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A Comparative Meta-Analysis Identifying Salivary Cytokines, IL-6, IL-8 and TNFα, and Lactate Dehydrogenase as Potential Non-Invasive Diagnostic Biomarkers of Oral Squamous Cell Carcinoma

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Abstract

Diagnostic screening of oral squamous cell carcinoma (OSCC) generally involves detection of biomarkers in invasive biopsy samples. Extensive investigations have detected a wide molecular signature, present in saliva that may be indicative of OSCC and stage of development. This meta-analysis assesses the potential of salivary cytokines, IL-1α, IL-6, IL-8 and TNFα, and lactate dehydrogenase (LDH) as suitable non-invasive biomarkers for OSCC and to identify those distinguishing between different stages of disease, as well as other oral pathologies. Electronic searches were conducted across databases such as PubMed, Web of Science and Scopus among others. The identified studies were screened, and eligibility was assessed using specific inclusion and exclusion criteria. Data from 12 included studies was then entered into RevMan5 for analysis. Heterogeneity of the outcomes were high, so subgroup analyses were conducted to establish the cause. Salivary IL-6, IL-8, TNFα and LDH are all significantly elevated in OSCC patients' saliva compared to healthy controls: IL-6 (HR=1.75; 95% CI: [0.84, 2.66]; P=0.0002); IL-8 (HR=2.20; 95% CI: [0.11, 4.30]; P=0.04); TNFα (HR=0.80; 95% CI: [0.46, 1.15]; P<0.00001); LDH (HR=8.71; 95% CI: [4.52, 12.90]; P<0.0001). IL-1α, on the other hand, was not significantly different between saliva samples from OSCC patients and healthy controls. Salivary IL-6, IL-8 and LDH were also significantly elevated in OSCC. This is ideal for establishing the presence of OSCC and highlights the applicability of these cytokines and LDH as biomarkers for the disease.

Keywords: OSCC; Salivary Biomarker; IL-6; IL-8; TNFα; LDH; Diagnosis

Abbreviations: OSCC: Oral Squamous Cell Carcinoma; LDH: Lactate Dehydrogenase; EMT: Epithelial-to-Mesenchymal Transition; CSC: Cancer Stem Cell; VEGF: Vascular Endothelial Growth Factor; TAM: Tumour Associated Macrophages; ECM: Extracellular Matrix; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; NOS: Newcastle-Ottawa Scale; TRAF6: TNF Receptor Associated Factor 6; EGF: Epidermal Growth Factor; HIF: Hypoxia Inducible Factor; CAFs: Carcinoma-Associated Fibroblasts

Introduction

Oral squamous cell carcinoma (OSCC) is a multifactorial malignancy of the oral cavity and is one of the most common cancers involving the head and neck. OSCC makes up over 90% of malignancies of the oral cavity and is associated with a high mortality rate and a stark increase in incidence in recent years [1,2]. Current diagnostics rely on histological examination following an invasive excisional biopsy from the suspected tumour site and it can only be identified during a direct visual clinical check-up [3]. OSCC commonly presents through a range of molecular mutations, which result in uncontrolled cell growth. Areas of hyperplasia develop into dysplastic lesions, which progress to carcinoma in situ and then invasive carcinoma [4]. Etiological factors for OSCC include a genetic predisposition and exposure to carcinogens such as human papilloma virus infection, tobacco, chronic inflammation and alcohol [5]. Prior studies

have elucidated that pro-inflammatory cytokines, such as IL-1α, IL-1α, IL-6, IL-8 and TNFα, are elevated in the saliva of OSCC patients [6], but their viability as biomarkers has not been fully determined. The mechanism of action of these pro-inflammatory cytokines however, is understood in a variety of malignancies. For example, IL-6 can drive tumour migration and invasion through crosstalk with STAT3 and Rac1 signaling in OSCC, which drives the loss of E-cadherin and its replacement with N-cadherin [7]; a characteristic sign of epithelial-to-mesenchymal transition (EMT), essential for tumour migration and invasion of adjacent tissues [8]. Additionally, TNFα has been found to increase malignant behaviour in tumour cells by increasing their potential for anchorage independent growth, as well as enhancing cancer stem cell (CSC)-like properties [9].

TNFα also increases tumour invasion by augmenting the expression of key matrix metalloproteases (MMPs) like MMP-9 [6], driving both invasion and migration. Furthermore, both IL-1α and TNFα can induce NF-κB-dependent IL-8 production [10], capable of augmenting the angiogenic potential of tumour associated macrophages (TAMs) by enhancing vascular endothelial growth factor (VEGF) production [11]. Concurrently, the expression of IL-8 CXC receptors (CXCRs), CXCR1 and CXCR2 have been reported to be increased in OSCC cells. The presence of both IL-8 receptors enables augmentation of the production and release of MMP-7 and MMP-9, increasing the proliferation, invasion and migration of OSCC cells [12]. Furthermore, LDH has been demonstrated to act as an oncogene to facilitate cell growth and metastasis in OSCC in vitro and in vivo. This occurs through LDH driving an acidic tumour microenvironment as a consequence of increased lactate production from pyruvate in aerobic glycolysis. This enables the breakdown and reorganization of the extracellular matrix (ECM) and the production of actin filaments, key to enhancing EMT [13]. By utilizing these cytokines or LDH as salivary biomarkers, this would provide a non-invasive diagnostic screening method that would enable OSCC to be identified with minimal interference or discomfort to the patient. Thus, it would reduce the need to perform incredibly invasive, excisional biopsies for diagnosis; where it has been proposed that these excisional biopsies can further accelerate tumour progression [7].

Current diagnostics rely on histological examination following an excisional biopsy from the suspected tumour site to diagnose OSCC, which can only be detected currently during a direct visual clinical check-up [3]. These excisional biopsies are incredibly invasive for the patient and can cause a lot of discomfort, as well as potentially increasing the risk of lymph node metastases [7] and stimulating a wound-healing reaction alongside the tissue trauma, resulting in macrophage polarization towards the tissue reparative M2 phenotype. This phenotype also supports tumour development and progression, thus increasing the risk of lymph node metastases [7]. Additionally, tumour-associated IL-1α may be implicated in this because it has been linked to increased lymph angiogenesis and lymph node metastases through the activation of IL-1α/IL-1R and CXCR4/SDF-1α signaling, which drives production of MMP-9 and MMP-13 [14]. Using salivary biomarkers to diagnose OSCC would be non-invasive, minimizing the patient's discomfort. They could be used to screen for disease prior to it being visually detected during a check-up and identify the disease earlier. This could provide a treatment benefit and hopefully lead to better prognoses for the affected patients, helping to reduce the high mortality rates currently associated with the condition [1]. Thus, this study aimed to assess the potential of salivary cytokines (IL-1α, IL-6, IL-8, TNFα) and LDH as diagnostic biomarkers of OSCC, given their roles in tumour development, progression, migration and invasion.

Methods

Search Strategy

Screening of studies and data extraction for meta-analysis were conducted using a standardized table to collect appropriate information and data, allowing for direct comparisons to be made between studies. The types of studies included in this meta-analysis were full journal publications that were case-control studies, which employed an appropriate number of controls in comparison to the OSCC group. A systematic search was performed on the following online databases: PubMed (1895 to 15th January 2021), Web of Science (1970 to 15th January 2021), Google scholar (whole database up to and including 15th January 2021), Medline (whole database up to and including 15th January 2021) and Scopus (1960 to 15th January 2021). These databases were searched using Boolean search strategies with the terms "oral squamous cell carcinoma" or "OSCC", "IL-1α", "IL-6", "IL-8", "TNFα", "lactate dehydrogenase" or "LDH", "saliva", "biomarker" or "salivary biomarker". Additional searches were performed on the references of existing reviews to retrieve relevant articles.

Eligibility Criteria – Included and excluded studies

The inclusion criteria for studies in this meta-analysis were: 1) English language publication, 2) studies that investigated cytokine or enzyme levels of unstimulated saliva, reporting the mean and standard deviation of the specific cytokines or enzymes present in the saliva samples of OSCC patients and healthy controls. Additionally, there were inclusion criteria relating to the participants in the studies and these included 3) subjects who were 18+ years old, male or female, and with histologically confirmed OSCC prior to any chemo- or radiotherapy treatment and/or surgical intervention, 4) Participants had to have given consent to take part in the individual studies. The exclusion criteria for the studies in this meta-analysis included: 1) Non-English language publication, 2) studies that didn't investigate the saliva of OSCC patients, 3) studies that did not report mean and standard deviation, 4) studies with no comparison to healthy controls, 5) Blood or serum cytokine levels were not permitted and 6) no other meta-analyses or reviews. The exclusion criteria relating to participants in this meta-analysis included 7)

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participants who had previously been treated for/were currently being treated for OSCC by chemo- and/or radiotherapy treatment or surgical intervention prior to partaking in the investigation and 8) participants who had previously been diagnosed with OSCC and were now in remission that were included in the healthy control group. No requirements were made for the number of participants in the included studies - provided there were a suitable number of controls. The types of primary outcome measures for this meta-analysis relate to the quantity of the specific cytokines (pg/ml of IL-1α, IL-6, IL-8, TNFα) and LDH (U/L) present in the saliva of OSCC patient and healthy controls. These cytokines are clinically relevant to OSCC as they are key to the instigation and maintenance of chronic inflammation associated with tumour development, progression, migration and invasion [15]. Levels with statistical significance between OSCC patients and healthy controls ($p<0.05$) were deemed clinically important as suitable salivary biomarkers.

Data extraction (outcomes)

The titles and abstracts were screened individually to ascertain their eligibility for inclusion. Studies which did not satisfy these inclusion criteria were excluded from this meta-analysis. Full copies of the studies were obtained and read in-depth to determine whether they were eligible for inclusion. Included is a Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) flow chart to give more details regarding this process for the meta-analysis [16], (Figure 1). A quality assessment study was conducted on each of these included studies using the modified Newcastle-Ottawa Scale (NOS) [17]. The data was then extracted and checked for accuracy prior to entry into Review Manager version 5.4.1 (The Cochrane Collaboration).

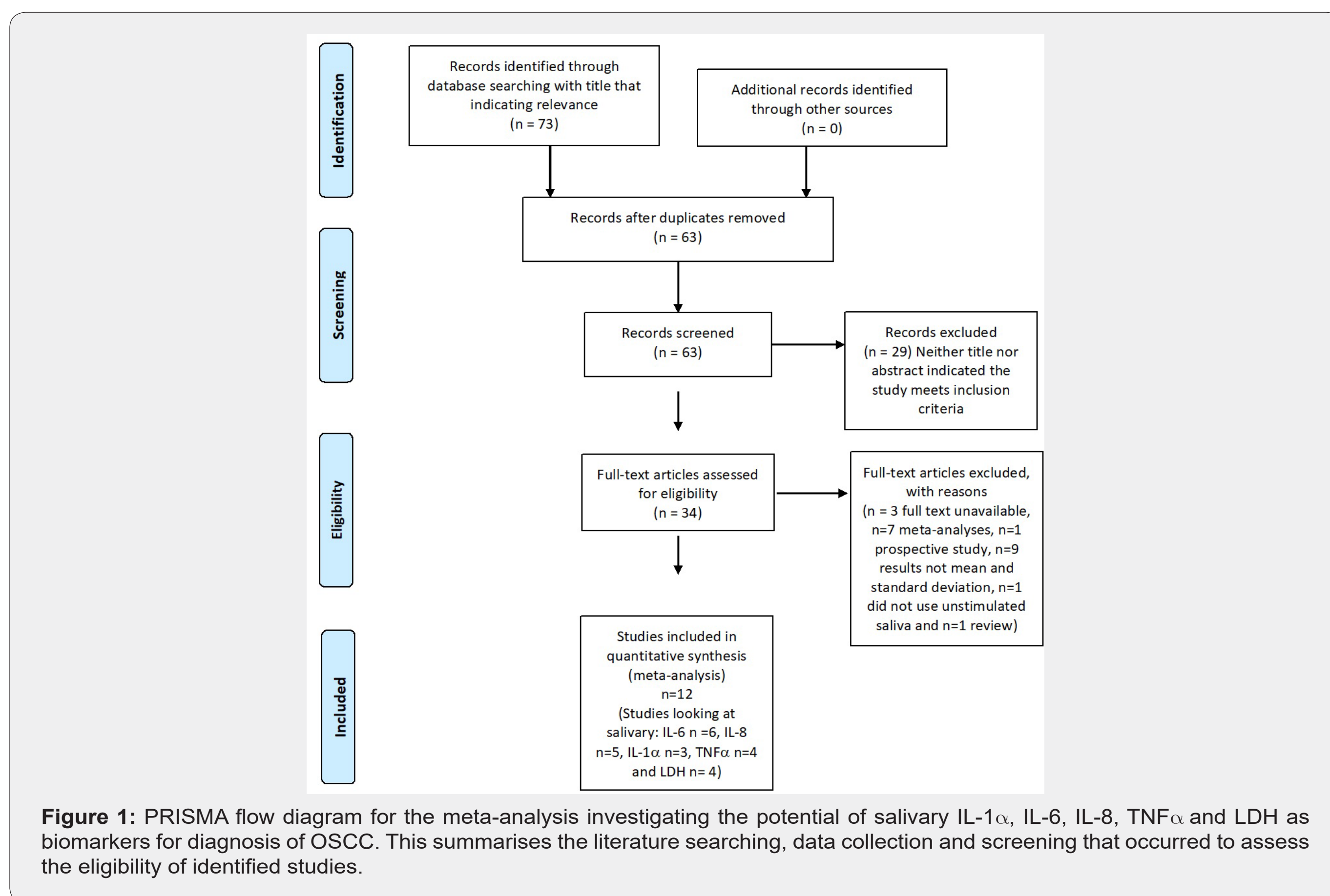

Figure 1: PRISMA flow diagram for the meta-analysis investigating the potential of salivary IL-1α, IL-6, IL-8, TNFα and LDH as biomarkers for diagnosis of OSCC. This summarises the literature searching, data collection and screening that occurred to assess the eligibility of identified studies.

Assessment of risk of bias in included studies

This quality assessment study was conducted on each of the included investigations using the modified NOS, as recommended by the Cochrane Collaboration for non-randomized controlled studies [17]. Criteria for this assessment of bias are related to the study population, the validity of the study, whether any confounders, such as comorbidities, were identified and whether any statistical adjustment was used to account for these potential confounding factors. Study population assessments were based on whether all the study groups derived from a similar source or population and whether attrition did not significantly differ across the study groups. Study validity determined whether the exposure and outcome measures were appropriate and whether the investigators were blinded to the endpoint assessment. Additional confounders included if any funding sources were disclosed and if

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there were any obvious conflicts of interest present in the studies. Bias was assessed and reported by inspection of study results and outcomes to ensure that there were no discrepancies.

Quantitative data synthesis and analysis

Each meta-analysis for the specific cytokine or LDH was performed using a random effects model in RevMan5 (RevMan, 2020) and forest plots were produced. The characteristics of the included studies can be seen in (supplementary Table 1) and the levels of the specified cytokines or LDH can be seen in (supplementary Tables 2 & 3), respectively. In addition, all the included studies reported continuous outcomes, so standardized mean difference was calculated in RevMan for analysis; similar assessment measures were employed by all studies (i.e. using quantitative ELISAs or enzyme activity assays). Heterogeneity was assessed using the statistic I2. This is used to highlight the percentage variation across the studies included in the analysis that is due to heterogeneity as opposed to chance. Low heterogeneity is indicated by an I2 ≤ 25%, moderate heterogeneity if I2 were 25-50% and high heterogeneity if I2 ≥ 50% [18]. Subgroup analyses were conducted in order to investigate the cause of heterogeneity for all of the specified cytokines and LDH levels in saliva.

Table 1: Modified NOS for appraisal of non-randomised controlled trials to review the author's judgement about each risk of bias for each included study.

Study (author, year)	Cheng [19]	D'Cruz and Pathiyil [20]	Deepthi [21]	Gholizadeh [22]	Kallalli [23]	Lee [24]	Lokesh, Kannabiran and Rao, [25]	Marton [26]	Rajkumar [27]	Rao [28]	Rhodus [29]	SahebJamee [30]
Study group	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Attrition	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Exposure measure	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Outcome measure	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Investigators blinded	N	N	N	N	N	N	N	N	N	N	N	N
Confounders identified	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Statistical adjustment	N	N	N	Y	N	N	N	Y	Y	N	Y	N
Funding source	Y	Y	Y	UC	Y	Y	Y	Y	UC	Y	Y	Y

Results

Description of Studies

This paper includes 12 studies, all of which were full journal publications. They include: Cheng [19], D'Cruz & Pathiyil [20], Deepthi [21], Gholizadeh [22], Kallalli [23], Lee [24], Lokesh, Kannabiran & Rao [25], Márton [26], Rajkumar [27], Rao [28], Rhodus [29] and SahebJamee [30]. Participants had to be at least 18 years of age to qualify in each included study.

Risk of bias in included studies

This was scored according to the presence of bias with yes (Y), no (N) or unclear bias (UC). Each study was assessed using the modified NOS. The results are shown in below Table 1. All studies were non-randomised, case-control studies. Smaller groups were used in some studies, so they may not necessarily be representative of the levels of the investigated salivary cytokines or LDH as seen outside of that population, but both OSCC and control group participants were taken from the same population. This was deemed appropriate and enabled direct comparisons to be made between the two groups. However, the assessors were not blind to the outcomes or whether a sample came from an OSCC patient or healthy control. The OSCC presence had to be histologically confirmed by an independent pathologist before the participant could be included in the group and this was often done. Additionally, no studies had incomplete data; no participants rescinded their consent once they had started the investigation and all relevant outcomes from the included studies were reported. Other potential sources of bias were identified in the included studies Table 1. Those that did not conduct a further statistical analysis on confounding factors (i.e. smoking habits, age or gender); smoking has been found to increase the risk of OSCC [31]. These studies included: Cheng [19], D'Cruz & Pathiyil [20], Deepthi [21], Kallalli [23], Lee [24], Lokesh, Kannabiran & Rao [25], Rao [28] and SahebJamee [30].

Additionally, two studies did not state their source of funding so were of unclear risk (UC) and included Gholizadeh [22] and Rajkumar [27]. Funnel plots were produced and assessed to determine the presence of any publication bias. All funnel plots were symmetrical and, thus, it was deemed that no significant

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publication bias was present in the studies included in this meta-analysis (see Funnel plots included in Supplementary (Supplementary Figure 1a-e). Salivary levels of IL-6 are significantly elevated in OSCC patient samples. Six included studies investigated the levels of IL-6 in saliva samples from OSCC patients compared to healthy controls with a total of 383 participants, this data was entered into RevMan5 for analysis to assess the significance of any differences; used to help identify it as a potential biomarker for OSCC (Figure 2a). Overall, IL-6 levels in the saliva from OSCC patients' samples were significantly higher than IL-6 levels in the healthy controls (P < 0.0002). However, due to the high heterogeneity (I2=91%), a subgroup analysis was performed in order to identify this and then re-evaluate the significance without this cause. No significant change occurred in the heterogeneity and the p-value became (P = 0.001) upon the exclusion of Rhodus [29] (Figure 2b).

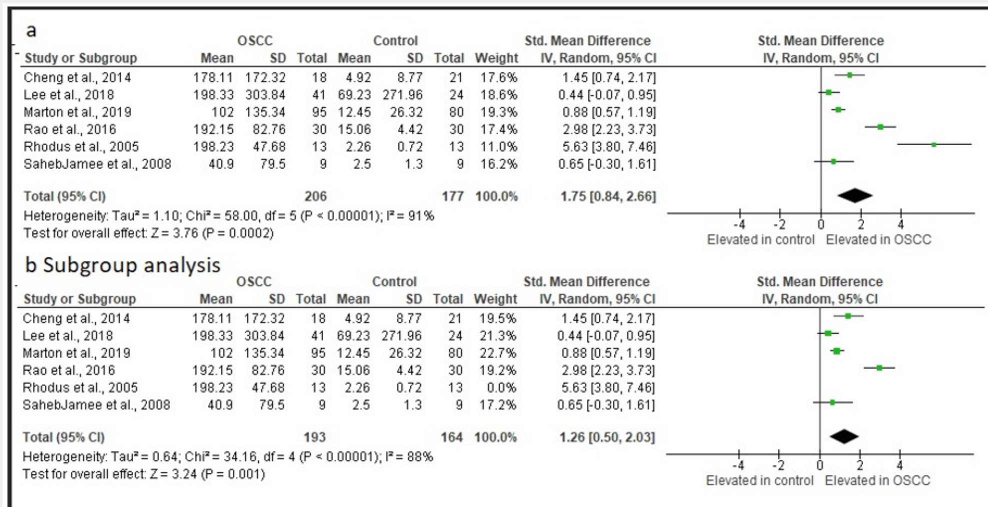

Figure 2: Elevated salivary IL-6 is associated with OSCC Forest plot reveals the Standard Mean Difference and 95% CI for the association of elevations in IL-6 in OSCC patients compared to healthy controls across the included studies (a & b). The green rectangles represent the standardised mean difference, and the weighting of each study is represented by the size of each rectangle where the horizontal lines are the 95% confidence intervals (CI). The vertical line indicated on zero, represents the line of no effect. The black diamond signifies the mean weighted overall standardised mean difference. The Forest plot P value reveals the statistical significance between IL-6 in saliva of OSCC patients.

Salivary levels of IL-8 are significantly elevated in OSCC patient samples

Five included studies investigated the levels of IL-8 in saliva samples of OSCC patients compared to healthy controls. They had a total of 348 participants and this data was entered into RevMan5 to analyze salivary IL-8 levels as a biomarker for OSCC (Figure 3a). IL-8 levels in saliva were significantly elevated in OSCC patients compared to healthy controls (P = 0.04). However, the I2 value was indicating that there was high heterogeneity, so a subgroup analysis was conducted to investigate this further. The heterogeneity was high, so a subgroup analysis was performed to identify this and then re-evaluate IL-8 as a biomarker of OSCC (Figure 3b). With the exclusion of Rajkumar [27], the heterogeneity became 86% and the p-value indicated a greater significant difference between IL-8 levels in the saliva of OSCC patients compared to healthy controls (P < 0.02) (Figure 3).

Salivary levels of TNFα are significantly elevated in OSCC patient samples

Four included studies investigated TNFα levels in saliva samples from OSCC patients compared to healthy controls with a total of 169 participants. This data was entered into RevMan5 for analysis of TNFα levels in saliva to be a potential biomarker for OSCC (Figure 4). This meta-analysis found salivary TNFα levels to be significantly greater in OSCC patients compared to healthy controls (p=0.0001) (Figure 4a). Once again, heterogeneity was high, so a subgroup analysis was performed to identify this and then re-evaluate TNFα as a biomarker of OSCC (Figure 4b). With the exclusion of Rhodus [29], the heterogeneity became 0% and the p-value indicated a greater significant difference between TNFα levels in the saliva of OSCC patients compared to healthy controls (P < 0.00001) Figure 4. Salivary levels of LDH are significantly elevated in OSCC patient samples compared to healthy controls . Four included studies investigated salivary levels of LDH in OSCC samples compared to healthy controls and had a total of 195 participants. This data was entered into RevMan5 to analyze LDH levels in saliva as a potential biomarker for OSCC (Figure 5a). Overall, levels of LDH were significantly greater in OSCC patients' saliva samples compared to samples from healthy controls (P < 0.0001). Despite the heterogeneity being very high (I2=97%), a subgroup analysis was conducted and with the exclusion of D'Cruz & Pathiyil [20], heterogeneity was 94% and significance was not greatly affected (Figure 5b). When Lokesh, Kanabiran & Rao [25] was excluded the heterogeneity increased to 98% and significance decreased (P = 0.03) (Figure 5c).

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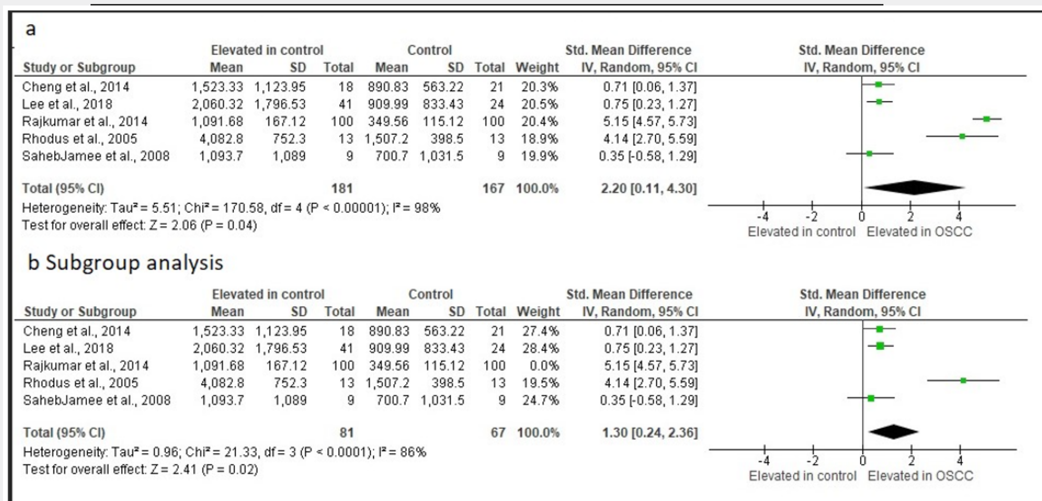

Figure 3: Elevated salivary IL-8 is associated with OSCC Forest plot reveals the Standard Mean Difference and 95% CI for the association of elevations in IL-8 in OSCC patients compared to healthy controls across the included studies (a & b). The green rectangles represent the standardised mean difference, and the weighting of each study is represented by the size of each rectangle where the horizontal lines are the 95% confidence intervals (CI). The vertical line indicated on zero, represents the line of no effect. The black diamond signifies the mean weighted overall standardised mean difference. The Forest plot P value reveals the statistical significance between IL-8 in saliva of OSCC patients. As a consequence of high heterogeneity in a, a sub-group analysis of IL-8 levels in between saliva samples from OSCC patients and that of healthy controls is presented in b.

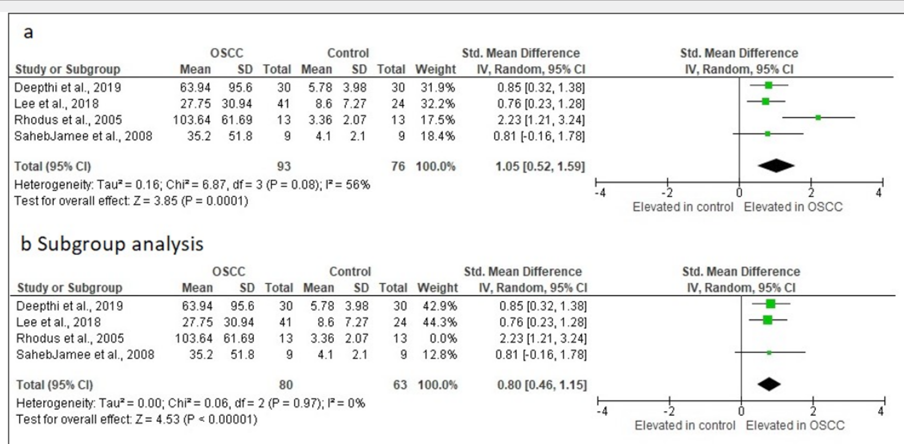

Figure 4: Elevated salivary TNFα is associated with OSCC Forest plot reveals the Standard Mean Difference and 95% CI for the association of elevations in TNFα in OSCC patients compared to healthy controls across the included studies (a & b). The green rectangles represent the standardised mean difference, and the weighting of each study is represented by the size of each rectangle where the horizontal lines are the 95% confidence intervals (CI). The vertical line indicated on zero, represents the line of no effect. The black diamond signifies the mean weighted overall standardised mean difference. The forest plot P value reveals the statistical significance between TNFα in saliva of OSCC patients. As a consequence of high heterogeneity (I2) in a, a subgroup analysis of TNFα levels compared between OSCC patient saliva and that of health control subjects is presented in b.

Salivary levels of IL-1α are not significantly different between OSCC patients and healthy controls

Three included studies investigated IL-1α and had a total of 109 samples from participants. They were entered into RevMan5 for analysis on the difference levels of IL-1α in samples from OSCC patients compared to healthy controls in order to assess its potential as a salivary biomarker for OSCC (see a 6). Overall, IL-1α levels in saliva were not significantly different in OSCC patient samples compared to healthy controls (p=0.09), but the black diamond in Figure 6a indicates a potential trend in elevated IL-1α levels in saliva of OSCC patients. Due to the high heterogeneity (I2 =95%), a subgroup analysis was conducted in order to establish what the cause of this could be and then to re-evaluate the potential of IL-1α as a salivary biomarker of OSCC (Figure 6b). However, when the study from Rhodus [29] was excluded, there was no significant difference between the levels of IL-1α in saliva between either group (p=0.97) and the I^2 value became 0% (Figure 6b). No meaningful difference occurred to the I2 or p-value when Lee [24] nor SahebJamee [30] were excluded.

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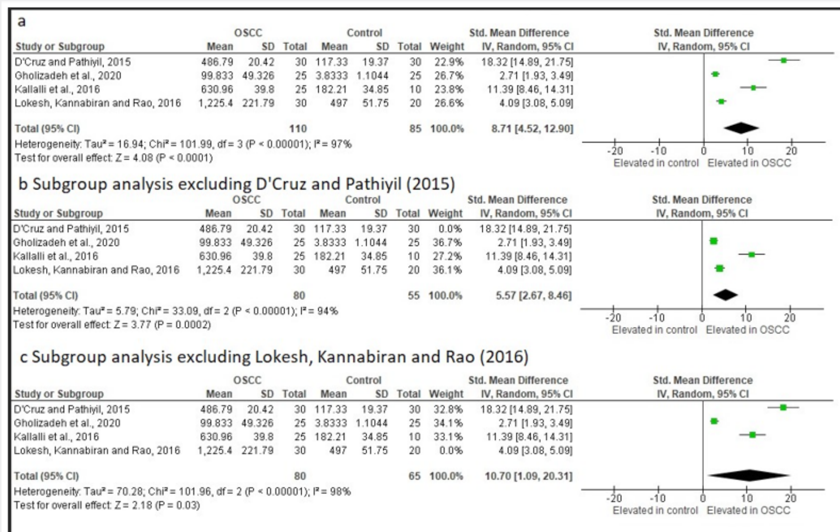

Figure 5: Elevated salivary LDH is associated with OSCC Forest plot reveals the Standard Mean Difference and 95% CI for the association of elevations in LDH in OSCC patients compared to healthy controls across the included studies (a, b & c). The green rectangles represent the standardised mean difference, and the weighting of each study is represented by the size of each rectangle where the horizontal lines are the 95% confidence intervals (CI). The vertical line indicated on zero, represents the line of no effect. The black diamond signifies the mean weighted overall standardised mean difference. The forest plot P value reveals the statistical significance between LDH in saliva of OSCC patients. As a consequence of high heterogeneity in a, a sub-group analysis of LDH levels in between saliva samples from OSCC patients and that of healthy controls is presented in b and c.

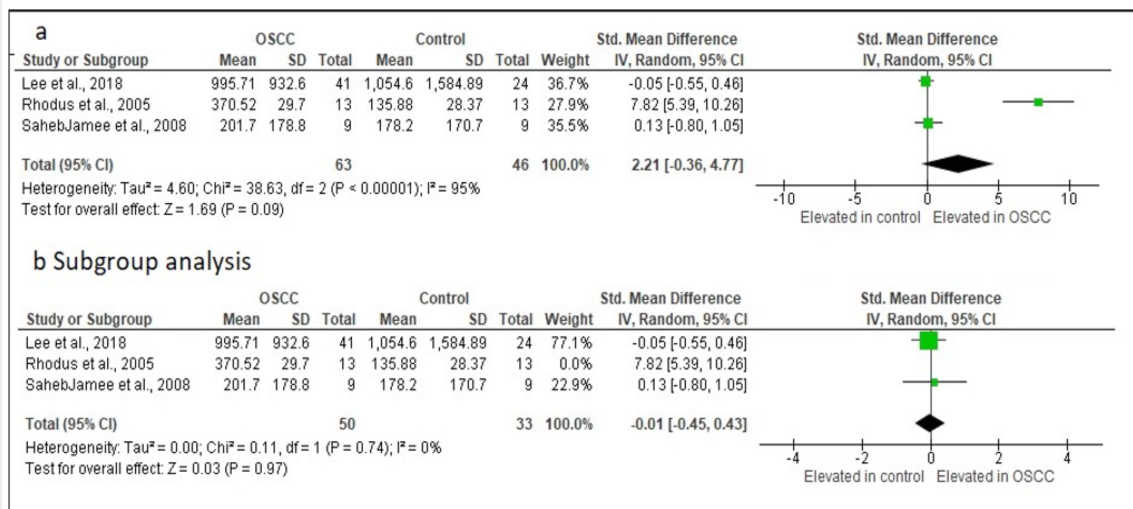

Figure 6: Elevations in salivary IL-1α are not associated with OSCC Forest plot reveals the Standard Mean Difference and 95% CI for the association of elevations in IL-1α in OSCC patients compared to healthy controls across the included studies (a & b). The green rectangles represent the standardised mean difference and the weighting of each study is represented by the size of each rectangle where the horizontal lines are the 95% confidence intervals (CI). The vertical line indicated on zero, represents the line of no effect. The black diamond signifies the mean weighted overall standardised mean difference. The forest plot P value reveals the statistical significance between IL-1α in saliva of OSCC patients. As a consequence of high heterogeneity (I2) in a, a subgroup analysis of IL-1α levels compared between OSCC patient saliva and that of health control subjects is presented in b.

Discussion

This meta-analysis aimed to investigate the potential of specific cytokines and LDH in saliva as non-invasive biomarkers for diagnosis of OSCC. The results of this meta-analysis indicated that IL-6, IL-8, TNFα and LDH levels in saliva were significantly elevated in OSCC patient samples compared to the healthy controls and therefore have good potential as salivary biomarkers of OSCC. IL-6 was significantly elevated in OSCC saliva samples compared to

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healthy controls as seen in Figure 2. IL-6 is involved in key tumour progression events; in particular, TAM IL-6 production drives EMT through crosstalk with STAT3 and Rac1. This interaction induces replacement of E-cad with N-cad, a key aspect of EMT. In addition, IL-6/STAT3 signaling activates NF-αB transcriptional activity to increase apoptotic resistance, as well as enhancing tumour cell proliferation [7] and OSCC migration [32]. Crucially, other studies have identified that salivary IL-6 levels are significantly elevated in OSCC compared to other oral pathologies, such as chronic periodontitis and oral lichen planus [19]. Thus, utilising salivary diagnostics focusing on IL-6 could differentiate between these other oral pathologies, as well as identifying OSCC. TNFα was also significantly elevated in OSCC patients' saliva compared to healthy controls. TNFα may upregulate its own production, alongside driving the production of other pro-inflammatory cytokines [33]. TNFα ligation and signal transduction activates TNF receptor associated factor 6 (TRAF6)-dependent JNK and PI3K signalling, which in turn activates NF-αB (p65/p50) and the production and increased activity of the oncogenes c-Fos and c-Jun [33]. The increased activity of the Fos and Jun dimer, AP-1, also drives the production of TNFα as a positive feedback mechanism, already described in breast cancer [34].

Aside from increasing proliferation, progression, angiogenesis and inflammation, TNFα can induce RANKL production on OSCC cells, which can initiate osteoclast development, and alongside IL-6 and IL-17, activate osteoclast-mediated bone loss seen in OSCC [35]. TNFα production can also be enhanced in OSCC, through increased neutrophil recruitment, driven by IL-8, which induces TNFα [36]. Levels of TNFα in saliva have been found to be significantly different in OSCC patients compared to other oral pathologies, including potentially malignant oral lesions, which can be a precursor to OSCC transformation [29]. This may enable TNFα levels in saliva to distinguish between OSCC and pre-malignant lesions. In addition, IL-8 was also significantly elevated in the saliva of OSCC patients. IL-8 is a chemokine that can induce VEGF production in TAMs, resulting in increased angiogenic activity [37]. IL-8 also upregulates the expression of chemokine receptors CXCR1 and CXCR2 [12], which in conjunction with high IL-8 levels (as seen in saliva of OSCC patients), results in increased ERK-dependent release of MMP-7 and MMP-9, known to promote proliferation, migration and invasion in OSCC [12]. Thus, salivary levels of IL-8 could differentiate between potentially malignant oral lesions and OSCC [29]. Importantly, Rajkumar [27] identified statistically significant differences in the levels of IL-8 in saliva of OSCC patients between earlier cancer stages I-II and later stages III-IV. This is incredibly beneficial because it would allow for different stages of disease to be determined using IL-8 levels in saliva.

Furthermore, the elevated levels of salivary LDH of OSCC patients (Figure 5) facilitates the adaptation of tumour cells to anaerobic conditions, driven by cytokines such as IL-6 and TNFα and LDH can also activate PI3K signaling, which can further enhance cancer progression through increasing proliferation and survival [38]. This results in hypoxia inducible factor (HIF) activation, driving production of LDHA, GLUT-1 transporter, epidermal growth factor (EGF) and VEGF [13]. LDH turns pyruvate (the end product of glycolysis) into lactic acid to produce ATP, and also drives the increased expression of GLUT-1 on the cell surface facilitating increased glucose uptake. This enables OSCC cells to maintain the increased rates of proliferation seen in cancer [13]. Additionally, EGF forms a positive feedback loop, enhancing LDHA production and augmenting tumour adaptation to anaerobic conditions commonly seen in solid tumours [13]. EGF can also enhance tumour proliferation, through downstream upregulation of cyclin D1 to allow cell cycle checkpoint advancement [39]. OSCC cell LDHA knockout resulted in suppressed proliferation, EMT, invasion and migration, highlighting the crucial influence of LDH in tumour progression and metastasis [13]. In addition, studies by Lokesh, Kannabiran & Rao [25] and D'Cruz & Pathiyil [20] both identified that LDH was significantly different in tumours of varying differentiation; poorly differentiated tumours expressed the greatest levels of active LDH and vice versa. If this could be directly correlated to the stage of OSCC (i.e. stage I-IV) then LDH could be an incredibly valuable biomarker for identifying the presence and stage of disease progression.

Salivary IL-6, IL-8, TNFα and LDH have been found to be significantly elevated in OSCC patients compared to other oral pathologies such as chronic periodontitis, oral submucous fibrous, oral lichen planus and oral premalignant lesions [19,23,29]. Thus, they present suitable salivary biomarkers of OSCC and could potentially be used to differentiate between the other aforementioned inflammatory pathologies. The difference in IL-1α in OSCC saliva samples and healthy controls was not statistically significant and was confirmed by the subgroup analysis (Figure 6b). However, Figure 6a does show a trend towards significance, so more research may be necessary to completely explore salivary IL-1α as a biomarker of OSCC. The lack of a statistically significant difference in IL-1α between its levels in saliva from OSCC patients and healthy controls, could be linked to the stage of OSCC in patient samples. Indeed, IL-1α was produced in greater quantities in later stage, aggressive tumours [11]. Additionally, an investigation on the influence of IL-1α on cancer progression, through the interaction of carcinoma-associated fibroblasts (CAFs) on OSCC cells, found that OSCC cell-derived IL-1α promoted the proliferation of CAFs and OSCC cells and upregulated the secretion of IL-8 [40]. Tumour-derived IL-1α was also found to cross-talk with TAMs, resulting in the polarization of macrophages to the pro-tumour M2 phenotype [14]. This shift in macrophage polarization promotes lymph angiogenesis by augmenting TAM VEGF production. IL-1α also increases lymph node metastasis via the upregulation of surface CXCR4 on TAMs, which bind SDF-1α [41]. This results in the ERK signaling pathway-dependent release of MMP-9 and MMP-13, which drive invasion and migration of cancer cells. This action of IL-1α could be directly related to the increased risk of lymph node metastases following excisional biopsies described by

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Weber [42], by further polarising the macrophages towards the M2 phenotype. Thus, with the action of IL-1α in OSCC being well understood, and alongside its greater prevalence in aggressive tumours, it is somewhat surprising that there was no significant difference between IL-1α in OSCC saliva samples compared to healthy controls. It is possible given the small sample sizes used in studies by Rhodus [29] and SahebJamee [30]; these studies may have had very few late stage or aggressive OSCC cases to collect saliva samples from for the investigation.

The statistical analyses performed in this meta-analysis produced highly significant evidence for IL-6, IL-8, TNFα and LDH as salivary biomarkers for OSCC. Despite some high heterogeneity values, this meta-analysis yielded highly clinically relevant outcomes, as most of the heterogeneity was likely related to different stages of OSCC in patients across the studies, expressing significantly different levels of these cytokines and LDH. Furthermore, if methods employed in each study were standardized, this also would have reduced heterogeneity; one example being the use of ELISAs to quantify cytokine levels in saliva, whereas Lee [24] used a Luminex bead-based multiplex assay. Over the last few years, there have been a number of studies investigating salivary cytokines as potential biomarkers for OSCC [4,43]. These share similar outcomes; all suggesting the potential of cytokines as salivary biomarkers for OSCC, but describe a lack of specific diagnostic levels, small sample sizes and lack of follow-up of these cytokine levels through treatment; effectively hindering any definitive assessment of their potential. The implications of this meta-analysis on practice are the clear potential of combined analysis of both salivary cytokines and LDH as biomarkers of OSCC to replace excisional biopsies currently being used; it has been established that LDH and the salivary cytokines (IL-6, IL-8 and TNFα) are involved in key aspects of tumour development, progression, invasion and migration [15]. As previously mentioned, excisional biopsies have been associated with an increased risk of lymph node metastases in OSCC [42]. By utilizing salivary biomarkers for diagnosis and prognosis of OSCC, it would be possible to completely eliminate this risk of lymph node metastases following excisional biopsies, presenting both a simple and non-invasive diagnostic/prognostic strategy that confers a benefit to the patient.

Excisional biopsy tissue trauma initiates a wound healing reaction, providing an environment resulting in macrophage polarisation towards the tumour-promoting M2 phenotype. This inhibits anti-tumour responses by inhibiting M1 macrophage activity through TGFα production and other anti-inflammatory cytokines such as IL-4, IL-10 and IL-13, resulting in accelerated tumour progression through a positive feedback loop of M2 macrophage polarisation [44]. As M2-like TAMs can produce IL-6 to drive EMT, as previously discussed, and TAMs have their angiogenic activity enhanced by IL-8, these cytokines, alongside TNFα and LDH, can enhance key cell survival and progression in OSCC and as such, offer themselves up as key prognosic indicators. Recently, Chohan [45], found that, in the case of excisional biopsy, $CD163^+$ TAMs and the expression of PD-L1 could serve as prognostic indicators of survival and possibly stage of cancer in OSCC, whereas $CD68^+$ TAMs were of no prognostic use. As a consequence of the suggestion that invasive excisional biopsy can accelerate tumour progression [7], there is a need for the adoption of non-invasive prognostic testing. The current investigation addressed this and found that the diagnostic testing of salivary levels of IL-6, IL-8, TNFα and LDH, but not IL-1α, could all serve as useful prognostic biomarkers for OSCC. The fact that the expression of these salivary markers are shared with inflammatory pathology such as CP, and that inflammatory mechanisms play an integral part in tumour development, would suggest that biopsy and saliva sampling may converge, where OSCC can be diagnosed by the non-invasive saliva sampling of IL-6, IL-8, TNFα and LDH, as well as detection of MMPs and the potential shedding of tumour-associated molecules, such as CD163 and PD-L1. Such a non-invasive diagnostic sampling approach may offer a definitive diagnosis of OSCC and its stage of development without the disadvantages of excisional biopsy and its potential to facilitate tumour progression.

Conclusions

This meta-analysis provides good evidence to support the potential of salivary IL-6, IL-8, TNFα and LDH as biomarkers for OSCC that have a great statistical difference between their respective levels in healthy controls and other oral pathologies. A crucial advantage of saliva-based diagnostics is the non-invasive nature of using saliva as a diagnostic tool compared to the current diagnostic methods.

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Supplementary Table 1: Characteristics of included studies.

	Region	Method	Number of Participants	Age (mean, range)	Stage	Outcomes
Cheng [19]	USA	ELISA	60	60.385, 32-78	I-IV	Salivary IL-6 levels significantly higher in OSCC patients than CP, OLP and healthy controls
D'Cruz and Pathiyil, [20]	India	ELISA	60	Not stated, but age-matched controls	I-IV	LDH levels in saliva of OSCC patients significantly higher than in healthy controls regardless of stage of the tumour
Deepthi [21]	India	ELISA	60	N/A, 20-74	I-IV	Sensitivity and specificity of TNFa in differentiating OSCC samples is 100% and 96.7% respectively. TNFa is a highly viable salivary biomarker
Gholizadeh [22]	Iran	ELISA	50	51.865, 40-64	Not specified	Salivary LDH levels in OSCC patients were significantly higher than in healthy controls. Sensitivity of 88% and specificity of 100% for detecting OSCC
Kallalli [23]	India	Semi-auto analyzer (ERBA-CHEM 5)	35	Not stated	Not specified	LDH levels in OSCC patient saliva was significantly higher than healthy controls, but the levels were not significantly different from OSMF, so could not distinguish between OSCC or OSMF
Lee [24]	Taiwan	Luminex Bead-based Multiplex Assay	65	X>55, range unknown	I-IV	IL-6, IL-8, IL-1a, and TNFa were all significantly elevated in the saliva of OSCC patients compared to healthy controls
Lokesh, Kannabiran and Rao [25]	India	Enzyme activity assay	50	N/A, 35-65	I-IV	Salivary LDH levels in OSCC patients were significantly higher than in healthy controls. LDH levels were significantly different between all stages of tumours, so could be used to identify the stage, as well as the presence of disease
Marton [26]	Hungary	ELISA	175	61.7, range unknown	I-IV	Salivary IL-6 levels in OSCC patients were significantly higher than in healthy controls. This trend was also seen with IL-6 mRNA expression
Rajkumar [27]	India	ELISA	200	N/A, 21-90	I-IV	IL-8 levels in the saliva of OSCC patients were significantly higher than healthy controls. Significant differences were also found between IL-8 levels in different stages of tumours
Rao [28]	India	ELISA	60	Not stated	Not specified	Salivary IL-6 levels were significantly higher in OSCC patients compared to healthy controls
Rhodus [29]	USA and China	ELISA	26	58, 46-70	I-IV	IL-6, IL-8, IL-1a, and TNFa were all significantly elevated in the saliva of OSCC patients compared to healthy controls and OMPL patients
SahebJamee [30]	Iran	ELISA	18	69.33, 57-86	Not specified	Salivary levels of IL-6 were significantly higher in OSCC patients compared to healthy controls. IL-1a, TNFa and IL-8 were not significantly different than the levels in the saliva of healthy controls

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Supplementary Table 2: Data extraction from included studies investigating IL-1α, IL-6, IL-8 and TNFα in saliva of OSCC patients.

Author and Year of study	Number of Participants	Cytokine/enzyme	OSCC (pg/ml)	Control (pg/ml)	P value
Cheng [19]	OSCC: 18	IL-6	178.11 (+/- 172.32)	4.92 (+/- 8.77)	<0.001
	Control: 21	IL-8	1523.33 (+/- 1123.95)	890.83 (+/- 563.22)	0.014
Lee [24]	OSCC: 41	IL-6	198.33 (+/- 303.84)	69.23 (+/- 271.96)	<0.001
	Control: 24	IL-8	2060.32 (+/- 1796.53)	909.99 (+/- 833.43)	0.001
		IL-1a	995.71 (+/- 932.60)	1054.60 (+/- 1584.89)	0.625
		TNFα	27.75 (+/- 30.94)	8.60 (+/- 7.27)	0.001
Rhodus [29]	OSCC: 13	IL-6	198.23 (+/- 47.68)	2.26 (+/- 0.72)	<0.0001
	Control: 13	IL-8	4082.80 (+/- 752.30)	1507.20 (+/- 398.50)	<0.0001
		IL-1a	370.52 (+/- 29.70)	135.88 (+/- 28.37)	<0.0001
		TNFα	103.64 (+/- 61.69)	3.36 (+/- 2.07)	<0.0001
Marton [26]	OSCC: 95	IL-6 (Stage I and II)	59.72 (+/- 105.23)	12.45 (+/- 26.32)	<0.001
	Controls: 80	IL-6 (Stage III and IV)	102.44 (+/- 135.34)		<0.001
SahebJamee [30]	OSCC: 9	TNFa	35.2 (+/- 51.8)	4.1 (+/- 2.1)	>0.05
	Controls: 9	IL-1α	201.7 (+/- 178.8)	178.2 (+/- 170.7)	>0.05
		IL-6	40.9 (+/- 79.5)	2.5 (+/- 1.3)	<0.05
		IL-8	1093.7 (+/- 1089.0)	700.7 (+/- 1031.5)	>0.05
Rao [28]	OSCC: 30 Controls: 30	IL-6	192.15 (+/- 82.76)	15.06 (+/- 4.42)	<0.001
Deepthi [21]	OSCC: 30 and Controls: 30	TNFa	63.94 (+/- 56.05)	5.78 (+/- 3.98)	<0.01
Rajkumar [27]	OSCC: 100 Controls: 100	IL-8	1091.68 (+/- 167.12)	349.56 (+/- 115.12)	<0.05

Supplementary Table 3: Data extraction from included studies investigating LDH in saliva of OSCC patients.

Author and Year of study	n	Cytokine /Enzyme	OSCC (U/L)	Control (U/L)	P value
Lokesh, Kannabiran and Rao, [25]	OSCC: 30	LDH WD	1049.07 (SD 46.89)		
	Controls: 20	LDH MD	1309.50 (SD 68.79)		
		LDH PD	1586.20 (SD 203.20)		
		LDH mean	1225.4 (SD 221.79)	497.00 (SD 51.75	0.0001
Gholizadeh [22]	OSCC: 25 Controls: 25	LDH	99.833 (SD 49.3260)	3.8333 (SD 1.1044)	0.021
Kallalli [23]	OSCC: 25 Controls: 10	LDH	630.96 (SD 39.80)	182.21 (SD 34.85)	0.0009
D'Cruz and Pathiyil [20]	OSCC: 30	LDH WD	355.83 (SD 16.73)		
	Controls: 30	LDH MD	484.18 (SD 25.84)		
		LDH PD	620.35 (SD 18.69)		
		LDH mean	486.79 (SD 20.42)	117.33 (SD 19.37)	<0.001

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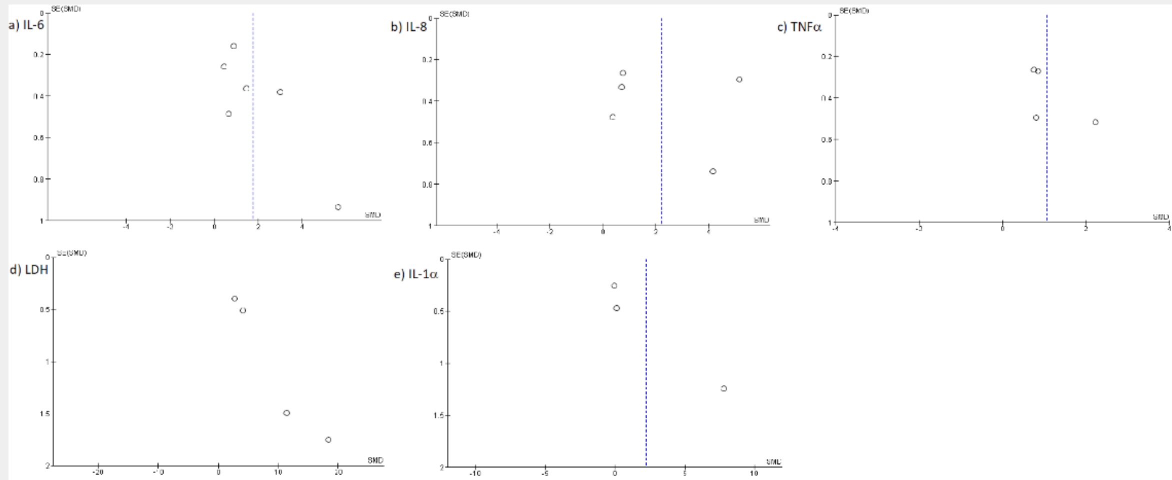

Supplementary Figure 1: Funnel plots for studies relating to cytokine secretion and LDH release evaluating publication bias. Standard error of the standardized mean difference (y axis) is plotted against its effect size (x axis) for a) IL-1α,b) IL-6, c) IL-8, d) TNFα and e) LDH.

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