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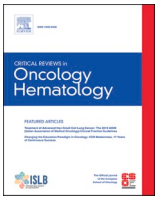

Correlation of Bcl-2 expression with prognosis and survival in patients with head and neck cancer: A systematic review and meta-analysis

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ABSTRACT

Head and neck cancer (HNC) is a growing disease, affecting more than 700.000 cases per year and ranking as the sixth most prevalent type of cancer worldwide. The impossibility of properly entering into apoptosis directly influences uncontrolled growth and consequently tumor development and progression. Bcl-2 emerged as a key regulator in the balance between cell apoptosis and proliferation in apoptosis machinery. This systematic review and meta-analysis aimed to review all published studies investigating changes in Bcl-2 protein expression assessed by immunohistochemistry (IHC) and related to prognostic and survival values of patients with HNC. After applying the inclusion and exclusion factors, we reached the number of 20 articles included in the meta-analysis. The random-effect pooled HR (CI95%) value of OS related to Bcl-2 IHC expression in tissues from HNC patients was 1.80 (CI95% 1.21–2.67) (p 0.0001) and DFS was 1.90 (CI95% 1.26–2.86 (p 0.0001). The OS value for the specific oral cavity tumors was 1.89 (1.34–2.67), while in the larynx it was 1.77 (0.62–5.06), and the DFS in the pharynx was 2.02 (1.46–2.79). The univariate and multivariate analyses of OS were respectively 1.43 (1.11–1.86) and 1.88 (1.12–3.16), while in DFS it was 1.70 (0.95–3.03) and 2.08 (1.55–2.80). The OS considering a low cut-off for Bcl-2 positivity was 1.19 (0.60–2.37) and DFS was 1.48 (0.91–2.41), while studies with a high cut-off demonstrated OS of 2.28 (1.47–3.52) and DFS of 2.77 (1.74–4.40). Our meta-analysis demonstrates that Bcl-2 protein overexpression can result in worse LNM, OS, and DFS in patients with HNC, however, it is not a reliable conclusion, due to the wide divergences between the original studies and the fact that many studies have a very high range of confidence and also a high risk of bias.

1. Introduction

Head and neck cancer (HNC) is a growing disease, affecting more than 700.000 cases per year and ranking as the sixth most prevalent type of cancer worldwide (Nix et al., 2005). This type of cancer includes all tumors that have originated in the mucosa of the oral cavity, oropharynx, hypopharynx, larynx, paranasal sinuses, and nasopharynx; and is most common histologically diagnosed as head and neck squamous cell carcinomas (HNSCC) (Pena et al., 1999; Aupérin, 2020). The mortality rate is very high, mainly because of their aggressiveness and frequent loco-regional metastases since the early stages (Cohen et al., 2018).

Apoptosis is a programmed cell death, which occurs intending to eliminate cells altered or senescent. DNA damages must be recognized, and when not repaired, damaged cells must be driven to apoptosis (Quentmeier et al., 2022). Programmed cell death, or its lack of

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functioning, is a milestone for oncology because cancer cells develop mechanisms to evade both apoptosis and growth inhibition signals. These mechanisms are described in several types of cancer and are associated with therapeutic resistance, prognosis and patient survival (Silva et al., 2022).

Proteins that regulate cell proliferation and apoptosis are closely related in a complex and multi-step pathway (Friedman et al., 2001). The impossibility of properly entering into apoptosis directly influences uncontrolled growth and consequently tumor development and progression (Fisher, 1994). In this complex protein machinery, Bcl-2 emerged as a key regulator in the balance between cell apoptosis and proliferation (Reed, 1995; Wilson et al., 2001).

Bcl-2 is a 26 kDa mitochondrial apoptosis regulatory protein that blocks programmed cell death (Solomon et al., 2016; Klatka, 2001). The Bcl-2 protein is a part of Bcl-2 genes family, which can be classified into anti-apoptotic or pro-apoptotic proteins (Kato et al., 2008). Bax and Bak are examples of pro-apoptotic proteins, while Bcl-2 and Bcl-X are examples of anti-apoptotic members of this family (Camisasca et al., 2009). Apoptosis is mainly regulated by the balance of pro- and anti-apoptotic proteins, so that overexpression of Bcl-2 is a central role in neutralize the function of pro-apoptotic proteins and consequently inhibits apoptosis (Yuen et al., 2001; Lazaris et al., 2000).

The first identification of Bcl-2 protein occurred in 1979 at a T-chromosomal translocation breakpoint of B-cell lymphoma (Popović et al., 2007; Boise et al., 1995; TTrask et al., 2002). Since then, its expression has been reported in hematopoietic malignancies and several solid tumors (Ghanem et al., 2001). In HNC, overexpression of Bcl-2 has been reported, but its role in the prognosis and survival of patients has shown variable and inconstant results (Wagener et al., 1996; Stoll et al., 2000; Prasad et al., 2012). Therefore, our systematic review and meta-analysis aimed to review all published studies investigating changes in Bcl-2 protein expression assessed by immunohistochemistry (IHC) and related prognostic and survival values of patients with HNC.

2. Material and methods

This systematic review was designed and filed by FFVS with registration in PROSPERO (CRD42022362303) and duly approved by all other authors involved. All terms indicated by the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) were used. The question elaborated according to the PICO framework was: "Does Bcl-2 protein expression predict prognosis in HNC?"; The population (P) refers to patients with HNC, intervention (I) refers to the assessment of Bcl-2 protein expression by IHC, comparison (C) refers to high versus low or positive versus negative Bcl-2 expression, outcome (O) refers to overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS).

2.1. Search strategy

The databases, Medline (via PubMed), Scopus, Cochrane, Web of Science, and Lilacs were used for screening studies from inception to October 2022. Grey Literature Database also was screened using the New York Academy of Medicine Grey Literature.

Thesaurus terms (eg, MeSH and EMTREE) and free text words were used to search the databases. The syntax was designed properly for each database, based on Medline screening: (“Head Neck Cancer” [All Fields] OR (“Oral Cancer” [All Fields] AND “Bcl-2″ [All Fields] AND (“Prognosis” [MeSH Terms] OR “Survival” [All Fields]). This search strategy was also used through manual search in journals involved with oral pathologies, otolaryngology, and oncology. The handling and elimination of duplicate articles from the references were performed using the EndNote 20 software by Clarivate™ (Philadelphia, PA, United States of America).

2.2. Eligibility criteria

An ad hoc review group composed of professionals qualified in medicine, oral medicine, molecular oncology and biostatistics was set up to carry out this systematic review and meta-analysis. The searches were performed by two experts (FFVS and GCVC) in two phases. A first, where the studies were selected through titles and abstracts, and a second, where the texts were selected after reading in full. After the individual analysis, the information was cross-referenced between the two specialists. Discordant cases were analysed by a third researcher (VCAC) and it was decided if the study would be included or not. Interobserver agreement was determined using the freeware Epidat 4.2 (SERGAS, Santiago de Compostela, Spain) using Cohen’s kappa coefficient (k).

Inclusion criteria: i) Original research published in English; ii) Evaluation of Bcl-2 expression using IHC in human tissues; iii) All subtypes of Head and Neck Cancer; iv) Analysis of the association between Bcl-2 overexpression that had some type of survival analysis: Overall Survival (OS), Disease-Free Survival (DFS) or Disease-Specific Survival (DSS); v) Studies that presented Hazard Ratios (HR) with the appropriate 95% Confidence Intervals (CI95%).

Exclusion criteria: i) Studies published in non-English language; ii) Reviews, case reports, case series and editorials; iii) Studies carried out in animals or in vitro; iv) Studies on Thyroid Cancer or subtypes where there are divergences in their classification as HNC; v) Comparison between different groups or diseases; vi) Studies demonstrated insufficient data, with no presence of HR CI95%.

2.3. Data extraction

The data extracted included: First author, year of publication, country where the study was performed, sample size, tumor subsite, recruitment period, type of comparison used (e.g. low versus high or negative versus positive), cut-off, magnification used for field analysis under a microscope, follow-up time of patients, staging edition used, type of survival analysis and HR (CI95%) as a prognostic value.

2.4. Quality assessment

All studies included in this meta-analysis underwent a risk of bias analysis using the parameters of the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) (Altman et al., 2012). All items included in the REMARK were duly analysed and classified by each of the two specialists (FFVS and GCVC) as adequate (A) or inadequate (I). The parameters used for this classification were: Sample size, clinical data, IHC, prognostic follow-up, statistical data and classical prognostic analyses. The sample size cut-off was considered 80 patients, considered through a sufficient relative average for the sample size to generate statistically significantly relevant results. Clinical information was considered inadequate when not informed, when not clearly described, or in studies where there were no direct comparisons of clinical-pathological variables with the expression of Bcl-2. The IHC was considered inadequate due to technical/parameters. The prognostic follow-up was considered inadequate when was not informed or insufficient for a faithful evaluation. Statistics were considered inadequate when statistical flaws were observed in the methodology and/or in the demonstrated results, as well as, in the absence of absolute values related to OR and/or HR related to prognosis or survival. The classic prognostic factors were considered inadequate when the study was limited information and analysis of clinical-pathological and prognostic values.

When the two experts disagreed, a debate was held so that they could reach a consensus. When the score was > 4, the studies were considered to be of high quality.

2.5. Statistical analysis

2.5.1. Statistical analysis for survival

Through the HR (CI95%) extracted from the participating studies, the impact of Bcl-2 expression on the aforementioned long-term outcomes (OS, DFS, and DSS) was estimated. Multivariate or univariate HR values were used, being differentiated and analysed together and also separately. When the HR data were not directly exposed in the studies, the referring study was duly excluded, according to the explicit inclusion and exclusion factors. The group analyses were performed using Review Manager software version 5.2.8 (Cochrane Collaboration, Copenhagen, Denmark; 2014). For the analysis of survival related to Bcl-2 expression, the natural logarithmic of the HR and its CI95% were entered into the software. The fixed effect was calculated by the inverse of variance test with a p-value lower than 0.05 as the threshold of statistical significance. Heterogeneity was also calculated according to the Q and I^2 methods. In addition, the results of the meta-analysis were summarized in forest plots, funnel plots and also subgroup/sensitivity analysis.

2.5.2. Statistical analysis for clinical-pathological aspects

It is extremely important to observe the Odds Ratio (OR) relative to the clinical-pathological characteristics of sick patients and relate it to survival, so that it is possible to measure data on the evolution and severity of such events with the survival rates of patients, being useful tools for coping with risk factors, as well as opening the possibility of comparisons between specific events and the prognosis of patients in different groups. For this reason, data were extracted as a total number of patients with high and low expression against the number of patients with high and low expression reporting the outcomes such as: Lymph node metastasis (LNM) - Grade 2–3 - Stage III-IV or T status 3–4. Data were pooled by the Mantel-Haenszel method to obtain a cumulative OR and respective CI95%.

3. Results

3.1. Study selection process and study features

The search strategy indicated in the methodology was applied and 2.005 articles were found in the databases. After removing duplicates, we are left with a number of 890 articles. A first manual exclusion by reading the titles and abstracts brought the number to 283, and a second manual exclusion brought the number to 231 articles. After applying the inclusion and exclusion factors, we reached the number of 20 articles included in the meta-analysis (Boonyaphiphat et al., 2012; Chen et al., 2008, 2010; Cullen et al., 2009; Gallo et al., 1996, 1999; Gasparini et al., 1995; Giotakis et al., 2019; Gomatos et al., 2007; Holgersson et al., 2010; Homma et al., 2001; Ito et al., 1999; Lee et al., 2021; Lo Muzio et al., 2005; Lovato et al., 2020; Michaud et al., 2009; Nichols et al., 2010; Shah et al., 2009; Thongsuksai et al., 2014; Trivedi et al., 2011) (Fig. 1). The statistical value κ was 0.89, considered an excellent level of agreement between reviewers.

The descriptive values of each study were organized through a table (Table 1). The studies were carried out in 8 different countries in Europe (Gallo et al., 1996, 1999; Gasparini et al., 1995; Giotakis et al., 2019; Gomatos et al., 2007; Holgersson et al., 2010; Lo Muzio et al., 2005; Lovato et al., 2020), North America (Cullen et al., 2009; Michaud et al., 2009; Nichols et al., 2010), and Asia (Boonyaphiphat et al., 2012; Chen et al., 2008, 2010; Homma et al., 2001; Ito et al., 1999; Lee et al., 2021; Shah et al., 2009; Thongsuksai et al., 2014; Trivedi et al., 2011) between the years 1995 (Gasparini et al., 1995) and 2021 (Lee et al., 2021). The total number of patients analysed was 1.913, ranging from 31 (Lovato et al., 2020) to 265 (Cullen et al., 2009) patients. The recruitment period

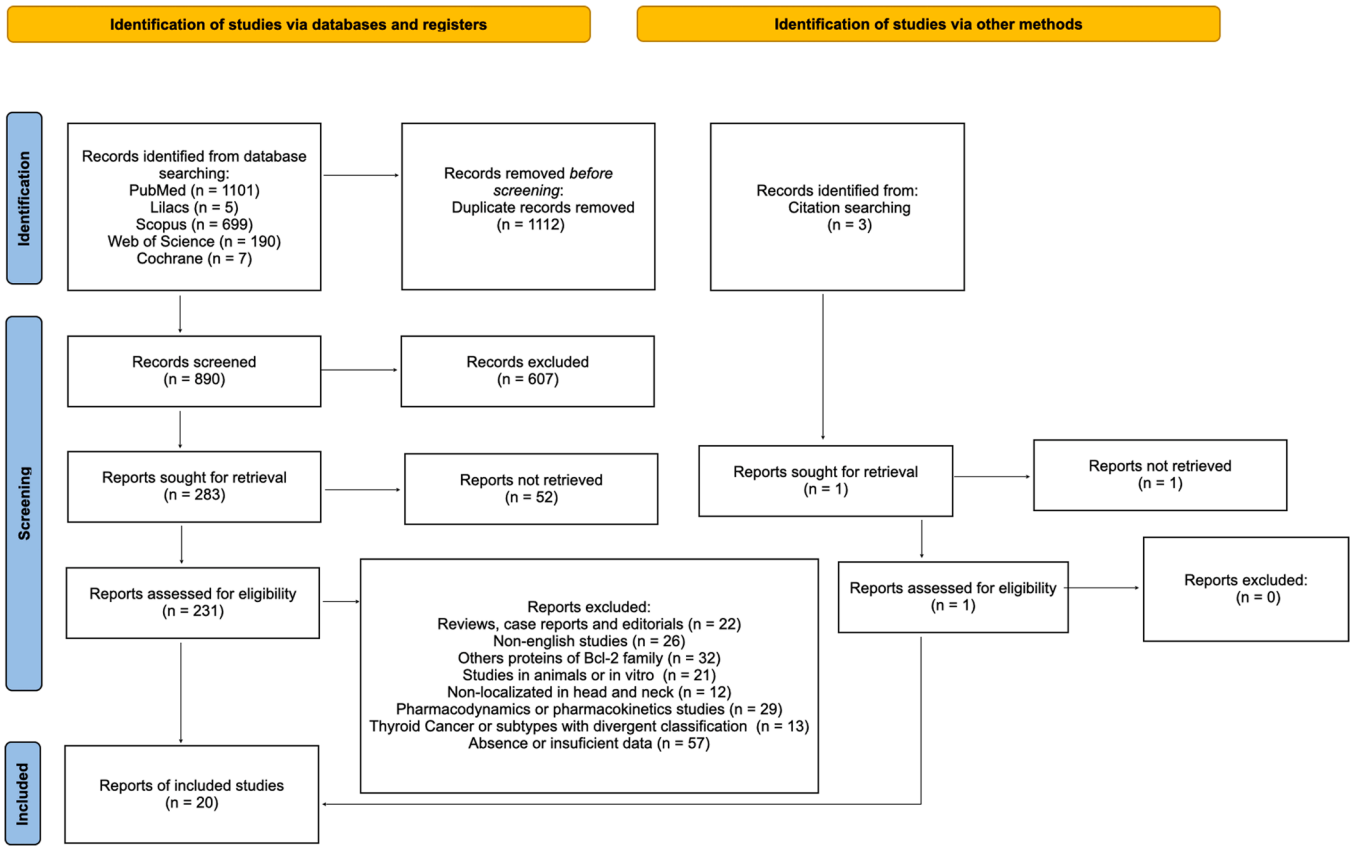

Fig. 1. Search strategy flow diagram. Flow diagram following PRISMA 2020 guidelines for new systematic reviews which included searches of databases, registers, and other sources.

Table 1
General Information. Information regarding the extraction of individual data from each study was included in the meta-analysis. *OS = Overall Survival; DFS = Disease-free Survival.

Authors	Year	Country	Subsite	Recruitment Period	Sample Size	Cut-Off	Follow-Up	Survival Analysis	Compared Data	REMARKS
Boonyaphiphat *et al.*	2012	Thailand	Larynx	2002–2004	94	5%	NI	OS	Positive x Negative	4
Chen *et al.*	2010	Taiwan	Pharynx	1996–2000	145	10%	NI	DFS	Positive x Negative	5
Chen *et al.*	2008	Taiwan	Pharynx	1996–2000	105	10%	NI	DFS	Positive x Negative	5
Cullen *et al.*	2009	USA	Pharynx, Larynx, Oral Cavity	NI	265	1%	2 Years	OS - DFS	Low x High	5
Gallo *et al.*	1996	Italy	Pharynx, Larynx, Oral Cavity	1986–1989	71	30%	54.2 Months	OS - DFS	Positive x Negative	6
Gallo *et al.*	1999	Italy	Pharynx, Larynx, Oral Cavity	1988–1991	85	30%	72 Months	OS - DFS	Positive x Negative	6
Gasparini *et al.*	1995	Italy	Pharynx, Larynx, Oral Cavity	1989–1993	73	25%	16 Months	DFS	Low x Moderate x High	5
Giotakis *et al.*	2019	Greece	Larynx	2005–2012	78	30%	80.9 Months	OS	Positive x Negative	6
Gomatos *et al.*	2007	Greece	Larynx	1992–1994	37	10%	50.5	OS	Positive x Negative	5
Holgersson *et al.*	2010	Sweden	Larynx	1978–1998	39	25%	NI	OS	Low x High	4
Homma *et al.*	2001	Japan	Larynx	1990–1994	62	30%	46.9 Months	OS	Positive x Negative	6
Ito *et al.*	1999	Japan	Pharynx, Oral Cavity	1984–1996	57	20%	2 Years	OS	Positive x Negative	6
Lee *et al.*	2021	Taiwan	Larynx	2012–2015	98	NI	68 Months	DFS	Positive x Negative	6
Lo Muzio *et al.*	2005	Italy	Oral Cavity	1995–2000	66	5%	72 Months	OS	Positive x Negative	6
Lovato *et al.*	2020	Italy	Larynx	NI	31	1%	43 Months	DFS	Positive x Negative	3
Michaud *et al.*	2009	USA	Pharynx	1996–2005	38	25%	46.5 Months	DFS	Positive x Negative	5
Nichols *et al.*	2010	USA	Pharynx	NI	68	25%	47 Months	OS - DFS	Positive x Negative	6
Shah *et al.*	2009	India	Oral Cavity	2000–2003	89	10%	2 Years	OS - DFS	Positive x Negative	6
Thongsuksai *et al.*	2014	Thailand	Pharynx	2002–2004	140	50%	5 Years	OS - DFS	Low x Moderate x High	4
Trivedi *et al.*	2011	India	Oral Cavity	2000–2003	135	30%	NI	OS - DFS	Positive x Negative	6

varied between 1978 (Holgersson et al., 2010) and 2015 (Lee et al., 2021) and the subsites analysed included the larynx, pharynx, and oral cavity. The expression of the Bcl-2 protein was evaluated through different cut-offs, ranging from 1% (Cullen et al., 2009; Lovato et al., 2020) to 50% (Thongsuksai et al., 2014) of cells stained to be considered positive. The individual statistical analyses of each study were performed univariate, multivariate, or both. Also, the types of comparisons varied between No/Low versus Moderate/High (or more than 2 groups) and Negative versus Positive analysis. In the end, we only managed to obtain a sufficient number of articles for meta-analysis regarding OS and DFS, thus analyzing DSS was impossible.

3.2. Quality Assessment within Studies

According to the cut-off point > 4 mentioned in the methodology, 35% of the studies were considered at high risk of bias (Boonyaphiphat et al., 2012; Gasparini et al., 1995; Gomatos et al., 2007; Homma et al., 2001; Lee et al., 2021; Lo Muzio et al., 2005; Michaud et al., 2009). Individually, the sample size was considered inadequate in 50% of the studies (Gallo et al., 1996; Gasparini et al., 1995; Giotakis et al., 2019; Gomatos et al., 2007; Holgersson et al., 2010; Homma et al., 2001; Ito et al., 1999; Lo Muzio et al., 2005; Michaud et al., 2009; Nichols et al., 2010). Clinical information was considered inadequate in 20% of the studies (Cullen et al., 2009; Gasparini et al., 1995; Gomatos et al., 2007; Trivedi et al., 2011). The IHC was considered inadequate in 30% of the studies (Boonyaphiphat et al., 2012; Cullen et al., 2009; Lee et al., 2021; Lo Muzio et al., 2005; Lovato et al., 2020; Thongsuksai et al., 2014). The prognostic follow-up was considered inadequate in 20% of the studies (Boonyaphiphat et al., 2012; Gasparini et al., 1995; Holgersson et al., 2010; Trivedi et al., 2011). The statistics were considered inadequate in 40% of the studies (Gallo et al., 1996, 1999; Gasparini et al., 1995; Gomatos et al., 2007; Homma et al., 2001; Lee et al., 2021; Michaud et al., 2009; Nichols et al., 2010). The classical prognostics analysis was considered inadequate in 65% of the studies (Boonyaphiphat et al., 2012; Chen et al., 2008, 2010; Gasparini et al., 1995; Giotakis et al., 2019; Gomatos et al., 2007; Homma et al., 2001; Ito et al., 1999; Lee et al., 2021; Lo Muzio et al., 2005; Lovato et al., 2020; Michaud et al., 2009; Thongsuksai et al., 2014) (Table S1).

3.3. Quantitative evaluation (Meta-Analysis)

3.3.1. Survival analysis

Fixed and random effect models are used. The grouped HR with a CI of 95% was evaluated for the survival analyses referring to OS and DFS, taking into account the heterogeneity according to the p-values and their respective Q tests. The random-effect pooled HR (CI95%) value of OS related to Bcl-2 IHC expression in tissue from HNC patients was 1.80 (CI95% 1.21–2.67); with Tau^2 heterogeneity = 0.39; Chi^2 = 47.00; df = 14; ($p < 0.0001$); I^2 = 70% (Fig. 2). For DFS the combined HR value with random effect was 1.90 (CI95% 1.26–2.86) with Tau^2 heterogeneity = 0.34; Chi^2 = 39.44; df = 11; ($p < 0.0001$); I^2 = 72% (Fig. 3).

3.3.2. Subgroups Analysis

We separately analysed the values of the studies in subgroups

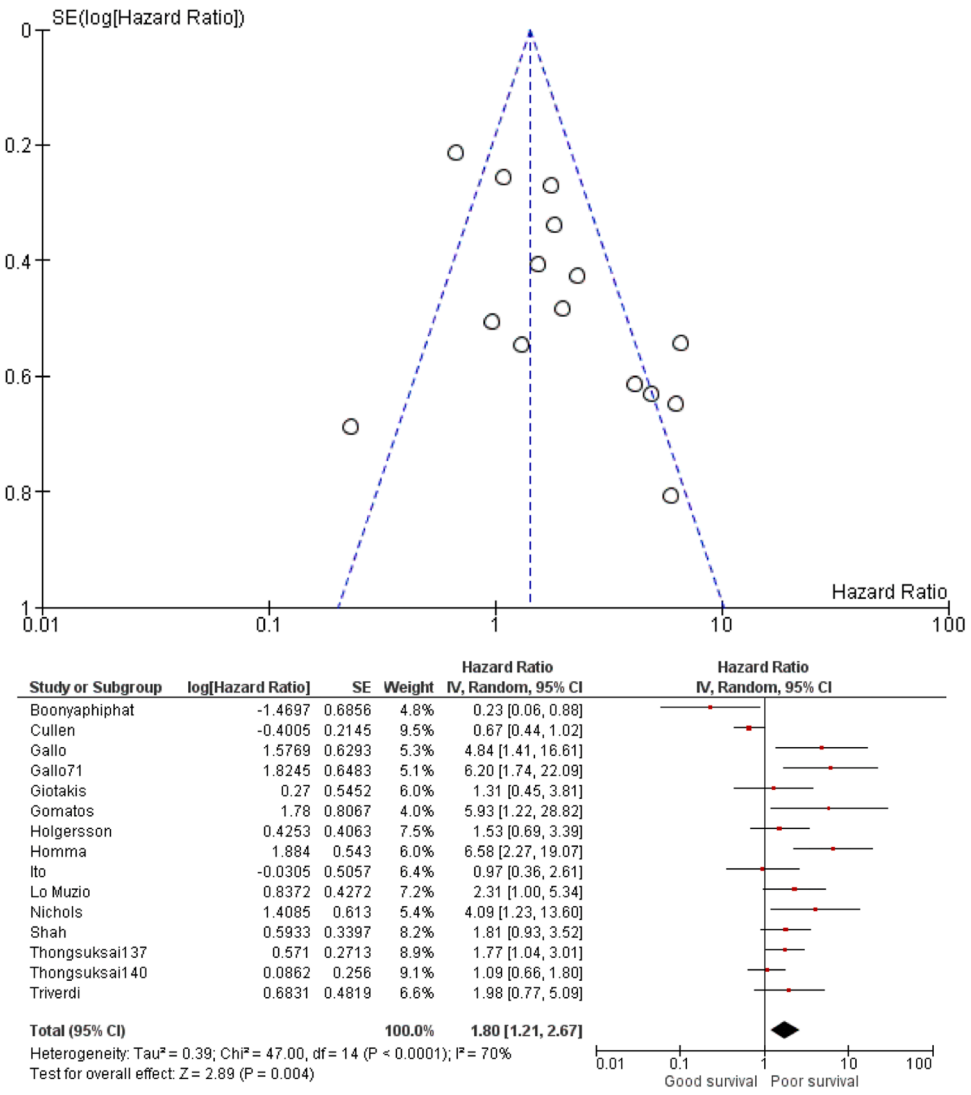

Fig. 2. OS meta-analysis of included studies. The Funnel Plot and the Forest Plot demonstrated meta-analysis results by comparing the included studies' HR and CI 95% values and considering heterogeneity between studies. *p = 0.0003.

according to the subsites of the tumors. The oral cavity, larynx and pharynx were analysed through a pooled HR with CI95% of random or fixed effect model (considering I^2 > or <50% for random or meta-analysis fixed effect). The OS value for the oral cavity was 1.89 (1.34–2.67) with zero heterogeneity (I^2 = 0%) (Fig. 4A), while for the larynx it was 1.77 (0.62–5.06) with high heterogeneity (I^2 = 77%) (Fig. 4B). Pharyngeal DFS was 2.02 (1.46–2.79) with low heterogeneity (I^2 = 29%) (Fig. 4C).

Subgroup analyses were also carried out according to the types of statistical analyses. The univariate and multivariate analyses for OS were respectively 1.43 (1.11–1.86; I^2 = 75%) and 1.88 (1.12–3.16; I^2 = 68%) (Fig. 5A; B), while for DFS it was 1.70 (0.95–3.03; I^2 = 76%) and 2.08 (1.55–2.80; I^2 = 0%) (Fig. 5C; D).

The cut-offs to consider the positivity of Bcl-2 expression considered by the authors varied a lot, for this reason, we performed an analysis referring to these subgroups. The average between the cut-offs was 20%, a reason why we divided the studies into two groups: A group comprising studies where cut-off was considered below 20% of expression; and a group that considered cut-off above 20%. Studies referring to the cut-off below 20% and above 20% showed an average OS of 1.19 (0.60–2.37; I^2 = 75%) and 2.28 (1.47–3.52; I^2 = 55%), respectively (Fig. 6A; B) and DFS of 1.48 (0.91–2.41; I^2 = 75%) and 2.77 (1.74–4.40; I^2 = 0%), respectively (Fig. 6C; D).

3.3.3. *Clinical pathological analysis*

Data were grouped by the Mantel-Haenszel method to obtain a cumulative OR and respective CI95% on each clinical-pathological aspect related to Bcl-2 expression. LNM showed an OR = 1.68 (95% CI 1.15–2.47) (Fig. 7A), while grading 2–3 showed an OR = 1.02 (0.67–1.55) (Fig. 7B). The staging III-IV demonstrated an OR = 2.06 (1.39–3.07) (Fig. 7C), while regarding the T status 3–4 demonstrated an OR = 1.27 (0.93–1.74) (Fig. 7D).

4. Discussion

4.1. *Meta-analysis*

Our meta-analysis showed a direct relationship between Bcl-2 overexpression and the worst prognosis and survival of patients, but when you analysed individually each study, these results are contestants. The analyses referring to OS and DFS demonstrate a very high heterogeneity, which directly influences the significance of the results of the meta-analysis in general.

Considering the divergent values and the high heterogeneity, our meta-analysis was considered extremely necessary to analyze different subgroups for more conclusive results. It is important to highlight that the analysis of OS in the larynx was not statistically significant and that

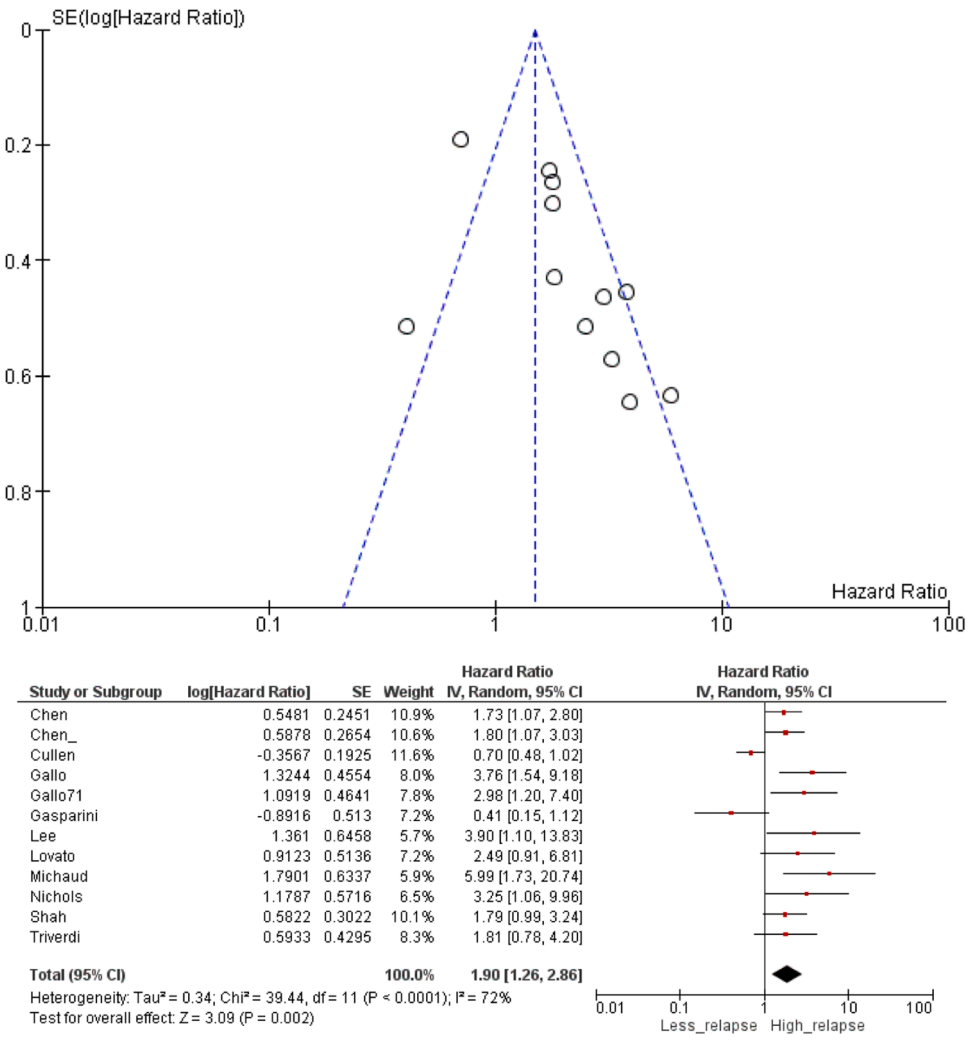

Fig. 3. DFS meta-Analysis of included studies. The Funnel Plot and the Forest Plot demonstrated meta-analysis results by comparing the included studies' HR and CI 95% values and considering heterogeneity between studies. *p = 0.002.

the study by Lee et al (Lee et al., 2021). was excluded from the analysis of subgroups referring to the cut-off for not specifying the parameters used to consider the positivity of Bcl-2 protein expression.

4.2. *Bcl-2 expression*

The Bcl-2 family of proteins plays an important regulatory role in cell fate. Individually, each protein in the family acts to inhibit or promote apoptosis (Chen et al., 2008; Holgersson et al., 2010). Bcl-2 is an anti-apoptotic protein whose expression has already been detected in several types of cancers, including lung, breast, cervical, prostate, and HNC (Lee et al., 2021; Lo Muzio et al., 2005). The biological effects of Bcl-2 expression in malignant neoplasms are divergent depending on the type of tumor (Chen et al., 2010).

Bcl-2 IHC expression was verified in all studies included in this review (Boonyaphiphat et al., 2012; Chen et al., 2008, 2010; Cullen et al., 2009; Gallo et al., 1996, 1999; Gasparini et al., 1995; Giotakis et al., 2019; Gomatos et al., 2007; Holgersson et al., 2010; Homma et al., 2001; Ito et al., 1999; Lee et al., 2021; Lo Muzio et al., 2005; Lovato et al., 2020; Michaud et al., 2009; Nichols et al., 2010; Shah et al., 2009; Thongsuksai et al., 2014; Trivedi et al., 2011). However, an important divergence in the categorization and cut-off is observed, where some studies consider the intensity of stained (Gasparini et al., 1995; Homma et al., 2001), others the semiquantitative gradation of stained cells (Gallo et al., 1996; Gomatos et al., 2007; Lovato et al., 2020), or a combination of the two classifications (Chen et al., 2008, 2010; Cullen et al., 2009; Giotakis et al., 2019; Holgersson et al., 2010; Nichols et al., 2010; Shah et al., 2009; Trivedi et al., 2011). In other words, some consider the expression only as a qualitative evaluation, in a simplified way (Gallo et al., 1996; Lovato et al., 2020), while others made a more systematic and quantitative evaluation, classifying according to the percentage as negative, low and high (Chen et al., 2008, 2010; Gasparini et al., 1995; Giotakis et al., 2019; Gomatos et al., 2007; Holgersson et al., 2010).

Bcl-2 overexpression in laryngeal carcinomas was found in some studies (Lee et al., 2021; Thongsuksai et al., 2014), however, in our meta-analysis, this overexpression did not show statistical significance. In OSCC this overexpression was commonly observed, even in poorly or moderately differentiated tumors (Thongsuksai et al., 2014). An important feature reported by Lee et al. was that intratumoral cells had higher Bcl-2 expression than peritumoral cells, going against the grain of Lo Muzio et al. who reported that cells located peripherally within the infiltrated tumor nest were more intensely stained (Lee et al., 2021; Lo Muzio et al., 2005).

4.3. *Risk factor, clinical-pathological aspects and prognosis*

Homma et al. associated Bcl-2 expression with some clinical aspects such as age and sex (Homma et al., 2001). However, in contrast, most studies have not observed a direct association between Bcl-2 protein

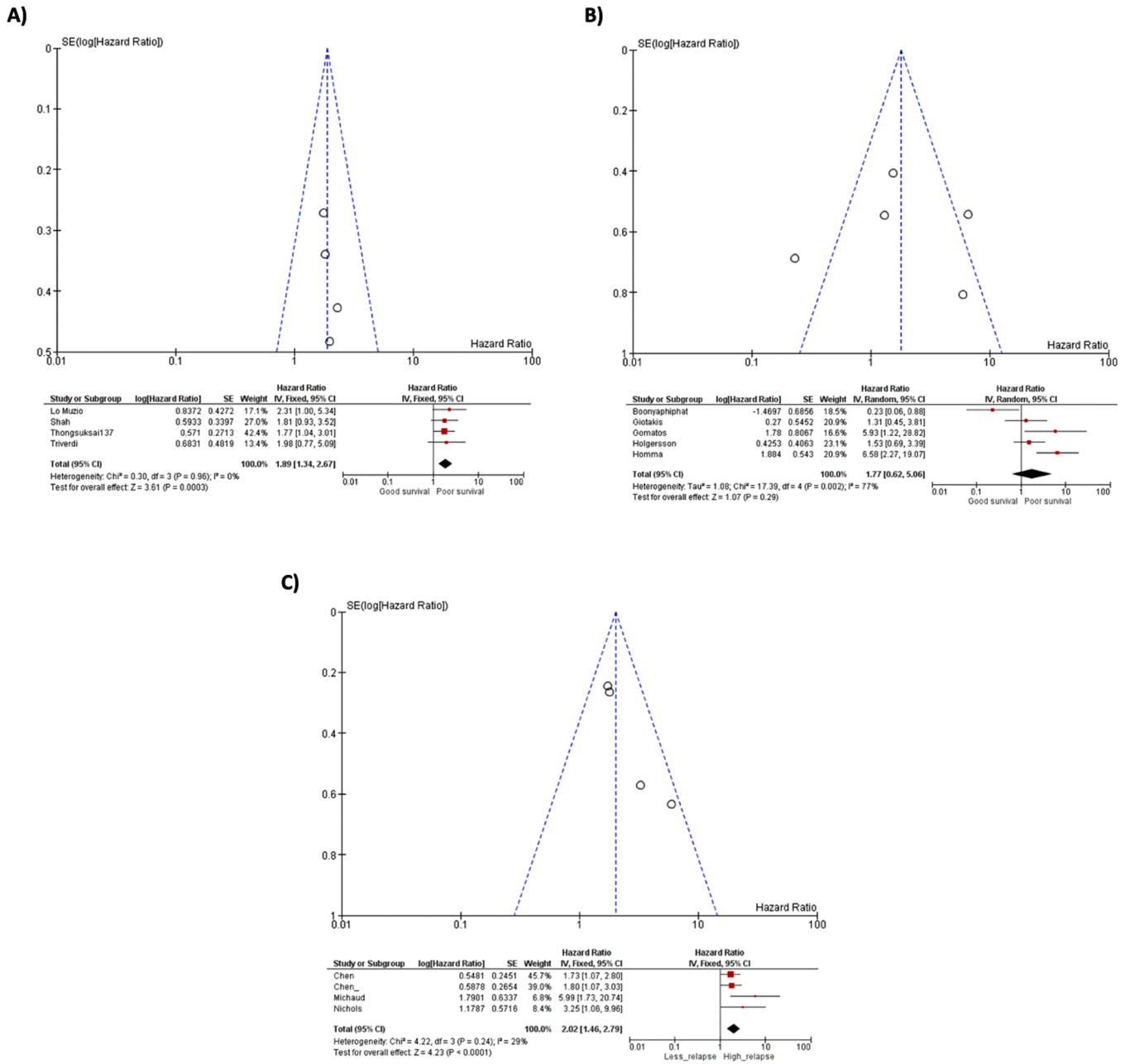

Fig. 4. Subgroup survival analysis referring to subsites. (A) OS meta-analysis referring to Oral Cavity Cancer; *p = 0.96 (B) OS meta-analysis referring to Larynx studies *p = 0.29; (C) DFS meta-analysis referring to Pharynx Cancer *p < 0.0001. The Funnel Plot and the Forest Plot demonstrated meta-analysis results by comparing the included studies' HR and CI95% values and considering heterogeneity between studies.

expression and classic clinical features such as TNM stage and histological grade (Chen et al., 2008, 2010; Gallo et al., 1996, 1999; Giotakis et al., 2019; Ito et al., 1999; Nichols et al., 2010).

Bcl-2 overexpression was considered an independent risk factor (Gasparini et al., 1995; Gomatos et al., 2007; Lee et al., 2021) and an independent predictor of survival (Gomatos et al., 2007; Michaud et al., 2009), which means no association with any other clinicopathological characteristic or with any other biological marker (Cullen et al., 2009; Gasparini et al., 1995; Lee et al., 2021). An intriguing fact was reported by Trivedi et al., who found the expression of Bcl-2 as an independent risk factor in tongue tumors, but not in other subsites of the oral cavity (Trivedi et al., 2011).

Lovato et al., contrary to other authors' claims, identified Bcl2 overexpression in T3-T4 patients compared to T1-T2 and considered that Bcl-2 expression is associated with the Maspin expression pattern (Lovato et al., 2020). Also, in this study, they observed that the subgroup of patients with non-nuclear Maspin expression and Bcl-2 positive had a lower DFS when compared to the subgroup with nuclear Maspin expression and Bcl-2 positive, not considering Bcl-2 expression an independent risk factor, raising the hypothesis that the nuclear expression of Maspin could influence the apoptotic process and regulate Blc-2 functions (Lovato et al., 2020).

In the study by Gallo et al., 70% of patients positive for Bcl-2 also had a simultaneous mutation in the p53 gene (Gallo et al., 1999). Furthermore, a statistically significant association was also found between Bcl-2 expression and tobacco exposure (Gallo et al., 1999).

Some authors consider that positive Bcl-2 expression is associated with an unfavorable prognostic biomarker, in agreement with the results of our meta-analysis, suggesting a more aggressive treatment (Chen et al., 2008, 2010; Michaud et al., 2009; Trivedi et al., 2011). But

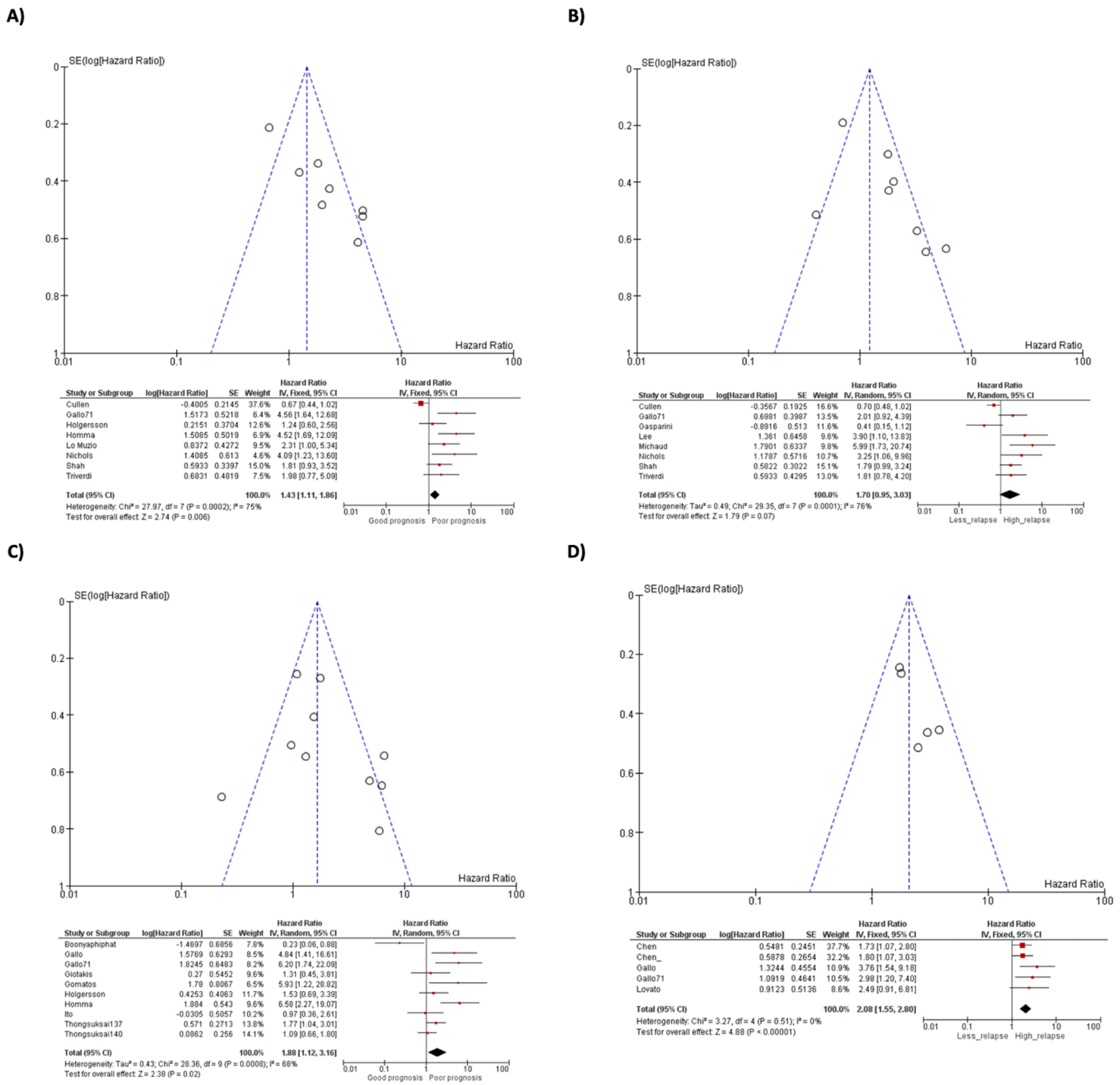

Fig. 5. Subgroup survival analysis referring to univariate and multivariate analysis studies. (A) The OS related to univariate analysis *p = 0.006; (B) DFS related to univariate analysis *p = 0.07; (C) The OS related to multivariate analysis *p = 0.02; (D) DFS related to multivariate analysis *p < 0.00001. The Funnel Plot and the Forest Plot demonstrated meta-analysis results by comparing the included studies' HR and CI95% values and considering heterogeneity between studies.

considering the heterogeneity of our meta-analysis, some other studies associated contrary conclusions, like Lo Muzio et al., that consider the negative expression of Bcl-2 associated with aggressive biological behavior and worst prognosis in OSCC (Lo Muzio et al., 2005).

Lee et al., Thongsuksai et al., and Ito et al. indicated in their study that Bcl-2 expression is in no way related to aggressiveness and prognosis in HNSCC (Ito et al., 1999; Lee et al., 2021; Thongsuksai et al., 2014), contradicting the results where the positive or negative expression of Bcl-2 was considered an important prognostic factor (Chen et al., 2008, 2010; Michaud et al., 2009; Trivedi et al., 2011).

Although Bcl-2 overexpression has been associated with primary tumors in several studies (Lee et al., 2021; Lo Muzio et al., 2005). Chen et al. make a connection between the overexpression of Bcl-2 protein with a blockade of apoptosis in experiments performed in cell lines derived from HNSCC already in the initial stage of carcinogenesis (Chen et al., 2008, 2010).

Most of the studies included in this meta-analysis established no association of overexpression of Bcl-2 with recurrences (Lee et al., 2021), but Cullen et al. and Gasparini et al. in a univariate analysis associate a significantly higher risk of relapse and primary disorders in Bcl-2 positive patients (Cullen et al., 2009; Gasparini et al., 1995). Galo et al. also described a relationship between post-treatment recurrence and positive Bcl-2 and, moreover, stated that recurrence occurs more frequently in stage I and II oral cavity tumors than in laryngeal and pharyngeal tumors (Gallo et al., 1996).

Studies have suggested that Bcl-2 status is closely related to the success of chemoradiation treatment of HNC culminating in a better prognosis (Gallo et al., 1996; Lee et al., 2021; Michaud et al., 2009).

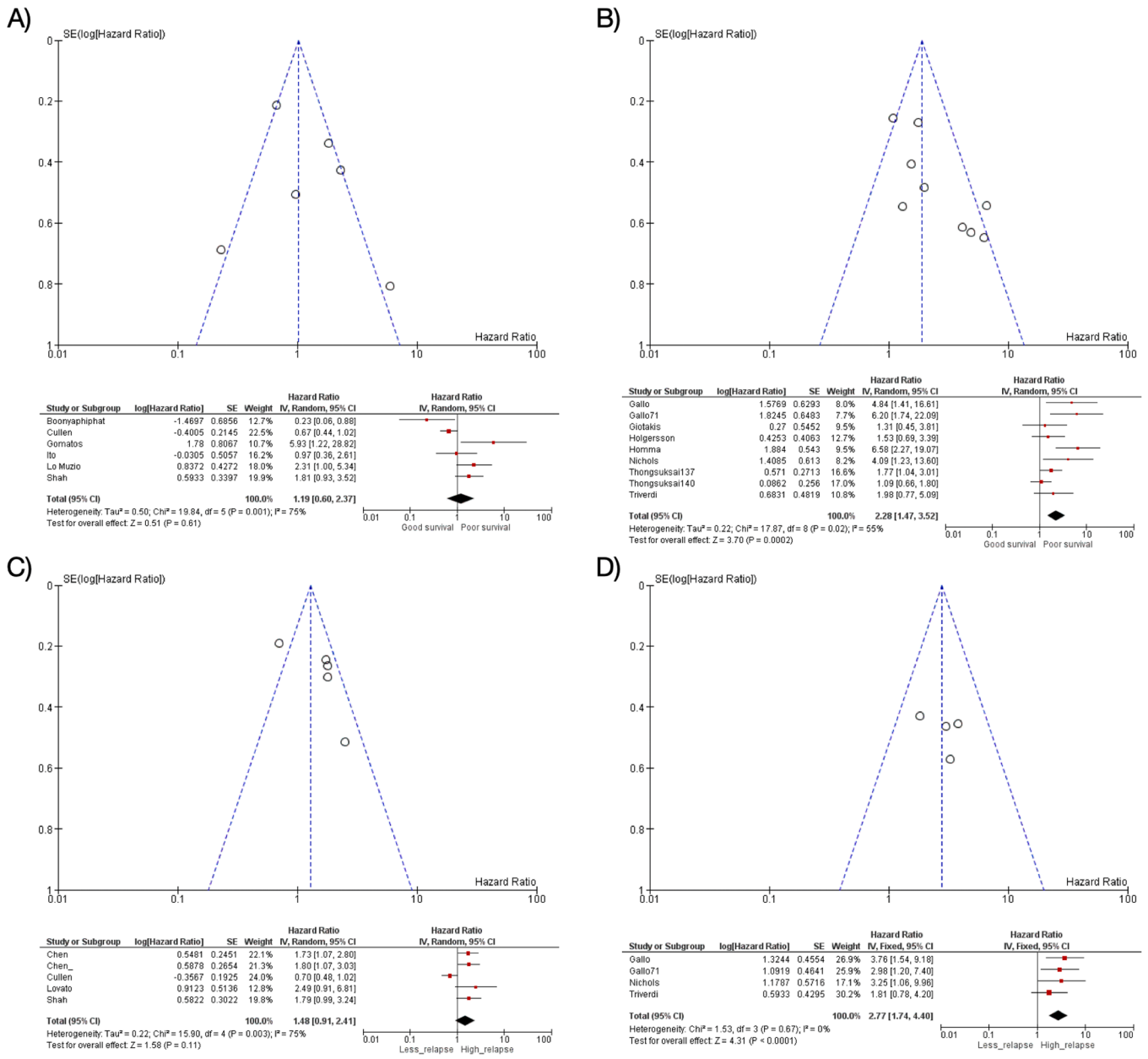

Fig. 6. Subgroup survival analysis referring to different cut-offs used as positivity/overexpression parameters for Bcl-2 protein in each study. (A) The Funnel Plot and the Forest Plot specific to OS for low-cut-off studies *p = 0.61; (B) The Funnel Plot and the Forest Plot specific to OS for high-cut-off studies *p = 0.0002; (C) The Funnel Plot and the Forest Plot specific to DFS for low-cut-off studies *p = 0.11; (D) The DFS-specific Funnel Plot and the Forest Plot for high-cut-off studies *p < 0.0001.

When considering the response to tumor treatment, Gasparini et al. significantly associated higher response rates to therapy in Bcl-2 positive patients (Gasparini et al., 1995). On the other hand, Michaud et al. associated high endogenous Bcl-2 expression with a worsening response to chemotherapy treatment with cisplatin (Michaud et al., 2009).

4.4. DFS and OS

The relationship between Bcl-2 expression and patient survival showed some differences in the studies included in this meta-analysis. For some studies, no significant differences were found from OS or DFS related to Bcl-2 expression, either positive or negative (Giotakis et al., 2019; Lovato et al., 2020).

Cullen et al., Boonyaphiphat et al., and Ito et al. noticed a better OS in tumors with positive expression of Bcl-2 (Boonyaphiphat et al., 2012; Lo Muzio et al., 2005). In another way, Chen et al. revealed that survival was worse in patients with Bcl-2 positive expression (Chen et al., 2010). Giotakis et al. and Thongsuksai et al. showed no significant association between Bcl-2 expression and survival in a multivariate analysis (Thongsuksai et al., 2014).

Gallo et al. in 1996 and 1999 reported that Bcl-2 expression was the most important indicator for OS and DFS within 5 years in patients with early-stage HNC, treated mainly with radiotherapy (Gallo et al., 1996, 1999). There was an association in the studies by Gallo et al. between the simultaneous detection of Bcl-2 protein expression and p53 gene mutation in HNSCC and patient survival, while Boonyaphiphat et al. considered that p53 expression had no association with survival (Boonyaphiphat et al., 2012; Gallo et al., 1999).

Giotakis et al. also compare results at an advanced stage, where the OS Bcl-2 positive group had a significantly longer survival time. While in

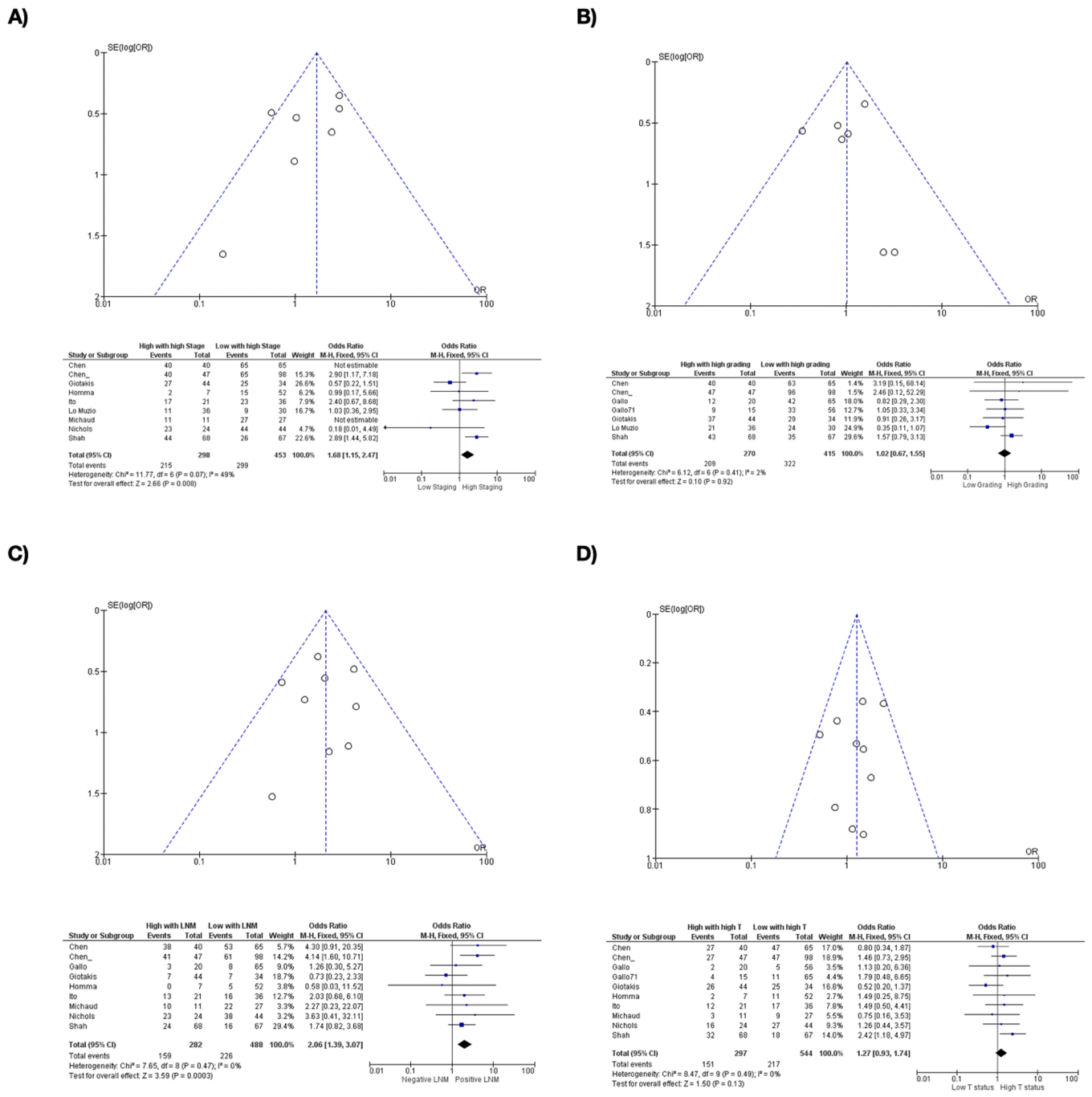

Fig. 7. Meta-analysis comparing clinical-pathological aspects with the expression of Bcl-2 in included studies. (A) Meta-analysis related to LNM *p = 0.008; (B) Meta-analysis related to Grading 2–3 *p = 0.92; (C) Meta-analysis related to staging III-IV *p = 0.0003; (D) Meta-analysis related to T status 3–4 *p = 0.13. The Funnel Plot and the Forest Plot demonstrated meta-analysis results by comparing the included studies' OR and CI95% values and considering heterogeneity between studies.

the early stage, no significant difference was observed between Bcl-2 positive and negative patients (Giotakis et al., 2019).

4.5. Importance and limitations

This systematic review and meta-analysis is a pioneering review relating Bcl-2 expression to patient prognosis and survival. Despite being relatively discussed a long time ago, the relationship and use of this protein as an indicator of survival are still very much questioned.

The articles included in this meta-analysis were mostly studies with high-quality scores, and even though there are articles published more than two decades ago, the inclusion of recent articles was extremely important to improve statistical power and prioritize studies that use precise analytical methods for reliable expression analysis.

The findings of this systematic review and meta-analysis are supported by solid evidence, and a reliable number of patients/studies are included, but some limitations must be mentioned and considered.

The main limitation concerns the exclusion of several studies that did not present absolute values of HRs. Some of them, even in the presence of Kaplan-Meier with survival analysis, did not have the clear values necessary for reliable statistical analysis and therefore had to be excluded. Another important limitation is the absence of absolute values

referring to LNM in the major part of the studies.

Another common limitation in studies related to HNC concerns the heterogeneity of lesions related to this type of cancer, as they represent a huge variety of tumor subsites and anatomical structures. For this reason, we analysed differentiating the subsites in the statistical analysis of the subgroups, to try to fill some gaps.

The studies included in our meta-analysis had quite varied qualities and quite clear individual flaws, and even some were described by the authors themselves. This could be represented through our risk of bias analysis, which was 35%, a number considered high for a meta-analysis (Table S2).

The different commercial brands and dilutions of the anti-Bcl-2 antibodies used may have generated deviations between the results. In addition to variations in positivity parameters and techniques used for detecting and grading positivity. Differences between the marking cut-off points can also generate important deviations.

Analysis of protein expression may also vary based on the ethnographical distribution of patients, which would explain the instability of some results observed in studies carried out in different parts of the world. Other limitations may be related to the differences between the number of samples, the tumors' individual clinical characteristics, the ambiguities in the semiology, and the distinction between the prognostic and survival values.

Even with all these limitations exposed, the study demonstrated total reliability of the results, with wide application in the field of molecular oncology, although more accurate and concordant IHC reports are still needed to validate this biomarker for use in clinical practice.

5. Conclusion

The results of this systematic review and meta-analysis point in a good direction in considering the analysis of Bcl-2 expression as a predictor of prognosis in patients with HNC. Our meta-analysis demonstrates that Bcl-2 protein overexpression can result in worse LNM, OS, and DFS in patients with HNC, however, it is not a reliable conclusion, due to the wide divergences between the original studies and the fact that many studies have a very high range of confidence and also a high risk of bias.

The lack of clarity in the methods and statistical results of some studies, as well as the methodological divergences of evaluation and parameters that characterize a Bcl-2 overexpression are barriers that need to be overcome when we consider the individual failures of each study. There is also an urgent need for new studies that correlate the expression of the Bcl-2 protein with clinical-pathological, prognostic, and survival aspects, which are also of paramount importance.

Considering the results of our meta-analysis, together with all the aspects mentioned in this conclusion, we were able to clearly observe the need for further studies that may shed light on the importance of analyzing Bcl-2 expression as a prognostic predictor in patients with HNC.

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CRediT authorship contribution statement

FFVS was responsible for designing the review protocol, conducting the search, screening potentially eligible studies, extracting and analyzing data, writing and revising the report, updating reference lists, and creating figures and tables. **GCVC** contributed to conducting the search, screening potentially eligible studies, and writing and revising the report. **VCAC** and **AILP** conducted the meta-analysis, analyzing data and interpreting results. **KCL** and **SLVS** were responsible for extracting data. **MPS, CMCP**, **MEPI**, and **JSP** were responsible for designing the review protocol and providing feedback on the report.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.critrevonc.2023.104021.

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