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Tumor budding is a prognostic factor in head and neck squamous cell carcinoma: A comprehensive meta-analysis and trial sequential analysis

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**TITLE:**

**Tumor budding is a prognostic factor in head and neck squamous cell carcinoma: a comprehensive meta-analysis and trial sequential analysis.**

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**Competing Interests**

The authors declare no competing financial interests.

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**Author Contributions**

FFVES was responsible for designing the review protocol, writing the protocol and report, conducting the search, screening potentially eligible studies, extracting and analyzing data, interpreting results, writing the report, updating reference lists, and creating tables. VCAC and AILP conducted the meta-analysis and meta-regression analyses and contributed to the design of the review protocol, writing the report, extracting and analyzing data, and interpreting results. MEP, MPS, JSP, and CMCP provided feedback on the report.

**ABSTRACT**

**Background:** Mortality is linked to tumor budding (TB) in certain neoplasms. TB as a relevant histopathological feature is conditioned by tumor site, a specific study on head and neck squamous cell carcinoma (HNSCC) is needed.

**Methods:** A comprehensive meta-analysis was undertaken to investigate the relationship between TB and HNSCC related outcomes, such as overall survival (OS), disease-free survival (DFS), disease-specific survival (DSS) and lymph-node metastasis (LNM). Additionally, trial sequential analysis (TSA) was performed to ascertain meta-analytic findings for DSS.

**Results:** Overall 42 studies were included comprising 6,652 patients. Patients harboring high TB reported an OS HR of 2.63 (95% confidential interval (CI) 2.04-3.39), DFS HR of 1.88 (95%CI 1.57-2.24) and DSS HR of 2.14 (95%CI 1.81-2.52). LNM studies harbored null heterogeneity and marked association with TB (OR = 4.48, 95%CI 2.97-6.76). There was no marked evidence of publication bias. TSA supported definitive results for DSS.

**Conclusion:** The study has provided compelling evidence that there is a significant association between TB and worse prognosis for HNSCC, highlighting the importance of considering TB in the management these patients.

**KEYWORDS:** Squamous Cell Carcinoma of Head and Neck, Tumor Budding, Prognosis, Proportional Hazards Models, Pathology, Metanalysis.

## 1. Introduction

Head and neck cancer is the seventh most common group of tumors and refers to diffrent cancer types that affect the anatomical sites of the upper aerodigestive tract [1, 2]. Among the subtypes of this cancer, head and neck squamous cell carcinoma (HNSCC) stands out and is responsible for more than 90% of cases [3]. The long-term outcomes vary greatly depending on the stage of initial diagnosis, the site of involvement, and other relevant risk factors [4-8]. While TNM staging system remains the gold standard for the prognostic assessment, there are features to look for at diagnosis that are indicative of an aggressive phenotype [9].

Initially called “sprouting”, tumor budding (TB) was first described in 1954 by Imai [11], but it entered conventional pathology only in the last two decades with the term as it is known today, being coined by Hase et al. in 1993 [12, 13]. TB is a morphological phenomenon of diffuse mucous infiltrative growth, characterized by the presence of isolated cells or clusters of tumor cells (up to 5 cells) scattered in the stroma for a variable distance from the invasive front of cancer [14-21]. These cells detach from the tumor mass and migrate into the adjacent stroma, representing the first step towards invasive growth followed by metastasis [22, 23]. The presence of TB is commonly associated with a more aggressive cancer phenotype, correlated with lymph node metastasis, recurrence, distant metastasis, and thus poor survival [14, 22-26].

In 2016, The International Tumor Budding Consensus Conference (ITBCC) agreed on a standardized and evidence-based scoring system for TB in routine practice [27]. Despite all advances and the ITBCC, the TB score continues to be a time-consuming assessment with a certain degree of subjective interpretation, which ultimately results in relatively high inter- and intra-observer variability [28]. In recent years, many studies have been published related to TB and HNSCC prognosis [29][31] [32]. Despite, TB is still not considered a well-established or validated prognostic factor during routine histopathological assessment [18]. Prompted by discussed

literature, this systematic review aims to provide a critical and exhaustive qualitative and quantitative synthesis of the role of TB in HNSCC prognosis.

## 2. Material and methods

Prospero database was originally accessed on 23/06/2022 and the protocol for this systematic review and meta-analysis was submitted and successfully registered: CRD42022341763. Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines were followed [35].

### 2.1 Search strategy and database screening

To identify eligible articles, the search strategy included the screening of different databases, such as PubMed, Web of Science, and Scopus. A combination of keywords and terms was set and adjusted for each database. Keywords and search strategies adapted for each database are reported in Supplemental Material table 1 (**Table S1**). Databases were accessed for their inception while the last screen was performed on 28/12/2022. Duplicated articles were removed manually after EndNote reference software import and automatic duplicates removal (Endnote X9.3.2, Clarivate Analytics).

### 2.2. Eligibility Criteria

#### 2.2.1 Inclusion Criteria

The resulting list from the above-mentioned search was screened for eligible articles, fulfilling the following inclusion criteria: 1. Any publication year; 2. English publication language; 3. Cohort studies including patients over 18 years old and histopathologic diagnosis of head and neck squamous cell carcinoma; 4. Sample histopathologic assessment of tumor budding was used to investigate its contribution to overall survival (OS), disease-specific survival (DSS), and/or

disease-free survival (DFS) expressed in terms of Hazard Ratios (HRs); and lymph-node metastasis (LNM) reported as Odds Ratios (ORs).

#### 2.2.2. Exclusion Criteria

Systematic review and meta-analysis, case reports, and case series were excluded. Cohort studies with missing information for which HRs or ORs could not be retrieved were also excluded. The same was applied to studies including animal models or patients with post-chemo- or radiotherapy assessment of tumor budding.

### 2.3. Focused PICO Question

For patients with head and neck squamous cell carcinoma, is TB associated with worse outcomes, such as OS, DSS, DFS or LNM?

Participants: Patients with head and neck squamous cell carcinoma, undergoing tumor surgical resection treatment or bioptic examination before any chemo- or radiation therapy.

Intervention: TB histopathologic assessment, indicating patients with a high number of TB.

Comparison: TB histopathologic assessment indicating patients with low TB.

Outcome: OS, DSS, DFS, or LNM reported as HRs or ORs.

### 2.4. Studies screening and inclusion

The resulting list from merged databases, after duplicate removal, was screened by two authors (FFVES and VCAC) on one's own. This screening was performed in the first instance by title and abstract reading and produced a list of articles for further full-text eligibility assessment. Reviewers' agreement was evaluated in this last step by k-agreement score calculation and a third author (MPS) was involved in case of disagreements. These steps generated a file of included studies based on the above-listed inclusion and exclusion criteria.

### 2.5. Data extraction

An ad-hoc extraction sheet was created and filled independently by two reviewers (FFVES and VCAC). In a joint meeting, the resulting filled sheets were merged and in the circumstance that discrepancy emerged, together with a third author (AILP), articles were re-screened. The following fields were provided on the Excel extraction sheet: 1. First author's name; 2. Country of population recruitment; 3. Year of publication; 4. Type of outcome (OS, DSS, DFS, or LNM); 5. Subsite; 6. Sample size; 7. TNM Staging and staging edition; 8. Cut-offs to separate high and low tumor budding and 9. Staining.

### 2.6. Assessment of risk of bias

Included studies underwent assessment of the risk of bias (RoB) by evaluating an ad hoc subfields derived from Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [37]. Briefly, this process included the rating of six parameters, such as 1. Samples; 2. Clinical data of the cohort; 3. Histopathology/Immunohistochemistry (in this case, this item was adapted to reflect hematoxylin and eosin [HE] identification of tumor budding); 4. Prognosis; 5. Statistics and 6. Conventional prognostic factors. Each item was given a score, such as 1. Adequate (A); Inadequate (I) or not-available (NA). Each adequate item increased the overall quality score of each study. Supplemental Material Table 2 (**Table S2**) depicts the exact thresholds chosen for each item based on previous reports [37].

This process was undertaken independently by two participants in the review process (FFVES and VCAC) and differences of opinion were solved in a joint meeting with a third author (AILP). A score greater than 4 was considered a low risk of bias. Medium and high risk of bias were merged as low risk of bias for sensitive meta-analysis.

### 2.7. Statistical analysis

Data were input under “binary data effect sizes”, measured as ORs and relative 95% confidence intervals (CIs) for LNM. Considering OS, DFS, and DSS, the collected data from single studies were pooled under “survival data” and input as HRs and relative 95% C.Is. Studies, for which HRs and 95% CIs were not available, the method of Tierney et al. was employed to estimate HRs and Standard Errors (SEs) from Kaplan-Meier survival curves; subsequently, CIs were estimated from SEs [38]. Pooled effect sizes were represented by a forest plot. Differences among studies were input as moderators, specifically “country”; “cut-off values” to distinguish patients with high and low tumor budding, if 5 buds or 10 buds; “publication year”; “staging” based if the included cohort was limited to early cancer stages or advanced; “staining” used to assess tumor budding; “subsite” and “risk of bias” according to REMARK guidelines. Meta-analyses were run by adopting a fixed or random effect model based on results from heterogeneity investigation. Heterogeneity was investigated by Cochran’s Q test and $I^2$ index. This index indicated low or high heterogeneity on a cut-off value of 50%, for which, in case of low heterogeneity, a fixed effect model was applied; while for values above 50%, such as high heterogeneity, a random effect model was employed [39]. Sensitivity analysis was performed by leaving one out method, indicating the influence of individual studies on the overall results [40]. Sensitivity analysis was furtherly performed by estimating pooled effect sizes for each moderator. At last, publication bias was inspected visually, by funnel plot and statistically, by evaluating trim and fill analysis [41], Egger’s test [42], and fail-safe N test [43].

**2.8 Trial sequential analysis**

Trial sequential analysis (TSA) was employed to account for an increased chance of random errors and to determine whether further studies would be necessary. TSA combines the a priori Information size with recurrent significance testing on gathering information [44]. The power of the meta-analytic findings was evaluated using TSA, and the results were adjusted to account for type I and type II errors. Stata 13 (StataCorp, College Station, TX, USA), with the

implementation of the R 4.2 software and metacumbounds commands. A fixed-effect model was used to calculate each cumulative z-value because the meta-analysis revealed moderate heterogeneity ($I^2$ <50%). A priori information size (APIS) was calculated using an a priori anticipated intervention effect, a 15% relative risk reduction (RRR), a 5% type I error, and a 20% type II error [45, 46]. We considered only DSS as an outcome of interest for TSA.

## 3. Results

### 3.1 Study selection

After removing the duplicates, our syntax resulted in 577 reports, from which 65 were included for full-text assessment. Then, 68 did not meet the pre-defined inclusion criteria and were removed. Finally, 42 were included in the systematic review for both qualitative evaluation and quantitative synthesis. **Figure 1** depicts a flow diagram representing comprehensively the study selection process.

### 3.2 Study characteristics

Supplemental Material Table 3 (**Table S3**) reports in detail a synopsis of each included study, whilst **Table 1** summarizes a global overview of the characteristics of the 42 studies [16, 17, 19, 28, 29, 47-83]. A total of 6,652 HNSCC patients were included in the meta-analysis. The population sample ranged between 33 [64] and 340 [49] cases, while the year of publication ranged from 2011 [17] to 2022 [47, 50, 56, 63, 65, 68, 73, 74, 83] in studies carried out among 15 different countries on 3 continents, America [57, 63, 73, 80, 83], Asia [29, 50, 51, 55, 56, 58, 59, 61, 63, 66-72, 75-79, 81, 82] and Europe [16, 19, 28, 47-49, 52-54, 60, 62, 65].

### 3.3 Quality assessment

According to the cut-off point >4 mentioned in the methodology, 19.05% (8/42) of the studies were considered at high risk of bias [16, 29, 51, 54, 55, 64, 71, 82], and 80.95% (34/42) were considered at low risk of bias. Considering the maximum score of 6 as the standard, only 14.29% (6/43) of the studies were classified as adequate in all evaluated parameters, in addition to low

risk of bias [28, 49, 57, 65, 72, 80] (**Table 1; Table S4**).

In relation to the sample size domain of the 42 included studies, 11 (25.6%) were considered inadequate [16, 29, 51, 55, 56, 58, 63, 64, 68, 81, 82]. Regarding the clinical data of the cohort, 13 (27.9%) reports were considered inadequate [19, 50, 52, 53, 56, 61, 62, 63, 66, 71, 73, 74, 78]. In terms of histopathology or immunochemistry analysis, among the 43 included studies, only 4 (9.3%) were considered inadequate [47, 53, 54, 60]. According to the prognosis evaluation, 8 (18.6%) reports were considered inadequate [19, 51, 58, 64, 70, 74, 76, 82]. Statistical analysis varied widely across the 42 studies included, specifically 14 (32.6%) reports were considered inadequate [16, 29, 54, 55, 59, 62, 66, 67, 69-71, 75, 78, 82]. Finally, regarding the inclusion the of classical prognostic factors 19 (44.2%) reports were considered inadequate [16, 17, 29, 48, 50, 52, 53, 55, 59, 64, 69, 71, 75-77, 79, 81, 83] (**Table S4**).

### 3.4 Meta-analysis and Trial Sequential Analysis

#### 3.4.1 Overall survival

Meta-analysis of the random effects model for OS showed a HR of 2.63 (2.04-3.39; p-value <0.001). Heterogeneity results showed high heterogeneity between studies ($I^2$ = 70.03%) (**Figure 2A**). The presence of moderate publication bias was highlighted by the trim and fill method, which found 7 trimmed studies graphically represented by the funnel plot. However, Egger's linear regression test also showed absence of publication bias ($p_{Egger}$= 0.132). Trimmed studies yielded a new HR of 2.13 (1.65-2.74; p-value <0.001) (**Figure 2B**). Fail-safe N test simulation showed that 941 unpublished negative (HR = 1) studies with as many cases and controls as the average number of the published studies were needed to yield a HR of 1.00. Meta-regression by year of publication found no statistically significant difference (p-value of the random effect model = 0.908), concluding that the effect size did not depend on the year of publication (**Figure S1**). Leave-one-out demonstrated a strong consistency in our results (**Figure S2**).

Considering subgroups analysis, when discussing the differences between continents, the

random effect model showed HR of 3.22 (2.48-4.19) in Asia with low heterogeneity ($I^2$ = 38.09%) while in Europe a lower HR was found (1.95, 1.43-2.67, $I^2$ = 60.63%) (**Figure S3**). As for the tumor subsite, the random effect model demonstrated a HR of 2.71 (1.55-4.74, $I^2$ = 77.11%) in laryngeal tumors, HR of 2.53 (1.71-3.73, $I^2$ = 64.93%) in oral cavity and HR of 2.88 (1.73-4.79, $I^2$ = 74.54%) when studies considered exclusively tongue tumors (**Figure S4**). According to the staging, random effect model HR was 2.57 (1.88-5.52, $I^2$ = 59.82%) for studies considering any cancer stage, while early stages yielded a HR of 3.33 (1.89-5.88, $I^2$ = 84.97%) (**Figure S5**). When considering the cut-off points used in each study to mark high TB, a random effect model demonstrated a HR of 2.70 (1.44-5.08, $I^2$ = 49.97%) in studies that considered 10 buds and a HR of 2.78 (2.04-3.79, $I^2$ = 72.55%) in studies that considered 5 buds as a cut-off point (**Figure S6**). Concerning the technique used, we evaluated the differences between the studies according to the staining method used. A HR of 2.97 (2.05-4.30, $I^2$ = 70.24%) was found pooling HE studies, HR of 1.55 (0.87-2.74, $I^2$ = 52.39%) in IHC studies and HR of 2.99 (1.94-4.60, $I^2$ = 62.22%) when HE was employed with IHC to score TB (**Figure S7**). Regarding RoB assessment, random effect model meta-analysis showed a HR of 2.66 (1.91-3.70, $I^2$ = 69.09%) when the score was 1-4, and higher RoB studies yielded similar HR (2.62, 1.71-4.00, $I^2$ = 71.29%) (**Figure S8**).

### 3.4.2 Disease free survival

Meta-analysis of random effects model showed a HR of 1.88 (1.57-2.24, p-value<0.001) for DFS. Heterogeneity results showed average heterogeneity between studies ($I^2$ = 62.88%) (**Figure 2**). The trim and fill method did not impute any study when we applied the random effects model. However, Egger's linear regression test showed the presence of publication bias ($p_{Egger}$ <0.001), with a funnel plot with a skewness to the right. Fail-safe N test showed the necessity of 829 unpublished negative studies with as many cases and controls as the average number of the published studies to reverse our conclusion. Meta-regression by year of publication did not find any statistically significant difference (p-value of the random effect model = 0.139), concluding

that the effect size did not depend on the year of publication (**Figure S9**). Leave-one-out method demonstrated a strong consistency across outputs in Supplementary Material Figure 2 (**Figure S10**).

Considering subgroup meta-analysis, fixed effect model showed similar HRs for Asia and Europe, respectively 2.09 (1.75-2.50, $I^2$ = 48.05%) and 1.94 (1.61-2.34, $I^2$ = 0%), while lowest HR was found from studies performed in America (HR = 1.18 (1.07-1.31), $I^2$= ) (**Figure S11**). As for the tumor subsite, the random effect model demonstrated a HR of 2.28 (1.46-3.58, $I^2$ = 72.27%) in laryngeal tumors, HR of 1.77 (1.36-2.30, $I^2$ = 70.33%) in the oral cavity and HR of 1.84 (1.42-2.37, $I^2$ = 0%) when studies considered exclusively tongue tumors (**Figure S12**). According to the staging, a HR of 1.96 (1.55-2.48, $I^2$ = 49%) was found pondering the studies that considered any stage of the disease, and HR of 1.81 (1.36-2.40, $I^2$ = 70.17%) for early stages tumors (**Figure S13**). When considering the cut-off points used in each study to mark high TB, a random effect model demonstrated HR of 1.73 (0.99-3.02, $I^2$ = 3.09%) in studies that considered 10 buds as the cut-off point and HR of 1.89 (1.55-2.30, $I^2$ = 66.42%) in studies that considered 5 buds as a cut-off point (**Figure S14**). Differences among the studies according to the staining method found a HR of 1.80 (1.55-2.09, $I^2$ = 0%) for HE, HR of 1.60 (1.14-2.26, $I^2$ = 70.82%) for IHC and HR of 3.39 (1.70-6.76, 62.79%) for both staining techniques (**Figure S15**). Regarding the RoB assessment, a HR of 2.00 (1.51-2.64, $I^2$ = 53.52%) was demonstrated when the score was 1-4, while in studies where the score was 5-6 a HR of 1.80 (1.45-2.24) was demonstrated with heterogeneity $I^2$ = 63.76% (**Figure S16**).

**3.4.3 Disease specific survival**

Meta-analysis of the fixed effects model showed a HR of 2.14 (1.81-2.52, p-value <0.001) related to DSS. Heterogeneity results showed low heterogeneity between studies ($I^2$ = 48.14%) (**Figure 3**). The presence of publication bias was highlighted by the trim and fill method, which found 3 new ¨unpublished studies¨ through the fixed effect model, graphically depicted funnel plot. On

the other hand, Egger's linear regression test showed absence of publication bias ($p_{Egger}$ = 0.121) with estimated HR of 1.96 (1.67-2.30; p-value <0.001). Fail-safe N test showed the need of 264 studies with the predefined conditions to reverse the meta-analytic finding. Meta-regression by year of publication found no statistically significant difference (p-value of the fixed effect model = 0.500), concluding that the effect size did not depend on the year of publication (**Figure S17**). Leave-one-out method reinforced our results as in Supplementary Material Figure 3 (**Figure S18**).

The statistical significance of the meta-analytic finding was confirmed by TSA since the cumulative z-curve (blue line) crossed the monitoring boundary (dotted red line). After the addition of the 11th study, the results of this meta-analysis were considered powered since the blue line crossed the APIS (vertical red line – 1990 patients) (**Figure 5**).

In the subgroups analysis, concerning continent groups, the fixed effect model showed a HR of 2.71 (1.56-4.68, $I^2$ = 20.06%) for Asia, while for Europe and America, HRs resulted respectively 2.51 (2.02-3.13, $I^2$ = 50.53%), and 1.50 (1.12-2.00; v )(**Figure S19**). As for the tumor subsite, the fixed effect model demonstrated HR of 1.53 (1.13-2.09, $I^2$ = 0%) in the oral cavity studies and HR of 2.31 (1.89-2.82; $I^2$ = 0%) when studies considered exclusively tongue tumors. One study considered laryngeal tumors (**Figure S20**). When considering any stage tumors, random effects model showed a HR of 2.18 (1.55-3.06, $I^2$ = 69.31% ), while, when pondering the studies that considered only early stages cancers, a HR of 2.53 (1.68-3.79, $I^2$ = 0%) was pooled (**Figure S21**). Accounting for cut-off points used to define high TB, a fixed effect model demonstrated a HR of 1.73 (0.99-3.02, $I^2$ = 4.59%) in studies that considered 5 buds as the cut-off point. In DSS evaluation only one study considered 10 buds as a cut-off (**Figure S22**). Still, on the technique used, we evaluated the differences between the studies according to the staining, where a HR of 2.39 (1.76-3.24, $I^2$ = 59.93%) was found for HE and 2.16 (1.14-2.26, $I^2$ = 0%) for both HE and IHC staining techniques. In DSS evaluation only one study considered IHC alone as a staining

technique (**Figure S23**). Regarding the RoB assessment, high RoB studies yielded a pooled HR of 2.61 (1.74-3.92, $I^2$ = 59.77%), versus HR of 2.12 (1.51-2.98, $I^2$ = 22.92%) for low RoB subgroup (**Figure S24**).

### 3.4.4 Lymph node metastasis

Meta-analysis of the fixed effects model showed an OR = 4.48 (2.97-6.76, p-value <0.001) related to LNM. Heterogeneity resulted in null heterogeneity among studies ($I^2$ = 0%) (**Figure S25**). The absence of publication bias was highlighted by the trim and fill method, which found 1 study graphically represented by the funnel plot. Trimmed studies and Egger's linear regression test also showed absence of publication bias ($p_{Egger}$ = 0.826) with estimated OR of 4.27 (2.86-6.36; p-value <0.001). Fail and safety to reverse significance = 80 studies with the opposite result. Meta-regression by year of publication found no statistically significant difference (p-value of the fixed effect model = 0.184), concluding that the effect size did not depend on the year of publication (**Figure S26**). Leave-one-out method demonstrated the strength of our findings as in Supplementary Material Figure 4 (**Figure S27**).

## 4. Discussion

TB is a histological feature representing the tumor cells along the tumor front, able to detach from the tumor core, as single or grouped into up to five tumor cells, invading the adjacent normal tissue or related stroma [57]. In our meta-analysis, TB presence was strongly associated with worse survival in HNSCC patients, with HR of 2.63 (2.04-3.39; p-value <0.001), HR of 1.88 (1.57-2.24; p-value <0.001) and HR of 2.14 (1.81-2.52; p-value <0.001), referring to OS, DFS, and DSS, respectively, in addition to OR of 4.48 (2.97-6.76; p-value <0.001) related to LNM. However, moderate heterogeneity emerged among subgroup analyses. First, standardization for TB assessment is required. An approach to evaluate TB was suggested by the ITBCC for colorectal cancer. To prevent any loss of prognostic information, the ITBCC advises conducting its evaluation using HE stained sections with a cut-off of five buds/field and supplying the total number of

buds. The ITBCC group proposed an evaluation by an area equivalent to 0.785 mm2 (20 objective lens with a 20 mm eyepiece field diameter), generating a conversion table for microscopes with various fields of view, to assure the standardization of field size. In this meta-analysis, included studies reported different ways of categorization, both 5 and 10 buds, assessed by HE, IHC and both methods, which in some cases were noticed to be source of heterogeneity, with controversial impact on pooled HRs. Indeed, tumor sample characteristics and examiner experience showed to impact TB assessment . Raboh et al. found a moderate agreement between HE and IHC [61] similarly for Mascitti et al. study [65]. In our meta-analysis, HE related OS yielded a higher HR in HE and both HE/IHC staining, while for DFS highest HR was found for IHC studies while no differences were found for DSS. Cytokeratin IHC was suggested to improve a more adequate TB visualization; on the other hand, cytokeratin staining may be misleading in presence of apoptotic bodies and cellular debris, which should not be considered [17, 78]. Angadi et al. and Sakata et al. comment on the fact of the IHC would have improved reproducibility compared to the HE, especially considering the examiner's experience [51, 69]. Recently, some authors proposed models using the combination of TB with IHC-based staining of proteins related to epithelial mesenchymal transition showing promising results [77-79].

Differences between the cut-off points among the studies were occasionally worrying when thinking about creating reliable histological parameters for prognosis [65]. In our meta-analysis a cut-off point of 5 or 10 buds did not show differences in pooled HRs. Despite this, many authors have been proposed novel TB models by integrating TB to other clinic-pathological variables. Boxberg et al., for example, investigated the compatibility of a new classification system that unites TB activity and cell nest size [53, 54]. Almangush et al. also created a model that combined TB and histopathological parameters, including tumor grade and depth of invasion [48, 49]. In a cohort of 211 patients with tongue cancer, Mascitti et al. explored and tested 5 different TB models and found that the use of TB alone with a cut-off point of 5 buds, resulted in better prognostication.

This systematic review and meta-analysis confirmed the prognostic role of TB, however, our results should be interpreted in the light of some limitations.

First of all, the publication bias exerted an improbable effect on our data synthesis, given the persistence of a strong association even when considering highly conservative assumptions regarding the number, size, and findings of potentially excluded or unpublished studies. Also, it is meaningful to note that the possibility of publication bias derived from unpublished or grey literature can be considered minor, owed to asymmetry tests of the funnel plot as the trim-and-fill method did not significantly alter our outputs.

Secondly, despite the high statistical power demonstrated by TSA, some authors are cautious in concluding that further studies on the topic are unnecessary. This is due to the potential imprecision in constructing monitoring boundaries for the quantitative synthesis, as there is no control groups in included studies to guarantee the genesis of new evidence, implying the adoption of "discontinuation rules". Therefore, the use of TSA should be simply interpreted as a method of detecting false positive or false negative results in our pooled analysis, rather than a proper indicator to truly discard the need for further studies [84].

Furthermore, although our study was still designed to overcome all anticipated limitations, when considering the studies individually, certain difficulties were observed and must be considered. The main limitation observed was the absence of absolute numerical values of HR or OR even in the presence of Kaplan-Meier plots. Specifically in these studies, data were extracted from the graphs through an approximation protocol. This approach could introduce some residual confounding, nonetheless when restricting our analysis to studies that adjusted for potential confounders (i.e., studies with a low RoB) the estimates remain close to our global outputs as ascertained by sensitivity analysis. The ethnic-geographical distribution of the studies could help explain some inconsistencies observed when comparing studies carried out in different parts of the world. Differences in TNM staging between studies are also an important factor to be

considered as a limiting factor, mainly because this is a prognostic and survival evaluation study. The aforementioned differences regarding the parameters for classifying TB and the techniques used in each study may also suggest deviations in the results. Differences between the number of samples, and test period, as well as ambiguities in the distinction between OS, DFS, and DSS, must also be considered. The existence of an unknown cofounders so strongly associated with the TB prognosis capacity in HNSCC is highly implausible.

Finally, in our subgroup analyses we delved into some factors accounted for study heterogeneity as in other cases characteristic able to enhance substantially the prognosis value of TB. In the situations where heterogeneity emerged, it should be viewed more as the rule rather than the exception as Berlin stated [85]. Moreover, in order to confront these statistic scenarios, random effects estimates were computed which are considered classically more conservative [86].

In conclusion, the consistent findings analyzed in this systematic review and meta-analysis indicate that TB may be useful as a prognostic histopathological marker for HNSCC. The practical applicability of this study aims to overcome any individual gaps that clinical studies may have left, and generate, based on strict consensus and reliable methods, a guiding light on the use of TB as a reliable prognostic histological marker in HNSCC.

**Tables**

**Table 1:** Main characteristic and quality scores of included studies.

DFS, disease free survival; DSS, disease specific survival; LNM, lymph node metastasis; OS, overall survival.

**Figure legends**

**Figure 1:** Flow diagram of study selection.

**Figure 2:** A) Forest plots of the association of tumor budding overexpression with overall survival (OS) (random-effect model). B) A funnel plot of estimated logHR against its standard error, graphically representing the analysis of "small study" effects on OS. The black circles represent the studies published and the black bubble represents the non-published studies estimated by

the trim-and-fill method.

**Figure 3:** A) Forest plots of the association of tumor budding overexpression with disease free survival (DFS) (random-effect model). B) A funnel plot of estimated logHR against its standard error, graphically representing the analysis of “small study” effects on DFS.

The black circles represent the studies published and the black bubble represents the non-published studies estimated by the trim-and-fill method.

**Figure 4:** A) Forest plots of the association of tumor budding overexpression with disease specific survival (DSS) (fixed-effect model). B) A funnel plot of estimated logHR against its standard error, graphically representing the analysis of “small study” effects on DSS.

The black circles repre-sent the studies published and the black bubble represents the non-published studies estimated by the trim-and-fill method.

**Figure 5:** Trial sequential analysis related to the association between tumor budding and disease-specific survival.

APIS, light green line (Z = 1.98); dashed red line (monitoring boundary); blue line (cumulative z curve); red line (sample size).

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