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Article type : Review

Prognostic value of the immunohistochemical detection of cancer-associated fibroblasts in oral cancer: a systematic review and meta-analysis

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**Running title:** Prognostic value of CAF in oral cancer.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jop.12623

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## Abstract

**AIM:** To perform a meta-analysis to assess whether the presence of cancer-associated fibroblasts (CAF) is a prognostic marker of oral squamous cell carcinomas (OSCC).

**METHODS:** Immunohistochemical studies assessing the prognostic relevance of CAF (alpha smooth muscle actin ( $\alpha$ -SMA)-positive fibroblasts) in patients with OSCC were systematically reviewed using Cochrane, Lilacs, PubMed, Scopus and Web of Science databases. The outcomes assessed were overall survival (OS) and disease-free survival (DFS). The meta-analysis was performed using the random and fixed effects model with adjusted hazard ratio (HR) and 95% confidence intervals (95% CI) as effect measures. The methodological quality of the included studies was assessed using the Meta-Analysis of Statistics Assessment and Review Instrument (MAStARI) tool and the evidence quality was assessed by the Grading of Recommendation, Assessment, Development, and Evaluation (GRADE) system.

**RESULTS:** The presence of high levels of CAF in the stroma of OSCC predicted shortened time to DFS (HR= 3.32, 95% CI: 2.09-5.26,  $p < 0.00001$ ) and an overall decrease in survival (HR: 2.16, 95% CI: 1.60-2.92,  $p < 0.00001$ ). Moreover, high presence of CAF was frequently reported in association with parameters that worsen the prognosis in OSCC, including advanced disease stage (TNM classification), recurrence, tumor grade, depth of invasion, vascular, lymphatic and neural invasion and extranodal metastatic spread.

**CONCLUSION:** The presence of CAF, as assessed by  $\alpha$ -SMA-positive fibroblasts in the stroma, indicates poor prognosis in patients with OSCC.

**Keywords:** oral cancer; cancer-associated fibroblast; alpha smooth muscle actin; prognosis.

## Introduction

Oral cancer, representing more than 90% of cases of oral squamous cell carcinomas (OSCC), is the eleventh most commonly diagnosed cancer worldwide, accounting for 300,000 new cases and 145,000 deaths per year (1). The prognosis of OSCC is widely variable, depending largely on clinical stage (TNM classification) and localization in the oral cavity. Overall, the 5-year survival rate is approximately 50%, which has remained unchanged over recent decades (2). Although advances in molecular biology have helped identify and characterize genes and molecular pathways involved in development and disease progression, little impact on predicting disease behavior, prognosis and treatment response has resulted (3). Therefore, markers for early detection, differentiating low and high risk groups, personalizing treatment plans and post-therapeutic monitoring are urgently required.

During OSCC invasion, tumor cells induce a series of modifications in the adjacent stroma, promoting an unique environment (commonly termed the tumor microenvironment) composed of an extracellular matrix scaffold, vascular structures and cellular components including adipocytes, muscle cells, mast cells, immune and inflammatory cells and fibroblasts. Some fibroblasts acquire an activated phenotype and are termed cancer-associated fibroblasts (CAF; also known as peritumoural fibroblasts, activated fibroblasts or myofibroblasts) (4). CAF are thought to have a variety of origins, including transformation from resident fibroblasts, epithelial cells and pericytes or differentiation from mesenchymal stem cells (5). There is no specific marker for CAF, but alpha smooth muscle actin ( $\alpha$ -SMA) is the most used and reliable marker for detecting CAF (6). CAF are found in approximately 60% of OSCC, frequently in close contact with the tumor islands (7, 8), but are not found in tumor-free tissues and in the adjacent stroma of potentially malignant disorders of the oral mucosa (9). Moreover, in vitro studies have demonstrated that transforming growth factor beta (TGF- $\beta$ ) released by oral carcinoma cells induces CAF activation (8, 10), suggesting

that the emergence of CAF within tumor microenvironment is influenced by tumor cell invasion.

Previous studies have demonstrated that increased density of CAF in the stroma of OSCC correlated with higher mortality (7, 11, 12). Further analyses revealed that CAF promote tumorigenesis of OSCC cell lines via an enriched and specific secretome, which contains activin A, fibronectin type III domain-containing 1 (FNDC1), serpin peptidase inhibitor type 1 (SERPINE1), stanniocalcin 2 (STC2), among other proteins putatively related to tumorigenesis (13, 14). Importantly Marsh and collaborators (12) provided evidence that the presence of CAF in the stroma of OSCC is a stronger predictor of mortality than the classical TNM staging. However, other studies did not find a significant association between CAF and survival of OSCC patients (15, 16). The aim of this systematic review and meta-analysis was to verify the value of CAF for the prognosis of OSCC patients. The present study provides evidence that immunohistochemical detection of CAF ( $\alpha$ -SMA-positive cells) is an independent marker of shortened disease-free survival (DFS) and poor overall survival (OS) in patients with OSCC.

## **Materials and methods**

This systematic review adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Checklist as described in Moher et al (17).

### *Protocol and registration*

The review was registered at the international prospective register of systematic review (<http://www.crd.york.ac.uk/PROSPERO/>) and received the number CRD42017060787 ([http://www.crd.york.ac.uk/PROSPERO/display\\_record.asp?ID=CRD42017060787](http://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42017060787)).

### *Study design*

The systematic review and meta-analysis of human studies was undertaken to evaluate whether immunodetection of CAF ( $\alpha$ -SMA-positive fibroblasts) serves as a prognostic factor of the survival of patients with OSCC.

### *Eligibility criteria*

Inclusion criteria: Articles that assessed the relationship between the immunohistochemical presence of CAF and the survival of OSCC patients were selected for our systematic review and meta-analysis. The search was conducted without time and language restrictions. The PICOS (population, intervention, comparison, outcome, study design) format was used to construct the research question with the following inclusion criteria: (i) Population: patients with oral squamous cell carcinoma (OSCC); (ii) Intervention: CAF analysis by immunohistochemical detection with anti- $\alpha$ -SMA antibody; (iii) Outcome: OS and DFS; (iv) Study Design: observational studies in humans.

Exclusion criteria: Studies were excluded for the following reasons: (1) studies that did not fit PICOS strategy; (2) reviews, letters, personal opinions, book chapters and conference abstracts.

### *Information sources and search strategy*

Search strategies in the Cochrane, LILACS, PubMed, Scopus and Web of Science databases included the following terms: "oral squamous cell carcinoma" OR "OSCC" OR "oral cancer" OR "oral cancers" OR "mouth neoplasms" (MeSH Terms) OR "mouth neoplasm" OR "oral neoplasm" OR "oral neoplasms" OR "cancer of mouth" OR "mouth cancers" OR "mouth cancer" OR "cancer of the mouth" OR "tongue cancer" OR "squamous

cell carcinoma of the oral cavity" OR "tongue squamous cell carcinoma" OR "tongue squamous cancer" AND "cancer-associated fibroblast" OR "carcinoma-associated fibroblast" OR "CAF" OR "peritumoural fibroblast" OR "activated fibroblast" OR myofibroblast OR myofibroblasts.

The search was conducted on April 02, 2017. A partial grey literature search was also performed using Google Scholar and ProQuest. The selected references were checked and managed by a reference manager software (EndNote, Thomson Reuters, Virginia, USA). In addition, the reference lists of the selected articles were hand screened for potential relevant studies that could have been missed during the electronic database searches.

#### *Study selection*

The articles were selected in two phases. In phase 1, two authors (MRD and RDC) independently reviewed the titles and abstracts, and selected those that apparently met the inclusion criteria. In phase 2, the same authors read the full texts of the selected articles at phase 1 and excluded those that did not meet the inclusion criteria. Any disagreements in the first or second phases were resolved by discussion and mutual agreement between the two authors. If the two authors did not reach a consensus, a third author (ENSG) made the final decision.

#### *Data collection process and data items*

One author (RDC) collected the following information from the included articles: authors, year of publication, country, number of samples, localization, clinical stage, classification (score system), results and adjusted hazard ratio (HR) and 95% confidence interval (CI) for both OS and DFS. A second author (MRD) crosschecked all the retrieved information.

Disagreements were resolved by discussion and mutual agreement between the authors. When necessary, a third author (ENSG) made the final decisions.

#### *Risk of bias in individual studies*

Methodologically, the authors appraised all of the included studies according to a checklist based in Meta-Analysis of Statistics Assessment and Review Instrument (MAStARI) (18). Two reviewers (MRD and RDC) answered 9 questions for descriptive studies as Y for “yes”, N for “no”, U for “unclear” and NA for “not applicable”. After that, the risk of bias was categorized as high when the study reached up to 49% of a “yes” score, moderate when the study reached 50% to 69% of a “yes” score, and low when the study reached more than 70% of a “yes” score. Disagreements were solved by discussion between the three authors (MRD, RDC and ENSG).

#### *Measures for meta-analysis*

The OS and DFS meta-analysis was performed following the appropriate Cochrane Guidelines (19). Review Manager 5.3 (Rev-Man 5.3, The Nordic Cochrane Centre, Copenhagen, Denmark) was used to construct the forest plots of the meta-analysis, with the HR and 95% CI determined at a significance level of 5%, based on the adjusted OS and DFS original values of the selected articles.

#### *Risk of bias across studies*

We assessed heterogeneity by comparing variability among number of sample, localization, clinical stage, classification and outcomes for survival studies (HR and 95% CI).

### *Level of evidence*

The Grading of Recommendation, Assessment, Development, and Evaluation (GRADE) instrument (20) was used to assess evidence quality and grading of recommendation strength in the 12 studies included in the quantitative synthesis (11, 12, 14, 15, 21-28). This assessment was based on the study design, risk of bias, inconsistency, indirectness, imprecision and other considerations. Evidence quality was characterized as high, moderate, low or very low. The GRADE was assessed using the website <http://grade.pro.org>.

## **Results**

### *Study selection*

In the first phase, 374 studies were selected in the five electronic databases. Duplicate studies were removed, resulting in 200 different citations. Subsequently, comprehensive evaluation of titles and abstracts resulted in the exclusion of 167 citations, thereby remaining 33 studies for consideration into the second phase. Moreover, 15 citations were identified in the grey literature (Google Scholar and ProQuest). The expert (RDC) identified 2 additional studies. In the second phase, the full-text review was then conducted on the 35 first-phase selected citations, which lead to the exclusion of 15 studies. In the end of the two phases, 20 studies fulfilled the inclusion criteria, but only 12 articles performed multivariate analysis and made accessible the adjusted HR and 95% CI. A flow-chart detailing the processes of identification, inclusion and exclusion of the studies is depicted in Fig. 1.

### *Study characteristics*

The selected studies were published between 2007 and 2017 and were all written in English. They were conducted in 8 different countries: Brazil (7, 8, 14, 25), Japan (15, 21, 28), Israel



(16, 22, 23), China (24, 26, 29), Finland (30), England (12, 31), Croatia (27) and Norway (32). In addition, 2 studies were performed in collaboration, 1 between Finland and Israel (11) and 1 between Brazil and Finland (33). The selected articles were observational studies. The main features and findings of the studies are presented in Table 1.

#### *Risk of bias*

Based on the MASTARI assessment, 3 articles (8, 15, 29) were classified as carrying a high risk of bias, mainly because the answers for questions 3 and 4 (related to co-founding factors and outcomes, respectively) were “No”. Sixteen studies were classified as with moderate risk for bias (7, 11, 12, 14, 16, 21-24, 26-28, 30-33) and 1 study was classified as low risk of bias (25) (Table 2).

With respect of MASTARI question 3, many studies did not include the classical prognostic factors associated with OSCC survival in the analysis. Treatment protocol and status of the surgical margins, which is essential for postsurgical therapy, were the most common lacking parameters. MASTARI question 4 deals with the criteria of the outcomes, including the assessment of the immunohistochemistry. In most of the studies, except Kawashiri et al (21), the quantification of CAF was subjective and performed by a single examiner, leaving the possibility of great variability.

Regarding the risk of bias across studies, the selected studies used similar methods, which reduced the possibility of misinterpretation. These studies were suitable for grouping for meta-analysis, and two different subgroups, based on the outcome, were generated.

#### *Correlation between CAF and clinicopathological characteristics*

Although there was no standard score system to assess CAF density in the stroma of the tumors, all studies reported some positive relationship between presence of CAF and clinicopathological features of tumors. The main findings of each study are described in

Table 1. CAF were identified in the stroma of OSCC for the first time by Barth and collaborators in 2004 (34), but the first study demonstrating their prognostic significance in OSCC was reported by Kellermann and colleagues in 2007 (7). This study analyzed the presence of CAF in 83 squamous cell carcinomas of the tongue, and revealed that CAF are found both in stroma within the tumor and in the deepest invasive tumor front, representing the band of tissue between the invasive tumor front and adjacent normal tissue. High density of CAF in the stroma and in the invasive tumor front was significantly associated with clinical N stage, vascular and lymphatic invasion, pathologically confirmed lymph node metastasis and extracapsular lymph node spread (7). At the invasive front, density of CAF was also associated with perineural invasion. The univariate analysis for OS showed that patients with abundance of CAF at the invasive front had significantly poorer OS rate than those with negative or low frequency of CAF (7). No association with DFS was observed. The same group confirmed in a second study the association between presence of CAF and parameters of OSCC aggressiveness (8). Presence of CAF was significantly correlated with N stage, disease stage and cervical relapse of the disease after initial treatment. This study also demonstrated that the presence of CAF was significantly higher in tumors containing a high number of Ki67-positive tumor cells.

Kawashiri et al (21) found CAF mainly in highly invasive OSCC, and their presence was significantly associated with the mode of invasion, degree of differentiation of tumors and pathologically confirmed lymph node metastasis. Kaplan-Meier curve showed that CAF were significantly associated with poor survival of the patients, but the significance did not withstand multivariate correction analysis for cofounders.

A significant association between CAF and a histopathologic risk score system for OSCC proposed by Brandwein-Gensler et al (35), which includes worst pattern of invasion, pattern of lymphocytic infiltration and perineural invasion, by Vered et al (22). This study also showed a significant association of CAF with both OS and DFS, but at multivariate analysis only DFS was significantly influenced by density of CAF.

Bello et al (11) observed a significantly higher mortality, after adjustment for cofactors, in patients with tongue squamous cell carcinoma that had high density of CAF in the stroma. This study did not perform association analysis between CAF density and clinicopathological parameters.

A significant and positive correlation between density of CAF and depth of invasion and extranodal metastatic spread was reported by Marsh et al (12) in a cohort containing 282 OSCC. In this study, multivariate analysis revealed an independent and significant prediction of OS by CAF density, regardless of disease stage.

Besides CAF quantification, Dayan et al (23) characterized the inflammatory infiltrate in the microenvironment of 64 squamous cell carcinomas of the tongue. The density of CAF was inversely correlated with the density of the inflammatory infiltrate, but positively correlated with a more protumorigenic and anti-inflammatory infiltrate composed of regulatory T cells (Foxp3<sup>+</sup> cells), tumor-associated macrophages (CD163<sup>+</sup> cells) and potentially Treg-inducing immune cells (CD80<sup>+</sup> cells). Importantly, CAF density was significantly associated with high risk of locoregional recurrence (DFS) and a decrease in OS at multivariate analysis.

Fujii et al (15) reported that the presence of CAF is significantly correlated with the presence of CD163-positive macrophages in the tumor stroma, but no further significant associations with clinicopathological parameters and OS were observed. Kaplan-Meier analysis for OS revealed a significantly lower survival rate for patients who had focal distribution of CAF compared with patients who negative/scanty or abundant presence of CAF. However, only 13 patients were classified in the group with focal distribution of CAF.

Ding et al (24) revealed a significant association between high density of CAF and presence of cervical lymph node metastasis and higher TNM classification. This study also demonstrated a positive and significant correlation between the presence of CAF and expression of vimentin, N-cadherin and blood vessel density. In the multivariate regression

analysis, high CAF density was significantly associated with OS, showing this an independent prognostic factor.

Dhanda et al (31) observed that the presence of CAF at the tumor invasive front, but not in the tumor center, is significantly associated with lymph node metastasis, extracapsular spread from metastatic lymph nodes, and poor OS at univariate level. No multivariate analysis was investigated.

Almangush et al (30) evaluated 82 patients with early stage tongue squamous cell carcinoma and did not find any influence of CAF on patients' survival. No association between CAF and the histopathologic risk score system for OSCC proposed by Brandwein-Gensler et al (35) was observed. Similarly, Kelner et al (25) demonstrated that CAF did not influence the survival of patients with tongue squamous cell carcinoma at an early stage.

In a study with 178 tongue squamous cell carcinomas, Li et al (26) showed a significant association between high density of CAF and pathologic stage, T classification, N classification and recurrence. The survival curves showed that the frequency and the distribution of CAF were significantly associated with a poor OS and DFS, but multivariate analysis showed that the presence of CAF was an independent predictor only for shortened OS.

Luksic et al (27) found that a high density of CAF in OSCC is associated with poor OS by multivariate analysis. These authors also demonstrated that the presence of CAF in the tumor stroma was significantly associated with T stage, presence of occult neck metastasis, regional recurrence and distant metastasis.

The study of Matsuoka and collaborators (28) included 60 patients that received preoperatively a combination of radiotherapy, based on daily doses of 2 Gy, 5 times a week, for 15 days, and chemotherapy with S-1, an oral fluorouracil anti-cancer drug, concurrently administered at a dose of 80, 100 or 120 mg/day according to each patient's body surface area for 14 days from the initiation of radiotherapy. In this unique group, the presence of

CAF was significantly associated with advanced pT and pN stages and with OS and DFS by univariate, but not multivariate analysis. There was a positive and significant correlation between the densities of CAF and CD163-positive macrophages in the tumor microenvironment, but density of macrophages was not also associated with survival.

Bagordakis et al (14) found in the adjusted multivariate analysis that the abundant presence of CAF, either at the tumor center or at the tumor front, significantly influences locoregional relapse (DFS) of OSCC patients. Significant correlations, as revealed by Spearman's rank test, were observed between the density of CAF and type of treatment and between the presence of CAF in the invasive front and the development of a second primary tumor.

In the 86 OSCC analyzed by Lin et al (29), cases with higher histological grade, more invasive phenotype, lymph node metastasis and recurrence showed significantly higher numbers of CAF. In addition, samples with increased numbers of CAF demonstrated elevated tumor cell MMP-9 expression, higher peritumoral lymphatic vessel density and higher microvessel density.

Significant associations between CAF and clinical stage, tumor grade, perineural invasion and tumor thickness were reported by Akrish et al (15), but association of CAF with survival was not statistically significant. As reported by Almangush et al (30) and Kelner et al (25), the study of Sundquist et al (32) showed that the presence of CAF was not significantly associated with survival of tongue squamous cell carcinoma at early stage.

Parajuli et al (32) showed a significant association between tumors with rich areas of CAF and poor OS at univariate level. No multivariate analysis or clinicopathological correlations were investigated.

### *Survival analysis*

Overall Survival: Eleven studies reported data regarding the association between CAF levels and OS (11, 12, 14, 15, 21, 23-28). Six studies (11, 12, 23, 24, 26, 27) found that the presence of CAF is a significant marker for OS. Three studies (25, 30, 33) included only samples at early stage of tumor development (stage 1-T1N0M0 or 2-T2N0M0) and reported a lack of association between CAF and survival, suggesting that CAF are not prognostic indicators of survival in early stage OSCC. The adjusted HR + 95% CI derived from multivariate analysis were used for the meta-analysis. As shown in Fig. 2, the pooled HR was 2.16 (95% CI: 1.60-2.92,  $P$  for heterogeneity = 0.02,  $I^2$  = 52%), indicating that high levels of CAF is an independent prognostic factor for shortened OS.

Disease-Free Survival: Four studies were evaluated for DFS (14, 22, 23, 28). As shown in Fig. 3, the pooled HR derived from multivariate analyses was 3.32 (95% CI: 2.09-5.26,  $P$  for heterogeneity = 0.50,  $I^2$  = 0%), indicating that patients with high density of CAF demonstrated worse DFS. Three of the studies (14, 22, 23) found significant association between density of CAF and increased relapse of disease after initial treatment.

### *Level of evidence*

Based on GRADE analysis, the quality of the evidence for both OS and DFS studies was moderate, suggesting a moderate confidence in estimating the outcomes. The moderate risk of bias in most studies was the main factor responsible for the limited quality of evidence (Supplementary File 1).

## Discussion

The biological properties and functions of CAF in tumor progression and metastasis have been extensively reported in several studies (4-6, 36-38). Owing to the substantial weight of evidence indicating a pro-tumourigenic role, CAF have been suggested as a promising therapeutic target in various cancers. However, data on the prognostic value of CAF are limited, particularly in OSCC. In the current study, we systematically reviewed and meta-analyzed the literature to verify the association of CAF with clinicopathological features and survival of OSCC. We extracted data from 12 eligible studies comprising 1328 patients and pooled data for analysis. The combined results showed that CAF predicted poor OS and shortened DFS in OSCC patients. In addition, the presence of CAF in the stroma of the tumors was consistently associated with several clinicopathological features associated with tumor aggressiveness, including tumor stage, tumor grade, recurrence and histological features such as depth of invasion, vascular, lymphatic and neural invasion and extranodal metastatic spread. Furthermore, heterogeneity assumption tested by  $I^2$  metric and publication bias examination illustrated the robustness of our results, supporting CAF immunodetection as a biomarker for OSCC prognostication.

Consistently, 3 studies (25, 30, 33) that included early stage OSCC did not find any association with patient survival. As extensively reviewed by Kelner et al (25) and Sundquist et al (33), the activation (transformation from resident fibroblasts or other cells of the stroma) is dependent on the cross-talk with tumor cells during invasion. CAF are not found in the stroma of normal oral mucosa and dysplastic lesions (7, 9, 39), but are found in close contact with tumor islands (7, 8, 11, 22). Furthermore, in vitro studies demonstrated that cytokines and other factors released by oral carcinoma cells induce CAF activation (8, 10), suggesting that the emergence of CAF within tumor stroma is coordinated by tumor cell invasion. In support, meta-analysis of the available data showed that larger tumors (T2-T4, >2 cm) have significantly higher density of CAF compared with tumors classified as T1 (<2

cm) (81.7% of high CAF for T2-T4 vs. 18.3% for T1;  $p < 0.0001$ ). Taken together, the results indicated that immunodetection of CAF might not have prognostic value for OSCC at early stage of development. Due to the limited sample size, large-scale studies including tumors at early stage are still warranted to confirm those results.

We have found few studies investigating the prognostic significance of CAF in other cancer types by meta-analysis. Folgueira and colleagues (40) pooled 1408 breast cancer cases from 8 studies and showed that CAF expressing matrix metalloproteinase 13 (MMP13) and lectin, galactoside-binding, soluble, 1 (LGALS1) are positively associated with enhanced OR for axillary metastasis, one of the main prognostic factors in breast cancer, whereas CAF expressing caveolin 1 (CAV1) were negatively correlated with lymph node metastasis. In a meta-analysis mixing different solid tumors (breast cancer, colorectal cancer, esophageal cancer, gastric cancer, liver cancer, non-small cell lung cancer, oral cancer, pancreatic cancer and prostate cancer), Liu et al (41) found that the abundant presence of CAF in the stroma is associated with both poor OS and DFS. Notably, this meta-analysis included studies with different entities, increasing the probably selection bias, which call for further investigations on separate cancer types. To our knowledge, the current study was the first meta-analysis to explore the prognostic value of CAF in OSCC, and the findings are in line with those previous studies of other cancers.

This study has some limitations. First, although against the same epitope (1A4), antibodies from different companies were used; this may lead to differences in the quality and intensity of staining results. Second, no method or cut-off definition have been accepted and validated for evaluating CAF density. Indeed, several different systems were applied to group tumors containing low or high density of CAF. Third, the sample size was limited and we were obligated to exclude some studies of the meta-analysis because they did not performed multivariate analysis or did not report the adjusted HR and 95% CI. These could have been estimated from the survival curves, but we elected not to do because this



approach could induce heterogeneity and bias in the results. Fourth, the clinical heterogeneity across the studies, including differences on tumor site, tumor stage and other clinicopathological features, was profound. Furthermore, assessment and adjustment of confounders differed among the studies. Finally, although we have adopted random-effects model for OS because moderate heterogeneity was detected, the inherent heterogeneity of included studies still existed. From a statistical point of view, our data are consistent and provide evidence that CAF density is a potential prognostic marker for OSCC, but the results should be further expanded, assuming a clear cut-off score.

In summary, this meta-analysis showed that abundance of CAF is correlated with clinicopathological features that reflect aggressiveness and dissemination of this disease and, more important, it was associated with worse OS and DFS in OSCC, suggesting that CAF analysis can be considered as a useful prognostic biomarker and therapeutic target in OSCC. Large well-designed studies employing a standard evaluated method are necessary to obtain higher-quality evidence.

### **Acknowledgements**

MRD is supported by grants from the Coordination for the Improvement of Higher Education Personnel-CAPES, Brasilia, Brazil and Medical Research Center, University of Oulu, Finland. Research in Coletta's lab is funded by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP, São Paulo, Brazil; and Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Brasília, Brazil.

### **Conflict of interest**

Authors have declared no conflicts of interest.

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### Figure legends

Figure 1. Flow Diagram of literature search and selection criteria adapted from PRISMA (17).

Figure 2. High density of CAF is significantly associated with shorten overall survival (OS).

(A) Forest plot of hazard ratio for overall survival (OS) comparing patients with high presence of CAF in the tumor stroma compared with those with low presence. The meta-analysis revealed that CAF was associated with worse OS (HR: 2.16, 95% CI: 1.60-2.92,  $p < 0.00001$ ). The diamond represents the pooled HR performed by the random-effect model.

(B) Funnel plot of the studies used in the comparison of CAF density for OS.

Figure 3. The density of CAF is an independent risk factor for relapse of the disease

(disease-free survival-DFS). (A) Forest plot for DFS comparing low and high density of CAF.

High presence of CAF in the stroma of OSCC significantly predicted shortened time to DFS

(HR= 3.32, 95% CI: 2.09-5.26,  $p < 0.00001$ ). The diamond represents the pooled HR performed by the fixed-effect model. (B) Funnel plot with the distribution of the studies used for DFS meta-analysis.

**Table 1** Overview of the immunohistochemistry studies with CAF ( $\alpha$ -SMA positive fibroblasts) as a prognostic marker of oral squamous cell carcinomas.

Study	Year	No. of Samples	Localization	Clinical Stage	Classification	Summary of the Results
Kellermann et al (Brazil)	2007	83	Tongue	All	<ul style="list-style-type: none"> <li>• Negative</li> <li>• Scanty (&lt;50%)</li> <li>• Abundant (&gt;50%)</li> </ul>	Abundant expression in the tumor front associated with N stage, vascular, lymphatic and perineural invasion and poor OS
Kellermann et al (Brazil)	2008	34	All	All	<ul style="list-style-type: none"> <li>• Similar to Kellermann et al (2007)</li> </ul>	Associated with N stage, disease stage and cervical relapse of disease (regional recurrence)
Kawashiri et al (Japan)	2009	84	All (including lip)	All	<ul style="list-style-type: none"> <li>• Negative</li> <li>• Positive</li> </ul>	Association with tumor grade, mode of invasion and lymph node metastasis. No association with OS at multivariate analysis
Vered et al (Israel)	2010	50	Tongue	All	<ul style="list-style-type: none"> <li>• 0 (negative)</li> <li>• 0.5 (few cells attached to tumor islands/nests)</li> <li>• 1 (few concentric layers in several foci)</li> <li>• 2 (cells in many areas of tumor)</li> <li>• 3 (abundant throughout the tumor)</li> </ul>	Association with histological grade and DFS (local recurrence) at multivariate level. Association with OS did not resist to multivariate analysis
Bello et al (Finland and Israel)	2011	77 (128 blocks)	Tongue	All	<ul style="list-style-type: none"> <li>• Poor (negative)</li> <li>• Medium (less dense or CAFs not distributed throughout the entire tumor)</li> <li>• Rich (dense distribution throughout the tumor)</li> </ul>	Associated with shortened OS at multivariate level
Marsh et al (England)	2011	282	All (including lip)	All	<ul style="list-style-type: none"> <li>• Negative</li> <li>• Low (&lt;5%)</li> <li>• Moderate (5-50%)</li> <li>• High (&gt;50%)</li> </ul>	Associated with depth of invasion, extranodal metastatic spread and worse DSS at multivariate level



Dayan et al (Israel)	2012	64	Tongue	All	<ul style="list-style-type: none"> <li>• 0 (negative)</li> <li>• 1 (weak staining in &lt;50% cells)</li> <li>• 2 (weak staining in &gt;50% cells)</li> <li>• 3 (strong staining in &lt;50% cells)</li> <li>• 4 (strong staining in &gt;50% cells)</li> </ul>	Associated with worse DFS and OS at multivariate level
Fuji et al (Japan)	2012	108	All	All	<ul style="list-style-type: none"> <li>• Negative</li> <li>• Scanty (small number and scattered)</li> <li>• Focal (concentrated in an irregular and non-continuous focus)</li> <li>• Abundant (concentrated in a continuous focus)</li> </ul>	Lower OS for patients with focal classification. No withstood to multivariate level
Ding et al (China)	2014	50	Tongue	All	<ul style="list-style-type: none"> <li>• 0 (negative)</li> <li>• 1 (one-layer of cells around tumor islands/nests)</li> <li>• 2 (multilayer of cells in many areas of tumor)</li> <li>• 3 (dense overlapping throughout the tumor)</li> </ul>	Associated with tumor stage, lymph node metastasis and OS at multivariate level
Dhanda et al (England)	2014	102	All	All	<ul style="list-style-type: none"> <li>• Low</li> <li>• Intermediate</li> <li>• High</li> </ul>	Expression at the invasive front associated with lymph node metastasis, extracapsular spread in the lymph nodes and poor OS
Almangush et al (Finland)	2014	82	Tongue	I and II	<ul style="list-style-type: none"> <li>• Similar to Bello et al (2011)</li> </ul>	Not associated with survival
Kelner et al (Brazil)	2015	110	Tongue	I and II	<ul style="list-style-type: none"> <li>• Similar to Kellermann et al (2007)</li> </ul>	Not associated with survival
Li et al (China)	2015	178	Tongue	All	<ul style="list-style-type: none"> <li>• Similar to Vered et al (2010)</li> </ul>	Associated with pathologic stage, T stage, N stage, recurrence and OS at multivariate level
Luksic et al (Croatia)	2015	152	All	All	<ul style="list-style-type: none"> <li>• 0 (negative)</li> <li>• 1 (&lt;25%)</li> <li>• 2 (26-50%)</li> <li>• 3 (51-75%)</li> </ul>	High scores (2, 3, 4) were associated with T stage, presence of occult neck metastasis, regional recurrence, distant metastasis and

Matsuoka et al (Japan)	2015	60	All	All	<ul style="list-style-type: none"> <li>• 4 (&gt;76%)</li> <li>• Low (low percentage of positive cells and intensity of stain, &lt;4)</li> <li>• High (high percentage of positive cells and intensity of stain, ≥4)</li> </ul>	OS at multivariate level High expression associated with pTstage and pN stage. No association with OS and DFS at multivariate level
Bagordakis et al (Brazil)	2016	113	All	All	<ul style="list-style-type: none"> <li>• Similar to Kellermann et al (2007)</li> </ul>	Abundant expression associated with relapse (DFS) at multivariate level
Lin et al (China)	2016	86	All	All	<ul style="list-style-type: none"> <li>• Negative</li> <li>• Weak</li> <li>• Moderate</li> <li>• Strong</li> </ul>	Associated with histological grade, invasive phenotype, lymph node metastasis and recurrence
Akrish et al (Israel)	2016	65	Buccal mucosa, gingival, palate	All	<ul style="list-style-type: none"> <li>• Similar to Bello et al (2011)</li> </ul>	Associated with tumor stage, tumor grade, perineural invasion and tumor thickness, but not with survival
Sundquist et al (Finland and Brazil)	2017	60	Tongue	I and II	<ul style="list-style-type: none"> <li>• Similar to Bello et al (2011)</li> </ul>	Not associated with survival
Parajuli et al (Norway)	2017	111	All	All	<ul style="list-style-type: none"> <li>• Poor (up to 3 concentric layers of cells around tumor islands)</li> <li>• Rich (more than 3 concentric layers of cells around tumor)</li> </ul>	At invasive tumor front, CAF were associated with poor OS at univariate analysis

**Table 2** Analysis of the risk of bias of the articles included in the review was performed with the MASTARI (Meta-Analysis of Statistics Assessment and Review Instrument) critical appraisal tool (18).

Studies	Questions*									% Yes <sup>#</sup>	Risk of Bias
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9		
Kellermann et al (2007)	NA	Y	N	N	NA	Y	NA	Y	N	50	M
Kellermann et al (2008)	NA	Y	N	N	NA	U	NA	Y	N	33.3	H
Kawashiri et al (2009)	NA	Y	N	Y	NA	U	NA	Y	Y	66.6	M
Vered et al (2010)	NA	Y	Y	N	NA	N	NA	U	Y	50	M
Bello et al (2011)	NA	Y	N	N	NA	Y	NA	U	Y	50	M
Marsh et al (2011)	NA	Y	Y	N	NA	N	NA	Y	Y	66.6	M
Dayan et al (2012)	NA	Y	N	N	NA	Y	NA	U	Y	50	M
Fujii et al (2012)	NA	Y	N	N	NA	U	NA	U	Y	33.3	H
Ding et al (2014)	NA	Y	N	N	NA	Y	NA	Y	Y	66.6	M
Dhanda et al (2014)	NA	Y	N	N	NA	Y	NA	Y	N	50	M
Almangush et al (2014)	NA	Y	N	N	NA	Y	NA	Y	U	50	M
Kelner et al (2015)	NA	Y	Y	N	NA	Y	NA	Y	Y	83.3	L

Li et al (2015)	NA	Y	N	N	NA	Y	NA	U	Y	50	M
Luksic et al (2015)	NA	Y	Y	N	NA	Y	NA	U	Y	66.6	M
Matsuoka et al (2015)	NA	Y	N	N	NA	U	NA	Y	Y	50	M
Bagordakis et al (2016)	NA	Y	Y	N	NA	Y	NA	U	Y	66.6	M
Lin et al (2016)	NA	Y	N	N	NA	U	NA	Y	N	33.3	H
Akrish et al (2016)	NA	Y	N	N	NA	Y	NA	Y	U	50	M
Sundquist et al (2017)	NA	Y	N	N	NA	Y	NA	Y	U	50	M
Parajuli et al (2017)	NA	Y	N	N	NA	Y	NA	Y	N	50	M

- Q1. Is the study based on a random or pseudorandom sample?
- Q2. Are the criteria for inclusion in the sample clearly defined?
- Q3. Are confounding factors identified and strategies to deal with them stated?
- Q4. Are outcomes assessed using objective criteria?
- Q5. If comparisons are being made, was there sufficient description of the groups?
- Q6. Is follow up carried out over a sufficient time period?
- Q7. Are the outcomes of people who withdrew described and included in the analysis?
- Q8. Are outcomes measured in a reliable way?
- Q9. Is appropriate statistical analysis used?

\*Y=Yes, N=No, U=Unclear, NA=Not applicable (which was not considered on the percentage calculation)

# Risk of bias was categorized as high when the study reaches up to 49% score “yes”, moderate when the study reached 50% to 69% score “yes”, and low when the study reached more than 70% score “yes”.



