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Published online 2024 March 10. **Systematic Review**

The Relationship Between Serum Folate and Homocysteine with Head and Neck Cancer: A Systematic Review and Meta-analysis

Mona Moghaddam Vahed [1], Ayla Bahramian [2], Katayoun Katebi [2,*] and Fatemeh Salehnia [3]

[1]Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Oral and Maxillofacial Medicine, Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Research Center for Evidence Based Medicine (RCEBM), Tabriz University of Medical Sciences, Tabriz, Iran

[*]*Corresponding author*: Department of Oral and Maxillofacial Medicine, Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran. Email: k_katebi@yahoo.co.uk

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Abstract

Context: Early diagnosis of head and neck squamous cell carcinomas (HNSCC) is critical for preventing further disease progression. This study aimed to compare the serum folate and homocysteine levels in patients with HNSCC and healthy controls through a systematic review and subsequent meta-analyses.

Evidence Acquisition: The research question was: Is there a difference between serum folate and homocysteine levels (O) of patients with HNSCC (E) compared to healthy controls (C)? To conduct a systematic review, keywords were first identified and then searched in Medline/PubMed, Web of Science, ProQuest, EMBASE, Scopus, and Google Scholar databases within the period from January 2000 to November 2023. The searched studies were screened based on inclusion and exclusion criteria, and after assessing the quality of the selected articles using the Joanna Briggs Institute assessment checklist, 10 articles were finally included in the meta-analysis (nine articles for serum folate and eight for homocysteine). Due to the heterogeneity of studies, meta-analyses were conducted according to the random-effects model. Several meta-analyses were carried out because the selected articles were not uniform regarding smoking habits.

Results: Regardless of smoking conditions, the serum folate levels of the HNSCC patients were significantly lower than those of the control groups. Similarly, the serum homocysteine levels were significantly higher in the patient groups compared to the control groups.

Conclusions: The meta-analyses in this study showed an association between serum folate and homocysteine levels with HNSCC, indicating their possible use as biomarkers for the early detection of HNSCC.

Keywords: Folate, Head and Neck Squamous Cell Carcinoma, Homocysteine, Meta-Analysis

1. Context

Head and neck squamous cell carcinomas (HNSCCs) are the most common malignancies affecting the oral cavity, pharynx, and larynx (1, 2). Despite advancements in HNSCC treatment, it still exhibits a low 5-year survival rate of approximately 40 percent (3). Many potential biochemical markers associated with head and neck malignancies originate from the methionine cycle. Methionine, a crucial amino acid, is essential for normal human growth and participates in numerous metabolic pathways (4, 5). Homocysteine (Hcy), an intermediate metabolite in the methionine cycle, influences all methyl and sulfur groups involved in bodily metabolism. DNA methylation plays a crucial role in gene expression, thereby affecting phenotype changes (6). Folate is responsible for remethylating Hcy back to methionine (7). Elevated serum Hcy levels are often linked to folate deficiency (8).

Several studies have documented a significant correlation between the occurrence of HNSCC and decreased serum folate (9-16), as well as increased serum Hcy levels (9, 10, 12, 13, 15, 17, 18). In a systematic review encompassing four studies, HNSCC patients exhibited significantly lower serum folate levels compared to controls (19).

Early detection of HNSCC through biomarkers holds promise in preventing disease progression. Hence, this study aimed to explore the association between serum folate and Hcy levels and HNSCC. To the best of our knowledge, no meta-analysis has yet been conducted on

these associations.

2. Evidence Acquisition

The research question addressed whether there is a difference in serum folate and Hcy levels between patients with HNSCC and healthy controls.

2.1. Literature Search and Study Selection

To conduct a systematic review, literature searches were performed using MeSH and free terms based on the PECO (Population, Exposure, Comparator, and Outcomes) strategy across multiple databases, including Medline/PubMed, Web of Science, Google Scholar, ProQuest, EMBASE, and Scopus. Additionally, reference lists and citations of included articles were reviewed for further relevant studies. A comprehensive search was carried out, including ProQuest for dissertations, Google Scholar for conference papers, and https://greymatters.cadth.ca/ for gray literature. The search strategy involved various combinations of terms such as folic acid, folate, pteroylglutamic acid, folvite, folacin, vitamin B9, vitamin M, homocysteine, Hcy, 2-amino-4-mercaptobutyric acid, squamous cell carcinoma, head and neck, oral, HNSCC, OSCC, oral tongue, oral cavity, laryngeal, larynx, nasal cavity, nasopharyngeal, hypopharyngeal, mouth, oropharyngeal, salivary gland, lip, cervical tracheal, tracheal, neoplasm, cancer, biomarker.

The inclusion criteria for selecting studies were articles published in English until November 20, 2023, case-control or cohort studies involving newly diagnosed, untreated HNSCC patients with histopathologically confirmed diagnosis and measurement of serum levels of Hcy and folate, and no restriction on age. Exclusion criteria comprised letters to the editors, meta-analyses, or systematic reviews.

2.2. Data Extraction

Following the extraction of articles from the selected databases, two authors (MMV and KK) independently assessed them. Any discrepancies were resolved by a third author (AB). Articles were screened based on title, abstract, and full text, with those meeting the inclusion criteria selected for further analysis. Extracted data included primary author name, location, publication year, study design, sample size of cases and controls, type of sample specimens, serum folate and Hcy levels with standard deviation in HNSCC patients and control groups, and any subgroup data (e.g., smokers or non-smokers), along with confounding factors in each study.

2.3. Assessment of the Risk of Bias

The retrieved articles underwent evaluation by two independent authors (K.K and M.M.V) utilizing the Joanna Briggs Institute (JBI) assessment checklist comprising 10 items. This checklist aimed to assess the methodological quality of the selected articles and identify potential biases in design, conduct, and analysis, such as appropriate case-control matching, measurement reliability, identification of confounding factors, and utilization of suitable statistical analysis (20). Articles with a JBI checklist score of ≥ 7 were deemed high-quality. In instances of disagreement between the two evaluators, a third evaluator (A.B.) was consulted.

2.4. Statistical Analysis

Meta-analysis was conducted based on sample size, mean, and standard deviation. The heterogeneity among studies was evaluated using the I2 index and the chi-square test. Given the significant heterogeneity observed between studies and I2 values exceeding 50%, the random-effects model was employed for the meta-analyses (21). Publication bias was assessed using the Egger linear regression model (22) and the Begg and Mazumdar rank correlation test (23). The pooled mean difference between case and control groups for serum folate and Hcy levels, along with its 95% confidence interval, was utilized to identify associations between serum folate/Hcy and HNSCC occurrence. Statistical analyses were performed using STATA (version 12).

While most studies included in the meta-analysis assessed both smoking and non-smoking patients, the majority did not report serum folate and Hcy levels separately for these subgroups (9, 14, 17). However, most of these studies separated serum folate and Hcy levels between smoker and non-smoker controls (9, 10, 12-16). To approximate the confounding effect of smoking on serum folate and Hcy levels in relation to HNSCC, five meta-analyses were conducted for smoking and/or non-smoking conditions, along with a meta-analysis comparing smoker controls with non-smoker controls.

3. Results

3.1. Summary of Study Search and Characteristics

The literature search yielded 1 425 articles after removing duplicate papers within EndNote. Out of the 21 articles that underwent full review, 12 were included in the systematic review. However, only 10 studies were eligible for the meta-analysis, as two studies (18, 24) did not report means and standard deviations. Figure 1 illustrates the search results and screening process.

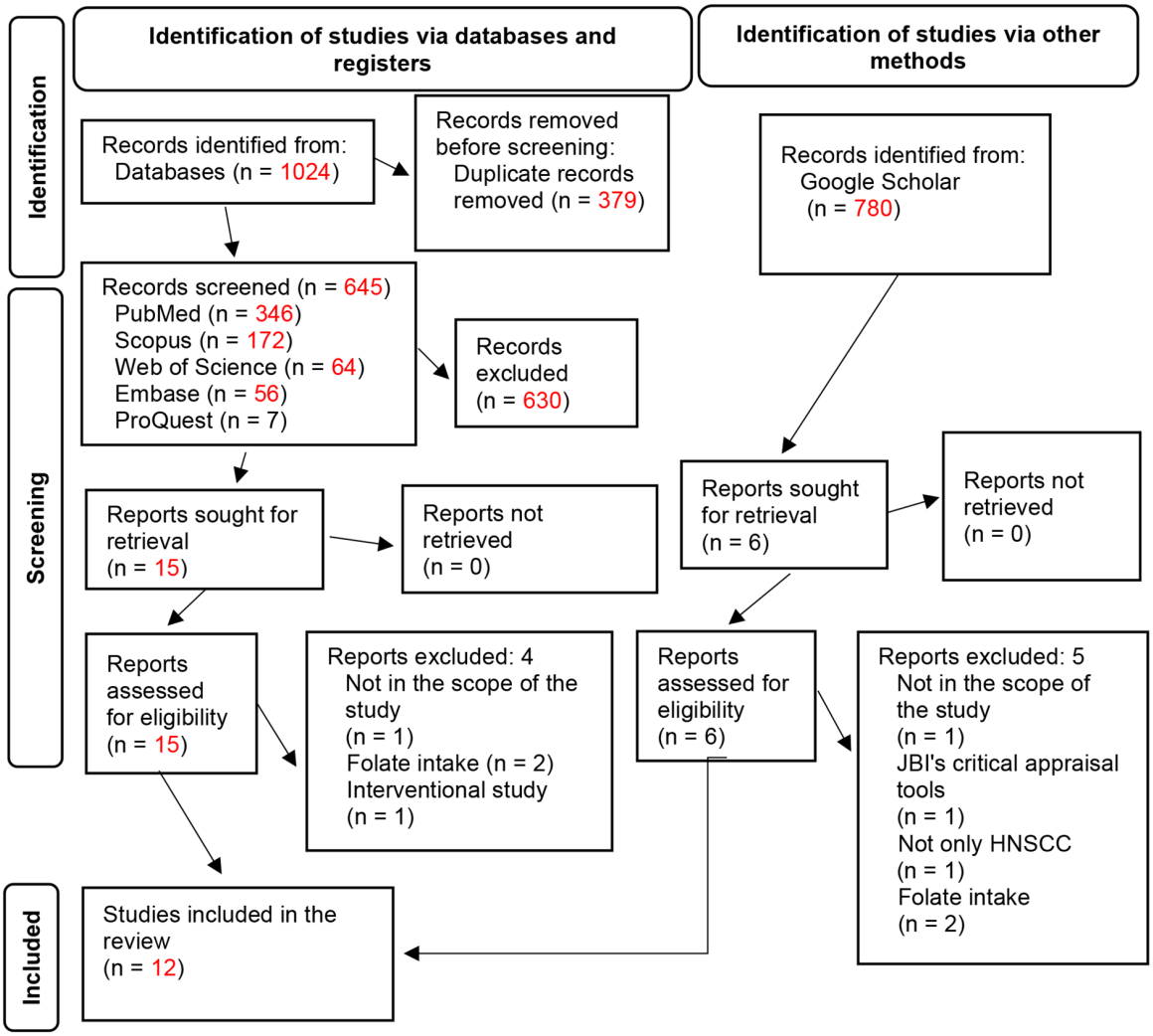


Figure 1. PRISMA flow diagram of the search results and selection process for inclusion in the systematic review

Of the selected articles, eleven were case-control studies, and one was a cohort study. These articles originated from European (n = 5), Asian (n = 6), and African (n = 1) countries. The types of squamous cell carcinoma cancers studied included comprehensive HNSCC (n = 6), laryngeal (n = 3), and oral cavity (n = 3). The sample sizes varied across the included studies in the meta-analysis, ranging as follows: for the relationship between serum folate levels and HNSCC (493 - 1240 cancer patients; 355 - 1342 controls) and for the relationship between serum Hcy levels and HNSCC (428 - 987 cancer patients; 327 - 1277 controls). A summary of the main characteristics of the included studies in the systematic review and meta-analyses is provided in Tables 1 and 2 for the folate and Hcy studies, respectively. In the majority of studies utilized for the meta-analysis, important confounding factors were either matched between patients and controls or adjusted for.

3.2. Assessment of the Risk of Bias

According to the JBI tool, out of the 12 included studies, 11 had a low risk of bias, while one study was identified with a moderate risk of bias (Table 3).

3.3. Meta-analysis for Folate

The results of various meta-analyses are presented in Table 4 and Figure 2. The number of studies included ranged from 6 to 9. In all meta-analyses concerning

Table 1. Summary of the Characteristics of the Research Included in the Systematic Review and Meta-analysis for the Serum Folate

Articles	Country	Study Design	Cancer Type	Sample Size (Patients)	Sample Size (Controls)	Folate Level in Patients, ng/mL	Folate Level in Controls, ng/mL	Matched Factors Between Patients and Controls and/or Adjustments
Akinmoladun and Arinola (18), 2019 [a]	Nigeria	Case-control	HNSCC	Total: 30; smoker: 19; nonsmokers: 11	Total: 30; smoker: 2; nonsmokers: 28	26.05 (Median)	30.82 (Median)	Not included in the meta-analysis
Chang et al. (11), 2016	China	Case-control	LSCC	Total: 60; nonsmokers: 60	Total: 30; nonsmokers: 30	3.35	4.40	Matched: No diseases affecting the outcome, no vitamin B intake
Erugula et al. (13), 2016	India	Case-control	OSCC	Total: 30; smokers: 30	Total: 30; smokers: 15; nonsmokers: 15	5.34	Smokers: 7.68; nonsmokers: 10.99	Matched: No diseases affecting the outcome, not receiving affecting drugs
Fanidi et al. (17), 2015	10 European countries	Cohort	HNSCC	Total: 516; smokers: 256; ex-smokers[b]: 145; nonsmokers: 105; unknown: 10	516 (matched control); smokers: 104; ex-smokers: 184; nonsmokers: 214; unknown: 14	12.5	12.9 (matched control)	Matched: Country, age, gender, not other cancer, date of blood collection
Gorgulu et al. (14), 2010	Turkey	Case-control	LSCC	Total: 60; smokers: 56; nonsmokers: 4	Total: 60; smokers: 30; nonsmokers: 30	5.8	Smokers: 7; nonsmokers: 7.1	Matched: Geographic area, age, low to moderate alcohol intake, normal renal function, no hepatic failure, not receiving affecting drugs, no folic acid and vitamin B12 intake, no nutritional deficiency
Nacci et al. (15), 2008	Italy	Case-control	LSCC	Total: 25; smoker: 13; ex-smoker: 12	Total: 80; smoker: 25; ex-smoker: 30; nonsmokers: 25	4.3; smoker: 4.6; ex-smoker: 3.8	7.9; smoker: 7.5; ex-smoker: 8; Nonsmokers: 8.1	Adjustment: Age, gender, alcohol intake, cardiovascular disease
Eleftheriadou et al. (12), 2006	Greece	Case-control	HNSCC	Total: 149; smoker: 131 nonsmokers: 18	Total: 150; smoker: 77; nonsmokers: 73	5.32	Smoker: 5.95; nonsmokers: 8.75	Matched: Geographic area, age, gender, no systematic alcohol intake, normal renal function, no folate intake
Almadori et al. (10), 2005	Italy	Case-control	HNSCC	Total: 144; smoker: 129; nonsmokers: 15	Total: 210; smoker: 90; nonsmokers: 120	4.87	Smokers: 9.1; nonsmokers: 9.7	Matched: Geographic area, age, gender, no habitual alcohol intake, normal renal function, no folate intake, no nutritional deficiency
Almadori et al. (9), 2002	Italy	Case-control	HNSCC	Total: 42; smoker: 39; nonsmokers: 3	Total: 210; smoker: 90; nonsmokers: 120	5.8	Smoker: 9.1; nonsmokers: 9.7	Matched: Geographic area, age, gender, low to moderate alcohol intake
Raval et al. (16), 2002	India	Case-control	HNSCC	Total: 214; smoker: 188; nonsmokers: 26	Total: 56; smoker: 28; nonsmokers: 28	11.083; smoker: 10.83; nonsmokers: 12.89	11.14; smoker: 11.45; nonsmokers: 10.83	Adjustment: age, area, education, income

Abbreviations: HNSCC, head and neck squamous cell carcinoma; LSCC, laryngeal squamous cell carcinoma; OSCC, oral squamous cell carcinoma.
[a] This article was not included in the meta-analysis due to the lack of data for means and standard deviations.
[b] Ex-smokers were regarded as non-smokers.

serum folate, the heterogeneity test yielded significant results (Q = 30.0 - 208.0, df = 5 - 8, P-value $<$ 0.001, I^2 = 83.3 - 96.8). Consequently, the outcomes were reported using the random effects model. The pooled mean differences between patients and controls across the studies ranged from -2.24 to -2.71 ng/ml of folate, all of which were statistically significant (P-value 0.003 - $<$ 0.001). The negative signs in all analyses indicate that the serum folate level in the HNSCC patient group was lower than that in the control group. Meta-analysis No. 6 (as depicted in Table 4 and Figure 2) compared smoking controls with non-smoking healthy individuals, revealing significantly lower serum folate levels in the smoking controls compared to the non-smoking controls (mean difference = -1.15 ng/mL, P-value = 0.05). All meta-analyses suggested no indication of publication bias, as the Egger test and Begg and Mazumdar test results were not significant (Table 4). The forest plots regarding folate from the meta-analyses are illustrated in Figure 2.

3.4. Meta-analysis for Homocysteine

The types of meta-analyses for serum Hcy were similar to those for serum folate (Table 5 and Figure 3). The number of studies included in different meta-analyses for Hcy ranged from 6 to 8. All meta-analyses for serum Hcy levels were conducted using the random effects model because all heterogeneity tests were significant (Q = 19.7 - 145.7, df = 5 - 7, P-value $<$ 0.001, I^2 = 74.7 - 96.2). The differences between the means of HNSCC and control groups for serum Hcy, pooled over studies, were significant in the meta-analyses (P-value $<$ 0.001) and ranged from 3.56 to 5.60 μM/L. This indicates that the serum Hcy level in the cancer group was higher than in the control group. Although the highest mean difference belonged to the meta-analysis in which

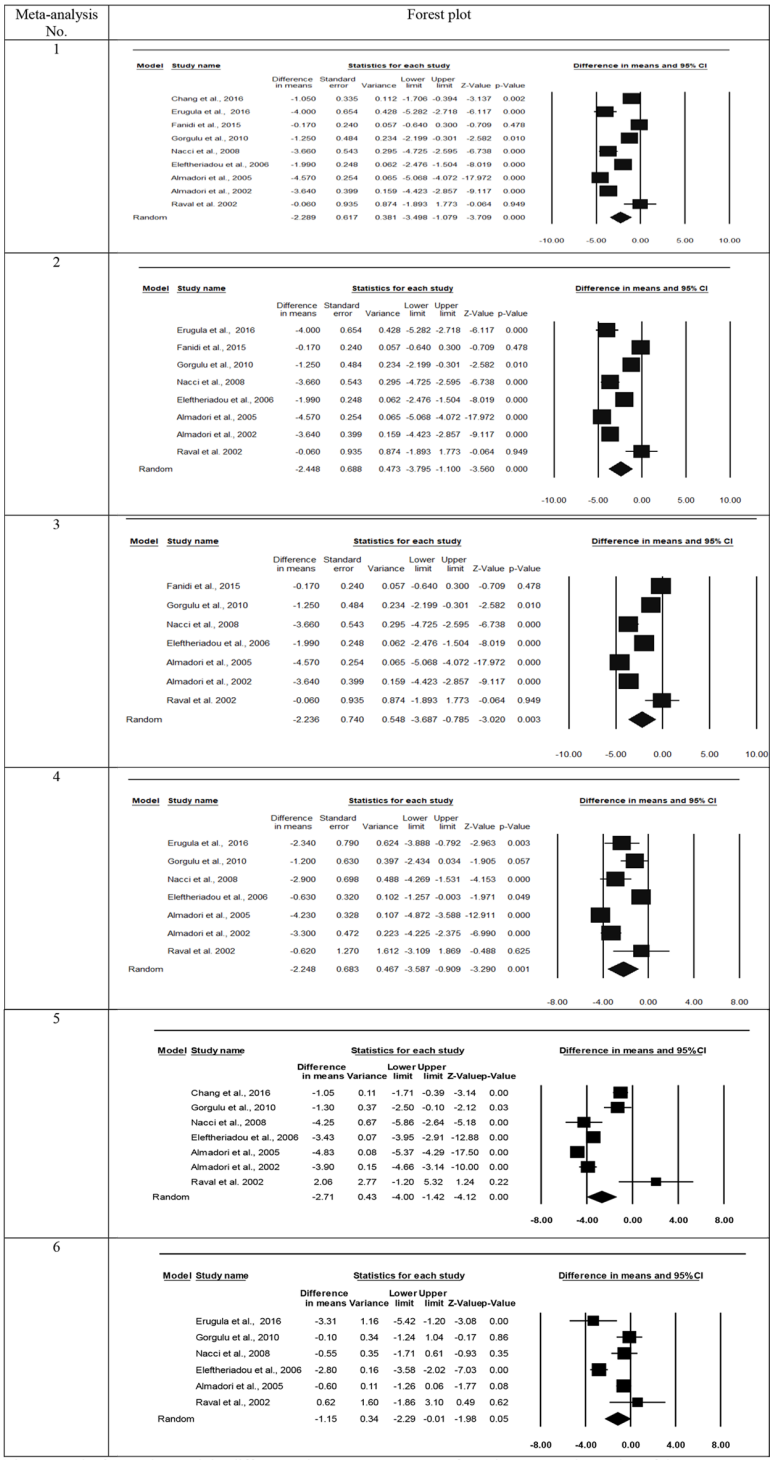


Figure 2. The forest plots and the differences between group means from the meta-analyses about folate.

Table 2. Summary of the Characteristics of the Research Included in the Systematic Review and Meta-Analysis for the Serum Homocysteine.

Articles	Country	Study Design	Cancer Type	Sample Size (Patients)	Sample Size (Controls)	Homocysteine Level in Patients, μM/L	Homocysteine Level in Controls, μM/L	Matched Factors between Patients and Controls and/or Adjusted for Confounding Factors
Akinmoladun and Arinola (18), 2019 [a]	Nigeria	Case-control	HNSCC	Total: 30; smokers: 19; nonsmokers: 11	Total: 30; smokers: 2; nonsmokers: 28	7.84 (Median)	8.44 (Median)	Not included in the meta-analysis
Palaskar et al. (24), 2022 [a]	aIndi	Case-control	OSCC	Total: 40; smokers: 17; nonsmokers: 23	40; Nonsmokers: 40	18.55 (Median)	16.85 (Median)	Not included in the meta-analysis
Bahramian et al. (25), 2023	Iran	Case-control	OSCC	21; Nonsmokers	21; Nonsmokers	3.71	2.01	Matched: Age, gender, normal renal function, no alcohol intake, no diseases affecting homocysteine, no vitamin B12 intake
Erugula et al. (13), 2016	India	Case-control	OSCC	Total: 30 ; Smokers: 30	Total: 30; nonsmokers: 15; smokers: 15	23.58	Smokers: 17.46; nonsmokers: 10.76	Matched: No diseases affecting the outcome, not receiving affecting drugs
Fanidi et al. (17), 2015	10 European countries	Cohort	HNSCC	Total: 516; smokers: 256; ex-smokers [b]: 145; nonsmokers: 105; unknown: 10	Total: 516 (matched control); smokers: 104; ex-smokers: 184; nonsmokers: 214; unknown: 14	10.8	10.2 (Matched control)	Matched: Country, age, gender, not other cancer, date of blood collection
Gorgulu et al. (14), 2010	Turkey	Case-control	LSCC	Total: 60; smokers: 56; nonsmokers: 4	Total: 60; smokers: 30; non-smokers: 30	11.5	Smokers: 9.7; non-smokers: 8.7	Matched: Geographic area, age, low to moderate alcohol intake, normal renal function, no hepatic failure, not receiving affecting drugs, no folic acid and vitamin B12 intake, no nutritional deficiency
Nacci et al. (15), 2008	Italy	Case-control	LSCC	Total: 25; smokers: 13; ex-smokers: 12	Total: 80; smokers: 25; ex-smokers: 30; nonsmokers: 25	20.57; smokers: 21.97; ex-smokers: 19.08	7.40; smokers: 7.84; ex-smokers: 7.40; nonsmokers: 6.88	Adjustment: Age, gender, alcohol intake, cardiovascular disease
Eleftheriadou et al. (12), 2006	Greece	Case-control	HNSCC	Total: 149; smokers: 131; nonsmokers: 18	Total: 150; smokers: 77; nonsmokers: 73	9.9	Smokers: 8.43; nonsmokers: 5.92	Matched: Geographic area, age, gender, no systematic alcohol intake, normal renal function, no folate intake
Almadori et al. (10), 2005	Italy	Case-control	HNSCC	Total: 144; smokers: 129; nonsmokers: 15	Total: 210; smokers: 90; nonsmokers: 120	13.4	Smokers: 9.1; nonsmokers: 8.7	Matched: Geographic area, age, gender, no habitual alcohol intake, normal renal function, no folate intake, no nutritional deficiency
Almadori et al. (9), 2002	Italy	Case-control	HNSCC	Total: 42; smokers: 39; nonsmokers: 3	Total: 210; smokers: 90; nonsmokers: 120	10.4	Smokers: 8.3; nonsmokers: 7.8	Matched: Geographic area, age, gender, low to moderate alcohol intake

Abbreviations: HNSCC: head and neck squamous cell carcinoma; LSCC: laryngeal squamous cell carcinoma; OSCC: oral squamous cell carcinoma.
[a] This article was not included in the meta-analysis due to the lack of data for means and standard deviations.
[b] Ex-smokers were regarded as non-smokers.

HNSCC patients (either non-smokers or non-smokers + smokers) were compared with the non-smoker controls (5.60 μM/L), in meta-analysis No. 10 (Figure 3), the serum Hcy level of patients (either smokers or smokers + non-smokers) was also significantly higher than that of the smoker controls (3.56 μM/L). Additionally, based on meta-analysis No. 12 in Figure 3, smoking controls had significantly higher serum Hcy levels than non-smoking healthy individuals (mean difference = 1.17 μM/L, P-value = 0.02). According to the Begg and Mazumdar test, all meta-analyses showed no indication of publication bias. Also, the Egger regression tests were not significant, except for meta-analysis No. 7 in Table 5 (P-value = 0.047). The forest plots from the meta-analyses regarding Hcy are displayed in Figure 3.

4. Discussion

Conflicting reports exist in the literature regarding the relationship of HNSCC with serum folate and Hcy levels. Most studies showed a significant association of HNSCC with folate (9-15, 17, 18) and Hcy (9, 10, 12, 13, 15, 17, 25). However, in others, no significant relationship of HNSCC with folate (16) and Hcy (14, 18, 26) was reported. The authors will discuss the results of different meta-analyses for folate and Hcy to verify the existence of an association between HNSCC and serum folate and Hcy levels.

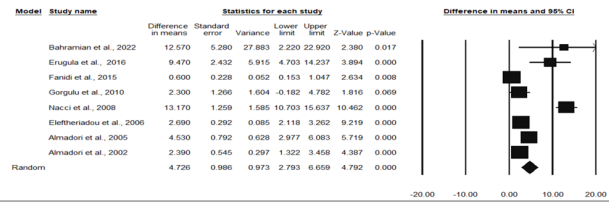
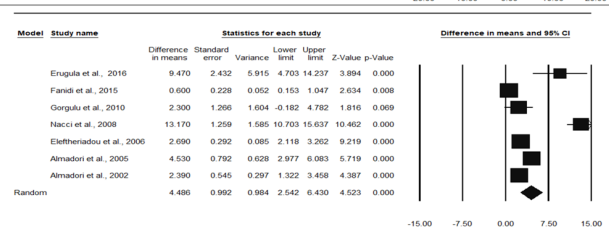
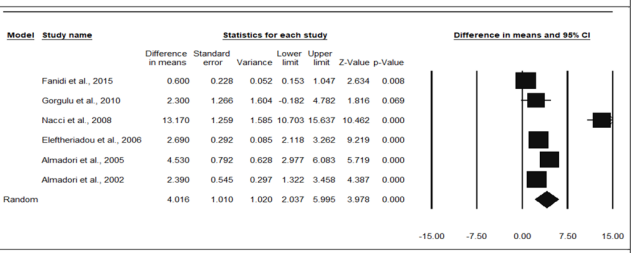
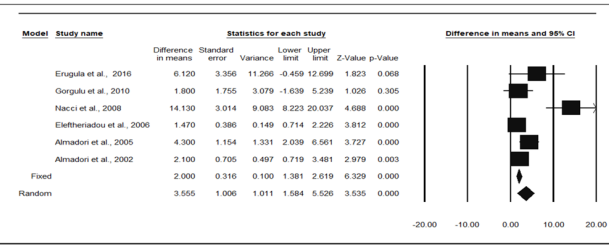
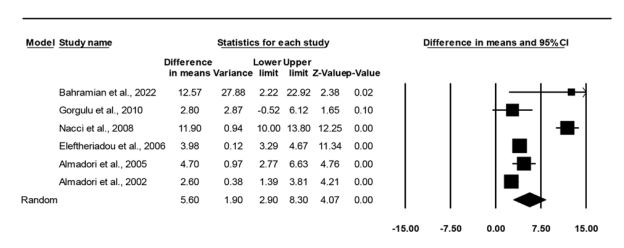
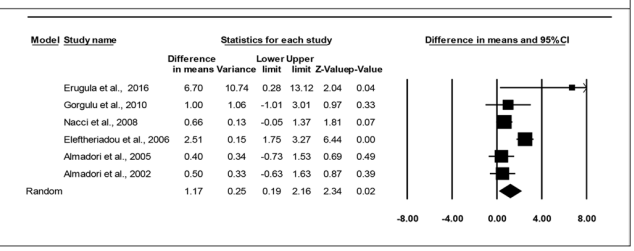
Meta-analysis No.	Forest plot
7	
8	
9	
10	
11	
12	

Figure 3. The forest plots and differences between group means from the meta-analyses regarding homocysteine.

Table 3. Risk of Bias of the Included Studies in the Systematic Review

Authors	Q1 [a]	Q2 [b]	Q3 [c]	Q4 [d]	Q5 [e]	Q6 [f]	Q7 [g]	Q8 [h]	Q9 [i]	Q10 [j]	Total [k], %	Risk of Bias [l]
Akinmoladun and Arinola (18)	N	N	U	Y	Y	Y	Y	Y	NA	Y	67	Moderate
Chang et al. (11)	Y	N	Y	Y	Y	Y	Y	Y	NA	Y	89	Low
Erugula et al. (13)	N	Y	Y	Y	Y	Y	Y	Y	NA	Y	89	Low
Fanidi et al. (17)	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	100	Low
Gorgulu et al. (14)	Y	Y	Y	Y	Y	Y	N	Y	NA	Y	89	Low
Nacci et al. (15)	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	100	Low
Eleftheriadou et al. (12)	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	100	Low
Almadori et al. (10)	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	100	Low
Almadori et al. (9)	Y	Y	N	Y	Y	Y	Y	Y	NA	Y	89	Low
Raval et al. (16)	N	Y	Y	Y	Y	Y	Y	Y	NA	Y	89	Low
Palaskar et al. (24)	Y	N	Y	Y	Y	Y	N	Y	NA	Y	78	Low
Bahramian et al. (25)	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	100	Low

Abbreviations: Y, yes; N, no; U, unclear; NA, not applicable; Q, question.
[a] Q1: Were the groups comparable other than the presence of disease in cases or the absence of disease in control?
[b] Q2: Were cases and controls matched appropriately?
[c] Q3: Were the same criteria used to identify cases and controls?
[d] Q4: Was the exposure measured in a standard, valid, and reliable way?
[e] Q5: Was the exposure measured the same way for cases and controls?
[f] Q6: Were confounding factors identified?
[g] Q7: Were strategies to deal with confounding factors stated?
[h] Q8: Were outcomes assessed in a standard, valid, and reliable way for cases?
[i] Q9: Was the exposure period of interest long enough to be meaningful?
[j] Q10: Was appropriate statistical analysis used?
[k] Total = ΣY/applicable items (not applicable (NA) items were excluded from the sum).
[l] The risk of bias was classified as low when the study reached a "yes" score of $\geq$ 70, moderate when the study reached a "yes" score of 50 to 69%, and high when the study reached a "yes" score of $<$ 49%.

Table 4. Results of Different Meta-analyses about the Relationship of Serum Folate Levels with Head and Neck Squamous Cell Carcinoma

No.	Meta-Analysis	Number of Studies	Heterogeneity Test			Publication Bias	
			Q [a]	P-Value	I^2 [b] (%)	Egger Test (P-Value)	Begg and Mazumdar Test (P-Value)
1 [c]	Patients vs. controls (with no regard to smoking conditions in both groups)	9	208.0	$<$ 0.001	96.2	0.80	0.68
2 [d]	Patients vs. controls (smokers or smokers + non-smokers in both groups)	8	194.9	$<$ 0.001	96.4	0.80	1.00
3 [e]	Patients vs. controls (smokers + non-smokers in both groups)	7	188.2	$<$ 0.001	96.8	0.95	0.88
4 [f]	Patients vs. smoker controls	7	71.6	$<$ 0.001	91.6	0.82	0.65
5 [g]	Patients vs. non-smoker controls	7	100.9	$<$ 0.001	94.1	0.39	0.65
6	Smoker controls vs. non-smoker controls	6	30.0	$<$ 0.001	83.3	0.88	0.57

[a] Cochran's measure of the heterogeneity of the studies.
[b] Measure of the inconsistency among the studies.
[c] Patients were compared with the controls without considering the smoking condition in both groups.
[d] Either smokers or smokers + non-smokers were compared with the controls (either smokers or smokers + non-smokers), excluding one study that had only non-smoker participants in both groups.
[e] Smokers + non-smokers were compared with the controls (smokers + non-smokers), excluding two studies that had solely smoker or non-smoker participants in either group.
[f] Either smokers or smokers + non-smokers were compared with the smoker controls.
[g] Either non-smokers or non-smokers + smokers were compared with the non-smoker controls.

4.1. Folate

The results of different meta-analyses showed significant differences between HNSCC patients and healthy controls. In meta-analysis No. 1, when HNSCC patients were compared with the control group without considering the group's smoking conditions, the serum folate level in patients was 2.29 ng/mL lower than in healthy individuals. The results did not considerably change when one or two studies with different smoking conditions were excluded from the meta-analysis; the

Table 5. Results of Different Meta-Analyses about the Relationship of Serum Levels of Homocysteine with Head and Neck Squamous Cell Carcinoma

No.	Meta-analysis	Number of Studies	Heterogeneity Test			Publication Bias	
			Q [a]	P-Value	I^2 [b] (%)	Egger Test (P-Value)	Begg and Mazumdar Test (P-Value)
7 [c]	Patients vs. controls (without regard to smoking conditions in both groups)	8	145.7	< 0.001	95.2	0.047	0.62
8 [d]	Patients vs. controls (smokers or smokers + non-smokers in both groups)	7	141.6	< 0.001	95.8	0.06	0.45
9 [e]	Patients vs. controls (smokers + non-smokers in both groups)	6	131.8	< 0.001	96.2	0.10	0.57
10 [f]	Patients vs. smoker controls	6	23.6	< 0.001	78.8	0.06	0.19
11 [g]	Patients vs. non-smoker controls	6	72.9	< 0.001	93.1	0.45	0.35
12	Smoker controls vs. Non-smoker controls	6	19.7	0.001	74.7	0.73	0.19

[a] Cochran's measure of the heterogeneity among the studies.
[b] Measure of the inconsistency among the studies.
[c] Patients were compared with the controls without considering the smoking condition in both groups.
[d] Either smokers or smokers + non-smokers were compared with the controls (either smokers or smokers + non-smokers), excluding one study that had only non-smoker participants in both groups.
[e] Smokers + non-smokers were compared with the controls (smokers + non-smokers), excluding two studies that had solely smoker or non-smoker participants in either group.
[f] Either smokers or smokers + non-smokers were compared with the smoker controls.
[g] Either non-smokers or non-smokers + smokers were compared with the non-smoker controls.

mean difference ranged from -2.24 to -2.71 ng/mL. These results indicate the significant association of serum folate level with HNSCC.

Meta-analysis No. 6 (Table 3 and Figure 2) revealed that smoking controls had significantly lower serum folate levels than non-smoking healthy individuals (mean difference of -1.15 ng/mL). Some substances in tobacco smoke interact with folate and lower serum levels in smokers (27). Although the serum folate level in the smoking controls was lower compared to the non-smoking control samples, the magnitude of this difference was lower than in cases when patients were compared with either smoking, non-smoking, or smoking + non-smoking controls (ranging from -2.24 to -2.71). Therefore, the higher reduction in the serum folate level of the HNSCC patients couldn't be attributed to the smoking conditions alone, and cancerous patients had lower folate than the controls, especially smoking controls, with a mean difference of -2.25.

The main risk factors for HNSCC are smoking (1, 28-36), alcohol (1, 30-32, 35, 37, 38), aging (1, 31), human papillomavirus infection (39, 40), and genetic factors (41), which may have contributed to the reduced folate levels and the onset of HNSCC. However, smoking is regarded as the primary risk factor for HNSCC. In a review study, Hashibe et al. (30) concluded that smoking accounts for 70% of HNSCC patients. In another review article, Whiteman and Wilson (38) reported that smoking contributed to a very high median population attributable fraction (PAF) of > 50% as the epidemiological measure for larynx cancer, and alcohol contributed to a high median PAF of 25 - 50% for oral cavity, pharynx, and larynx cancers.

As mentioned earlier, the serum folate level in HNSCC patients was significantly lower than in both smoking and non-smoking controls. Folate mediates one-carbon metabolism and plays a critical role in several pathways, such as DNA synthesis, amino acid homeostasis, and antioxidant generation, and its deficiency adversely alters these pathways (42, 43). DNA methylation modifies gene expression and transmits epigenetic information through DNA replication and cell division (42). Folate deficiency may result in abnormal methylation of DNA, consequently altering the expression of cancer suppressor genes (44). Additionally, low folate conditions may stimulate uracil production and decrease thymidine synthesis in the DNA sequence during cell division (42, 45), increasing the frequency of chromosomal breaks and, presumably, the risk of carcinogenesis (45).

4.2. Homocysteine

The trend of differences between HNSCC patients and healthy controls for Hcy was similar to serum folate. When HNSCC patients were compared with controls without considering the smoking conditions in both groups (meta-analysis No. 7), the serum Hcy level was 4.73 μM/L higher in the cancerous patients compared to the

controls. After excluding one or two studies with different smoking conditions, the results didn't change drastically, and the difference between patients and controls ranged from 3.56 to 4.49 μM/L Hcy. The lowest mean difference for Hcy (3.56 μM/L) belonged to meta-analysis No. 10, where the patients, smokers or smokers + non-smokers, were evaluated against the smoker controls. However, when non-smokers or non-smoker + smoker patients were compared with non-smoker controls, the mean difference between the two groups for Hcy rose to 5.60 (meta-analysis No. 11). These results also show the significant association of serum Hcy with HNSCC, without considering the smoking conditions of the control groups. Although smoking controls also had significantly higher Hcy levels than non-smoking individuals (mean difference = 1.17 μM/L), this difference was much lower than in the analysis where HNSCC patients were evaluated against healthy controls, regardless of the smoking situation (ranging from 3.56 to 5.60 μM/L). Thus, similar to the findings for serum folate level, the increase in Hcy level in cancerous patients couldn't be attributed solely to the smoking habit, and HNSCC patients had higher Hcy than the controls (especially the smoker controls).

Folate acts as a methyl donor in the methionine cycle, while Hcy serves as an intermediate metabolite within this cycle (15, 46). Hcy metabolism is crucial for regulating methionine availability and DNA methylation. It is synthesized from methionine through two cofactors: S-adenosylmethionine and S-adenosylhomocysteine. The level of Hcy is maintained through the remethylation pathway, which converts Hcy to methionine, and the transsulfuration pathway, which converts Hcy to cysteine (47). Consequently, alterations in Hcy metabolism lead to hyperhomocysteinemia, which is associated with increased free radicals, induced oxidative stress, and possibly increased risks of cancers and other diseases (13, 48). One reason for the elevation in Hcy levels in HNSCC patients might be the reduction of serum folate, which affects Hcy metabolism (49, 50). Thus, the positive relationship between Hcy and HNSCC may be linked to folate deficiency, resulting in the accumulation of Hcy in the blood serum (17).

4.3. Folate and Homocysteine as Possible Biomarkers

Despite advancements in the treatment of HNSCC through surgery, radiotherapy, and chemotherapy, the disease still carries a low overall 5-year survival rate of about 40 percent (3, 51). Clinical examinations and biopsies often fail to detect HNSCCs, such as oral squamous cell carcinoma, in the early stages (52). Therefore, early diagnosis of these malignancies can reduce morbidity and mortality. Identifying biomarkers would be beneficial in the early detection of these cancers (13, 53). Although Almadori et al. (10) stated that in patients with HNSCC, Hcy levels may not solely depend on folate levels but possibly are influenced by the heterogeneity of the HNSCC phenotype, our meta-analysis of included articles yielded similar results for serum folate and Hcy, both of which showed a significant relationship with HNSCC. Therefore, these biomarkers may prove useful in the early detection of HNSCCs.

There were some limitations in this research. The heterogeneity among the studies concerning confounding factors related to HNSCC patients and controls may have influenced the results.

4.4. Conclusions

The results of different meta-analyses in this study showed that the serum folate and Hcy levels of HNSCC patients were significantly lower and higher, respectively, than those of the control groups, regardless of the smoking condition in both groups. Although serum folate was significantly lower and serum Hcy was significantly higher in the smoking control groups compared to the non-smoking control groups, the magnitude of these differences was smaller when patients were compared with the healthy groups. In conclusion, our meta-analyses suggest a potential association of serum folate and Hcy levels with HNSCC. Therefore, these biochemical compounds may serve as biomarkers for the early detection of HNSCC onset in patients.

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Footnotes

Authors' Contribution: M.M.V: Acquisition of data, analysis and interpretation of data, and drafting of the manuscript; A.B: Study concept and design, critical revision of the manuscript for important intellectual content, and administrative, technical, and material support; K.K: Study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and study supervision; F.S: Acquisition of data and drafting of the manuscript. All authors read and approved the final manuscript.

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Data Availability: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

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