPV-positive compared to HPV-negative carcinomas (p = .017; Figure 4A), cases with a nodal ratio ≥50% (p < .001; Figure 4B), patients with more than one positive lymph node (p = .024; Figure 4C), and patients who underwent a modified radical neck dissection (p < .001; Figure 4D). Unilateral or bilateral neck dissection was performed (p = .144; Figure 4E), and the location of the primary tumor (p = .139; Figure 4F) did not influence the LBC results for neck lavage.

Prognostic relevance of neck lavage cytology results

When analyzing the prognostic relevance of neck lavage cytology results in our cohort of 56 patients with HNSCC, no significant difference in overall survival was found when comparing patients with suspicious and nonsuspicious neck lavage cytology (p = .932; Figure 5A). As expected, there was a trend toward a favorable outcome in HPV-positive cases compared to that in HPV-negative cases (p = .060; Figure 5B).

Neither the presence of histologically proven lymph node metastases (p = .515; Figure 5C) nor primary tumor localization significantly affected overall survival (Figure 5D). Significantly longer overall survival was observed in patients with UICC stage I than in those with UICC stage IV (I vs. IV, p < .001; Figure 5E).

DISCUSSION

HNSCC is one of the most common cancers worldwide, with approximately one million new cases and 500,000 deaths reported in 2020.[25] Late diagnosis, usually at an advanced tumor stage, with extensive cervical lymph node involvement and reduced general patient condition, remains a major challenge in the clinical management of HNSCC.[26] Most patients receive multimodal treatment with surgical tumor resection followed by radiation or radiochemotherapy. Therefore, histopathological information regarding the presence and location of lymph node metastases, with or without extranodal extension, is essential for adjuvant treatment planning. In this context, the time to histopathological diagnosis is of utmost importance because a delayed initiation of adjuvant treatment can significantly worsen patient outcome.[20]

Our pilot study aimed to investigate whether cervical lymph node status can be predicted via intraoperative wound irrigation (LBC) and subsequent cytological examination of the irrigation fluid via Pap and ICC staining in a cohort of 56 patients with surgically treated HNSCC with 93 neck dissections. High sensitivity (100%), specificity (93.1%), positive predictive value (80%), and negative predictive value (100%) were obtained for predicting the presence of lymph node metastases with ECS.

The technique of wound irrigation at the end of the surgical procedure is well established in clinical practice, which ensures that it does not extend the operation time or pose any additional risk to the patient when using the irrigation fluid for diagnostic purposes. Our

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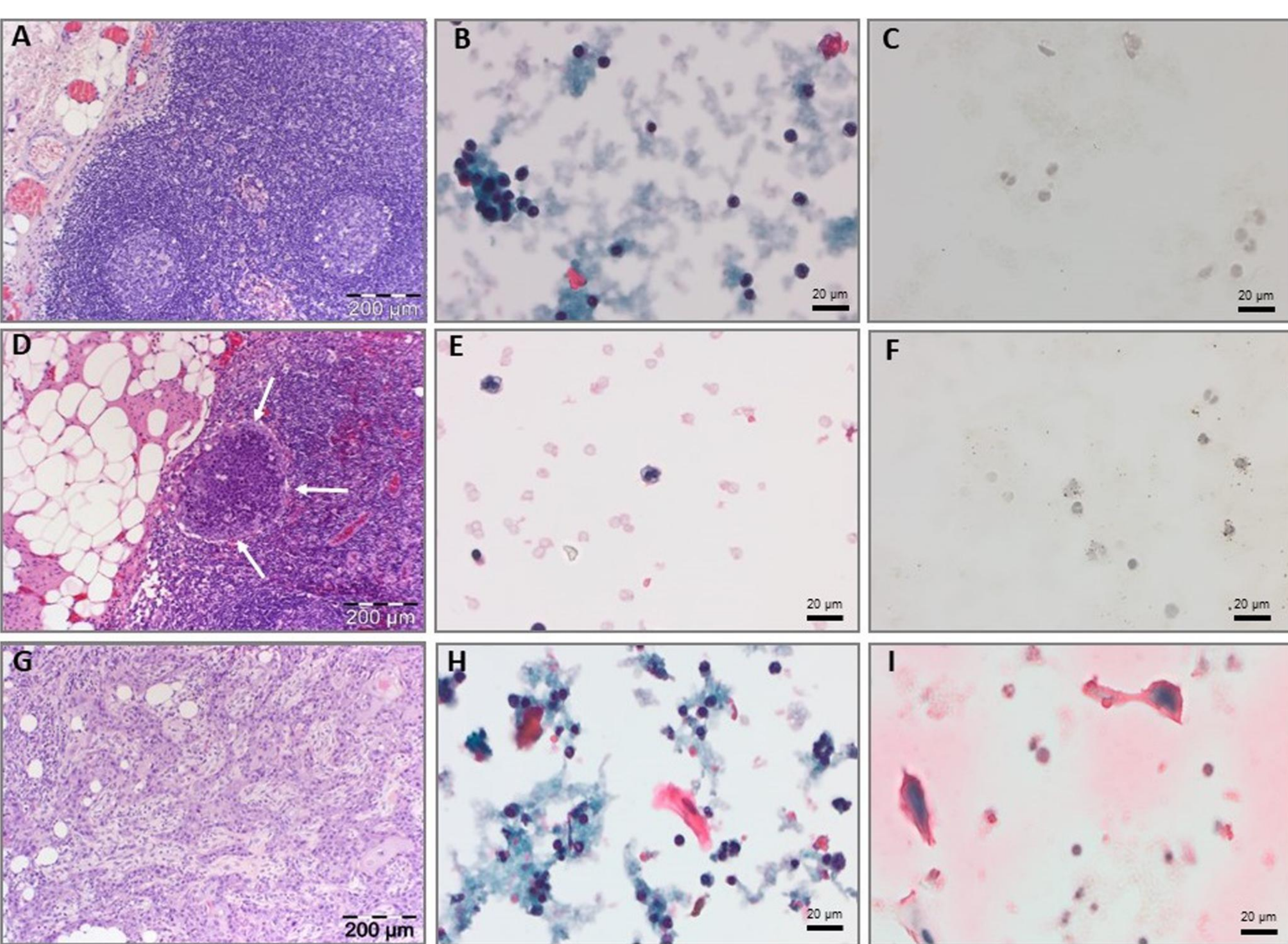

FIGURE 2 Examples of hematoxylin and eosin–stained (C, D, G), formalin-fixed paraffin-embedded tissue preparations and corresponding Papanicolaou (B, E, H) as well as immunocytochemical (C, F, I) staining of lavage cytology. Samples were obtained from a neck dissection without evidence of lymphatic metastasis (A–C), intranodal lymphatic metastasis (D–F), and extracapsular extension of nodal metastasis (G–I). Case 1 (A–C) and case 2 (D–F) were cytologically classified as nonsuspicious, and case 3 (G–I) was classified as suspicious. (D) Intranodal tumor cells are highlighted by white arrows. (A, D, G) Magnification 40×. (B, C, E, F, H, I) Magnification 20×.

technique for material collection is easily applicable to treating surgeons, and every irrigation fluid sample in our study was eligible for cytological processing and diagnosis. With respect to therapeutic management, the timely prediction of ECS-positive lymph node metastases via lavage cytology can facilitate the planning of adjuvant treatment and potentially enable an earlier start of mandatory chemoradiation. Notably, the presence of tumor cells in all neck lavage preparations of ECS-positive patients in our study emphasizes the importance of escalated adjuvant treatment in this clinical situation to decrease the risk of tumor recurrence due to persistent tumor cells in the surgical field, even after neck dissection is completed. In addition, the high relevance of quick and reliable information on the therapeutic schedule for the patient should not be overlooked. The long waiting period after surgery until histopathological results are available to guide further treatment places an emotional burden on patients and can aggravate existing mental illness symptoms.[27,28]

To date, no comparable study has reported the cytological examination of neck dissection irrigation fluid for diagnostic purposes, which emphasizes the novelty of our study. Only one other study has investigated lavage cytology in the field of head and neck surgery, albeit in a slightly different setting. Kinoshita et al. intraoperatively generated LBC samples by rinsing biopsied tissue fragments of head and neck lesions and showed that cytological analysis of these LBC preparations can provide a reliable intraoperative diagnosis in a couple of minutes. This technique was found to be even more reliable and accurate than frozen section histology.[29] In contrast to our study, no patients with HNSCC were included in that study.

In other surgical disciplines, more evidence exists regarding the potential diagnostic value of lavage cytology during surgical procedures; however, publications on this methodology are sparse. Over a 22-year period, Bando et al.[30] performed intraoperative peritoneal lavage cytology in 1297 patients with gastric cancer. This study demonstrated the validity of this technique for predicting survival and peritoneal recurrence of this type of cancer. In addition, Jamel et al.[31] conducted a meta-analysis of more than 20 studies involving nearly 8000 patients with gastric cancer and showed that negative peritoneal lavage cytology and a change in cytological status from positive to negative at the end of the surgical procedure significantly

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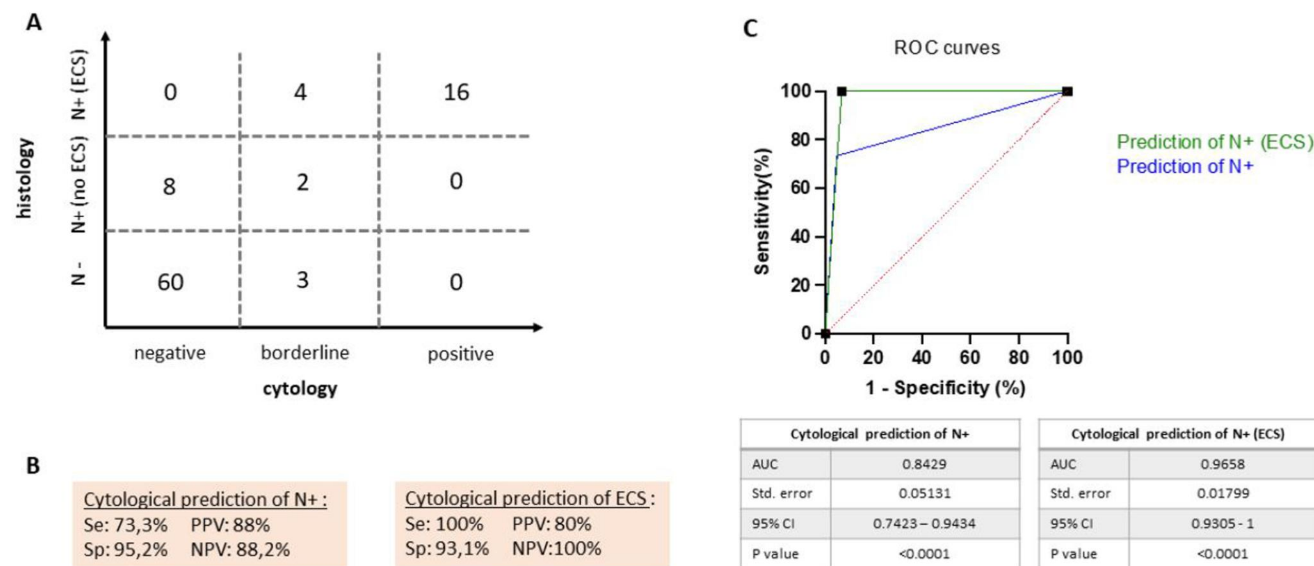

Cytological prediction of N+	
AUC	0.8429
Std. error	0.05131
95% CI	0.7423 – 0.9434
P value	<0.0001

Cytological prediction of N+ (ECS)	
AUC	0.9658
Std. error	0.01799
95% CI	0.9305 - 1
P value	<0.0001

FIGURE 3 Correlation of histomorphological and cytomorphological diagnoses (A) and diagnostic accuracy of cytological findings detecting lymph node metastases without and with ECS (B). (C) ROC curve analysis to determine the diagnostic quality of lavage cytology. AUC indicates area under the curve; ECS, extracapsular spread; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; Se, sensitivity; Sp, specificity.

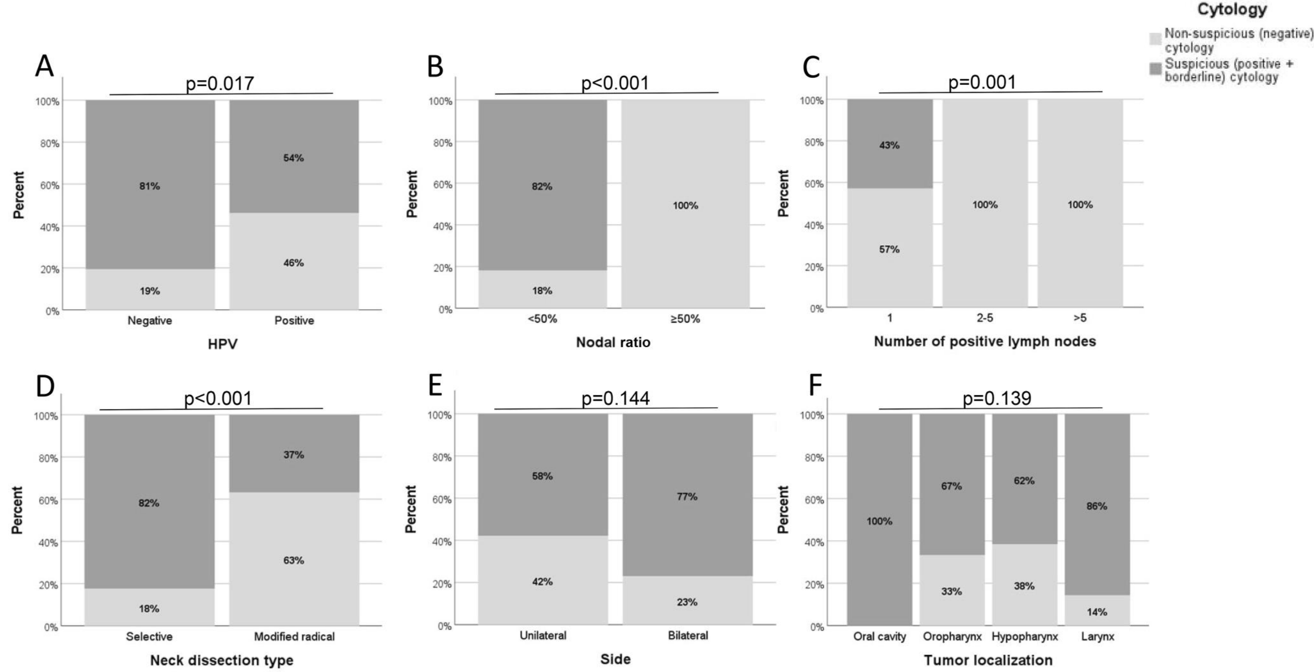

FIGURE 4 Correlation between suspicious (dark gray) and nonsuspicious (light gray) neck lavage cytology with clinical and histopathological features. (A) HPV status. (B) Nodal ratio (number of positive lymph nodes divided by the total number of resected lymph nodes per neck dissection). (C) Number of lymph nodes with metastasis per neck dissection. (D) Surgical technique used. (E) Location of neck dissection. (F) Tumor localization. HPV indicates human papillomavirus.

improved patient outcomes. Furthermore, Douligeris et al., in a study of 907 patients who underwent debulking surgery after neoadjuvant chemotherapy for locally advanced ovarian cancer, demonstrated that negative peritoneal cytology was associated with significantly improved progression-free survival, whereas overall survival showed a comparable nonsignificant trend.[32] Another meta-analysis, conducted by Bosanquet et al., indicated that positive intraoperative peritoneal lavage for colorectal cancer at the end of surgery was associated with worse overall survival, local/peritoneal recurrence, and overall recurrence in a cohort of 2580 patients.[33]

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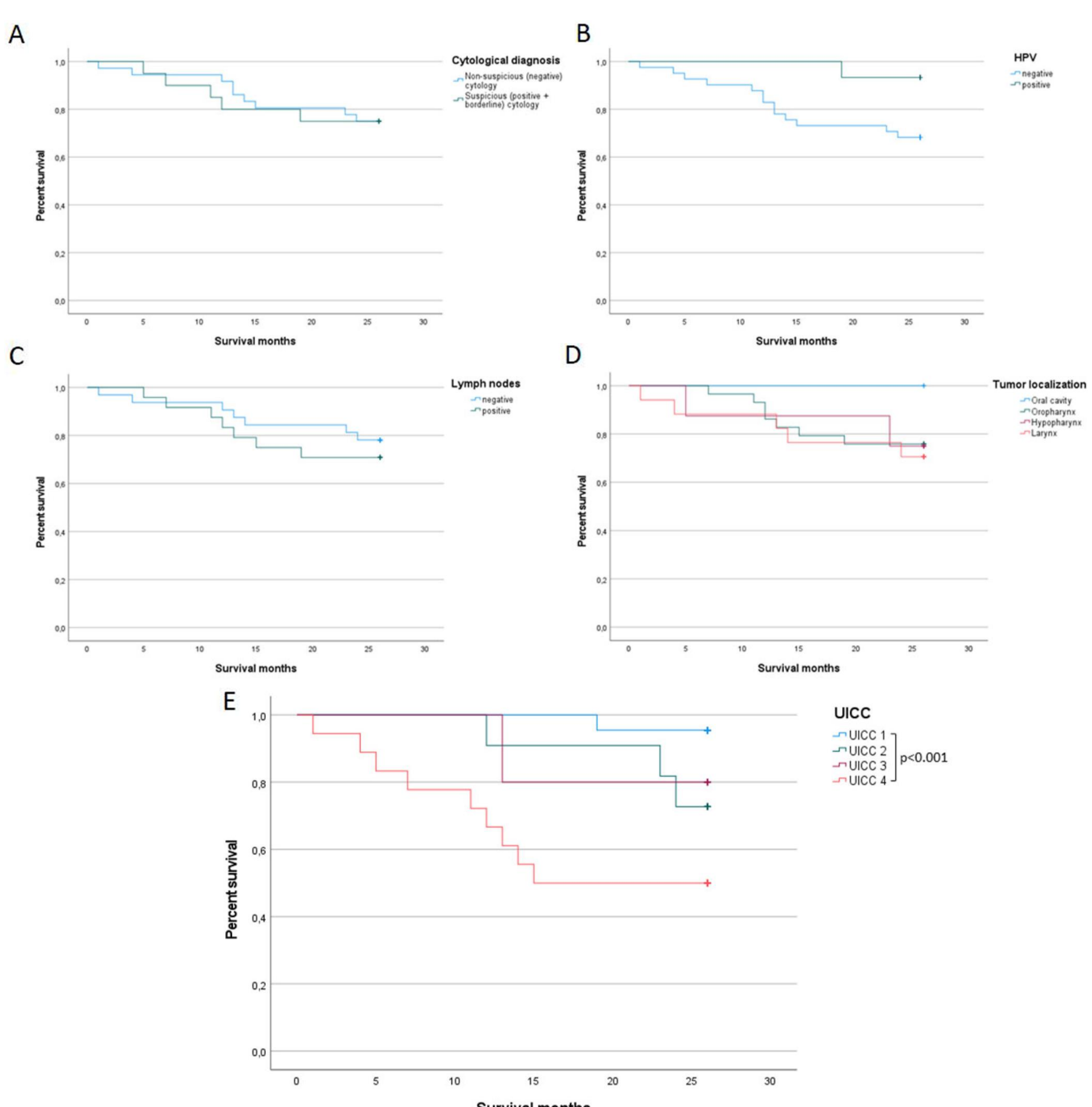

FIGURE 5 Impact of cytological findings (A), HPV status (B), lymph node status (C), tumor localization (D), and UICC stages (E) on patient survival. (A) Comparison of overall survival in patients with nonsuspicious (blue) versus suspicious cytology (green), (B) in HPV-positive (green) compared to HPV-negative cases (blue), (C) in cases with histologically positive (green) and negative lymph nodes (blue), (D) regarding primary tumor localization (oral cavity [blue], oropharynx [green], hypopharynx [violet], and larynx [red]), and (E) UICC stages (I [blue], II [green], III [violet], and IV [red]). HPV indicates human papillomavirus; UICC, International Union Against Cancer.

Together, these studies indicate the potential clinical benefit that intraoperative lavage cytology, as a diagnostic tool, can provide in terms of predicting outcomes. Nonetheless, all the aforementioned studies did not involve patients with HNSCC, and the lavage cytologies were performed in predefined anatomical cavities. In contrast, our study involved the irrigation of a surgically created cavity, which highlights a clear limitation in comparability.

Regarding the methodology used in our study, specifically the sampling of cytological materials, several limitations must be considered. To enable the valid identification of tumor cells in the neck lavage material, ICC staining targeting pan-CK (AE1/3) was used with Pap staining to confirm the epithelial differentiation of cells. Indeed, all cells identified as tumors and dysplastic squamous cells on the basis of cytomorphology showed a positive pan-CK

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staining signal in our study. There were no cases with discrepant cytomorphological or ICC findings, which raises the question of whether CK ICC is necessary. When we initially planned our study, we lacked information about the types of cells we would find in the neck lavage specimens and the extent of cell preservation. Accordingly, we decided to perform CK ICC in all cases to obtain additional information on the cells' biological origin and enable a better interpretation of the cellular composition in each specimen. In retrospect, it was probably not necessary to perform CK ICC to correctly classify neck lavage specimens because cytomorphology alone proved to be sufficient for cytological diagnosis. In addition, we found that in cases where neck dissection included level Ib (submandibular triangle) and the capsule of the submandibular gland was opened, cytological neck lavage preparations could contain acinar, ductal, and/or myoepithelial cells, which exhibited a positive pan-CK signal. It is important to carefully evaluate the morphological characteristics of cells to allow valid differentiation from tumor cells without hampering diagnostic sensitivity and specificity. Moreover, this study did not include neck dissections involving transcervical pharyngotomy. For example, when the pharynx is opened for laryngectomy or transcervical resection of laryngeal/hypopharyngeal tumors, there is a theoretical risk of false-positive findings in ICC staining because of potential contamination of the neck dissection wound with mucosal cells. Moreover, it is important to ensure sufficient hemostasis and a bloodless operation field before starting wound irrigation because an excessive number of erythrocytes necessitates elaborate clean-up steps and can complicate cytological evaluation. Additionally, care must be taken to avoid contamination of the irrigation sample with squamous epithelial cells from the skin because this can produce false-positive results when relying only on CK staining. All these aspects show that experienced cytopathologists must consider a learning curve when evaluating neck lavage cytological preparations. A close dialogue between the surgeon and the cytopathologist is needed to know what cell types can be expected in the cytological sample on the basis of the surgical technique and to finally decide whether liquid-based lavage cytology is suitable for pathological diagnosis, especially when considering possible therapeutic consequences.

Regarding the three cases in our study with positive cytology but no nodal metastasis, we hypothesized that cancer cells may have spread from the primary tumor to the lymphatic vessels without reaching the lymph nodes. In fact, the histopathology report showed lymphangitis carcinomatosis in two of those three cases. Two potential explanations are possible for the two cases with positive cytology and nodal metastasis without extranodal extension. First, these cases could have also shown lymphangitis carcinomatosis, which led to the presence of tumor cells in the neck lavage specimens (L1 status was confirmed in one of the two cases). Second, it is possible that the metastases were surgically opened during neck dissection, which led to contamination of the neck wound with cancer cells. However, no relevant statements were found in the surgical reports for these cases.

To date, evidence from a single study with a limited number of cases is clearly insufficient to change therapeutic algorithms. Nonetheless, the results of our study show that neck lavage cytology has great potential for predicting lymph node status in patients with HNSCC and motivate further evaluation of this technology in large-scale clinical trials. Future studies will need to determine whether the clinical application of neck lavage cytology can improve patient outcomes by facilitating timely and valid adjuvant treatment planning, which could not be proven in our study because of the limited number of patients and a comparably short follow-up period. In this context, a recent report on cytology-based cancer surgery of the head and neck demonstrated that LBC is not only a complementary diagnostic tool but can also be used to guide therapeutic decisions and surgical management of patients with HNSCC.[34]

In conclusion, our study showed that differential cytological analysis of neck dissection wound lavages can provide valid and timely information on the neck lymph node status of patients with surgically treated HNSCC, with an excellent correlation with histopathological findings, and may pave the way for faster planning of adjuvant treatment.

AUTHOR CONTRIBUTIONS

Hugo Rimbach: Methodology; validation; visualization; software; project administration; data curation; and writing–review and editing. **Maximilian Linxweiler**: Supervision; data curation; resources; project administration; software; formal analysis; validation; methodology; visualization; writing–review and editing; conceptualization; investigation; funding acquisition; and writing–original draft. **Sandrina Körner**: Methodology; software; formal analysis; and writing–review and editing. **Sigrun Smola**: Methodology and writing–review and editing. **Barbara Linxweiler**: Methodology; supervision; investigation; conceptualization; and writing–review and editing. **Stefanie Speicher**: Methodology; data curation; and writing–review and editing. **Johanna Helfrich**: Methodology; data curation; and writing–review and editing. **Erich-Franz Solomayer**: Supervision and writing–review and editing. **Mathias Wagner**: Methodology; validation; software; formal analysis; and writing–review and editing. **Bernhard Schick**: Writing–review and editing; conceptualization; investigation; and supervision. **Jan Philipp Kühn**: Writing–review and editing; writing–original draft; formal analysis; resources; data curation; supervision; visualization; validation; methodology; conceptualization; investigation; funding acquisition; project administration; and software.

ACKNOWLEDGMENTS

We thank Birgit Glombitza and Alexandra Prinz for their excellent technical assistance. This work was supported by a Homburger Forschungsförderungsprogramm grant received by JPK.

Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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ORCID

Maximilian Linxweiler https://orcid.org/0000-0002-5028-9282

Jan Philipp Kühn https://orcid.org/0000-0001-5692-8543

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How to cite this article: Rimbach H, Linxweiler M, Körner S, et al. Prediction of lymph node status in patients with surgically treated head and neck squamous cell carcinoma via neck lavage cytology: a pilot study. *Cancer Cytopathol*. 2024; 132(5):285-296. doi:10.1002/cncy.22800

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