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PhD Thesis

Liquid Biopsies for Precision Medicine in Colorectal and Head and Neck Cancers: Saliva and Blood Biomarkers

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Doctoral Programme in Dental Science

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DOCTORAL THESIS

LIQUID BIOPSIES FOR PRECISION MEDICINE IN COLORECTAL AND HEAD AND NECK CANCERS: SALIVA AND BLOOD BIOMARKERS

Óscar Rapado González

INTERNATIONAL PHD SCHOOL OF
THE UNIVERSITY OF SANTIAGO DE COMPOSTELA

PHD PROGRAMME IN DENTAL SCIENCE

SANTIAGO DE COMPOSTELA
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TESIS DE DOCTORADO

BIOPSIAS LÍQUIDAS PARA LA MEDICINA DE PRECISIÓN EN EL CÁNCER COLORRECTAL Y DE CABEZA Y CUELLO: BIOMARCADORES EN SALIVA Y SANGRE

Óscar Rapado González

ESCUELA DE DOCTORADO INTERNACIONAL DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

PROGRAMA DE DOCTORADO EN CIENCIAS ODONTOLÓGICAS

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TESE DE DOUTORAMENTO

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AUTHORIZATION OF THE THESIS DIRECTORS/TUTORS

Liquid Biopsies for Precision Medicine in Colorectal and Head and Neck Cancers: Saliva and Blood Biomarkers

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Quien no ha afrontado la adversidad no conoce su propia fuerza.
BEN JONSON

Puede ser un héroe lo mismo el que triunfa que el que sucumbe, pero jamás el que abandona el combate. Quizá ganen los impertinentes medio mundo, pero los constantes poseen el mundo entero.
THOMAS CARLYLE

No basta dar pasos que un día puedan conducir hasta la meta, sino que cada paso ha de ser una meta sin dejar de ser un paso.
JOHANN P. ECKERMANN

¿Qué sería la vida si no tuviéramos el valor de intentar algo?
VICENT VAN GOGH

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AGRADECIMIENTOS

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AGRADECIMIENTOS

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THESIS SUMMARY
RESUMEN DE LA TESIS
RESUMO DA TESE

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THESIS SUMMARY

This PhD thesis highlights the potential value of different biomarkers analyzed on liquid biopsies, such as saliva and blood, in colorectal and head and neck cancers, representing a step forward towards precision medicine. During carcinogenesis, tumors release different components (*e.g.*, cells, proteins, vesicles, and nucleic acids) into body fluids that could be used as biomarkers for cancer diagnosis, prognosis, and therapy selection. Therefore, liquid biopsies constitute a novel and minimally invasive strategy for identification and monitoring tumor biomarkers that could provide relevant dynamic information on the molecular landscape during the tumor evolution.

Among different types of liquid biopsy, saliva is considered as "*mirror of the body*" due to its composition varies in different physiological and pathological states. In addition, saliva presents numerous advantages as diagnostic medium because its collection is simple, easy, non-invasive, and cost-effective. This makes it an attractive biofluid for screening, diagnosis, and monitoring of diseases such as cancer. Ongoing advances in the saliva-omics (proteome, transcriptome, metabolome, genome, epigenome, and microbiome) have allowed the identification of different classes of molecules related to the malignant disease as promising biomarkers. In this line, many studies have focused on the identification of different salivary biomarkers in oral cancer but also in cancers distant to the oral cavity demonstrating the potential of saliva for detecting molecular alterations derived from tumor. In this context, the first objective of the present thesis (***Salivary Biomarkers for Cancer Diagnosis: A Meta-analysis)*** was to analyze the diagnostic accuracy of salivary biomarkers for the detection of non-oral malignant tumors to better define the value of saliva as an alternative liquid biopsy. To systematically evaluate the diagnostic accuracy of salivary biomarkers for detection of non-oral tumors we carried out a systematic review and meta-analysis. For that study, we considered eligible those

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studies that examined the potential of different salivary biomarkers for the diagnosis of non-oral tumors. The searches were performed in different electronic databases including PubMed, Embase, LILACS, and the Cochrane Library. To assess the overall accuracy, we calculated the diagnostic odds ratio (dOR), area under hierarchical summary receiver operating characteristic (ROC) curve (AUC), sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR), using a random- or fixed-effects model. Heterogeneity and publication bias were assessed. One hundred fifty-five study units from 29 articles with 11,153 subjects were included in this meta-analysis. The pooled sensitivity, specificity, PLR, NLR, dOR and AUC were 0.76 (95% confidence intervals (CIs), 0.74–0.77), 0.76 (95% CI, 0.75–0.77), 3.22 (95% CI, 2.92–3.55), 0.31 (95% CI, 0.28–0.34), 13.42 (95% CI, 12.28–15.96) and 0.85 (95% CI, 0.84–0.87), respectively. The results based on this analytic approach revealed that salivary biomarkers may be potentially used for non-invasive diagnosis of malignant non-oral tumors.

Taking in mind the promising value of saliva for detecting cancer and its advantages as a diagnostic fluid, we developed various studies to provide more evidence about the potential clinical utility of this fluid sample for cancer management. In this sense, the second objective of this thesis (***A Novel Saliva-Based miRNA Signature for Colorectal Cancer Diagnosis)*** was to characterize salivary microRNAs (miRNAs) in order to identify non-invasive markers for colorectal cancer (CRC) diagnosis. To carried out this study we designed biomarker discovery and validation phases. In the discovery phase, screening of 754 miRNAs was performed in saliva samples from 14 CRC and 10 healthy controls. Further, part of the differential expressed miRNAs were validated by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) in 51 CRC, 19 adenomas and 37 healthy controls. ROC curves and logistic regression models were performed to analyze the clinical value of these miRNAs. Twenty-two salivary miRNAs were significantly dysregulated in CRC patients vs. healthy individuals ($p < 0.05$) in the discovery phase. From those, five miRNAs (*miR-186-5p*, *miR-29a-3p*, *miR-29c-3p*, *miR-766-3p*, and *miR-491-5p*) were confirmed to be significantly increased in the CRC vs. healthy

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group ($p < 0.05$). This five-miRNA signature showed diagnostic value (72% sensitivity, 66.67% specificity, and AUC of 0.754) to detect CRC, which was even higher in combination with carcinoembryonic antigen (CEA) levels. After the first global characterization of salivary miRNAs in CRC, we identified a 5-miRNA panel as a promising tool to diagnose this malignancy, representing a novel approach for detecting cancer-associated epigenetic alterations using a non-invasive strategy.

Although there are no animal models that demonstrate specifically the origin of salivary miRNAs in cancer, scientific evidence indicates that tumor-derived exosomes could function as the shuttle between the remote tumor and the oral cavity leading to the identification of disease-associated miRNAs. Comparing with remote tumors to the oral cavity, in oral cancers there is a direct release of miRNAs into saliva due to the direct contact with cancerous lesions. In addition, miRNAs can also be released into the bloodstream like in remote cancers and, therefore, reach salivary glands in exosomes that could modify the salivary miRNA composition. Then, this direct release both in blood and saliva represents an opportunity to identify miRNAs in fluid samples with potential for oral cancer diagnosis. In this sense, another thesis objective (***miRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic Review and Meta-analysis***) was to systematically evaluate the overall diagnostic accuracy of blood and salivary miRNAs for detecting oral squamous cell carcinoma (OSCC). In this work, we performed a comprehensive meta-analysis based on given sensitivity and specificity values of miRNAs detected in liquid biopsies from patients with OSCC. The eligible studies were identified by searches through PubMed EMBASE, Web of Science, LILACS, Scopus, and the Cochrane Library. Study quality was assessed with the Quality Assessment for Studies of Diagnostic Accuracy-2 (QUADAS-2) tool, and sensitivity, specificity, PLR, NLR, dOR, and their corresponding 95% CIs, were calculated using a bivariate random-effect meta-analysis model. Meta-regression and subgroup analyses were performed to assess the heterogeneity. Twenty-five study units from 16 articles with 2,562 subjects were included. The sensitivity and specificity of blood and salivary miRNAs in the diagnosis of OSCC were 0.78 (95% CI, 0.76–0.80) and 0.82 (95% CI, 0.79–0.84),

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respectively, and the pooled PLR and NLR were 4.31 (95% CI, 3.38–5.51) and 0.25 (95% CI, 0.20–0.32), respectively. The overall AUC was 0.91 (95% CI, 0.88–0.93), with a dOR of 21.46 (95% CI, 13.37–34.45). These findings provide more evidence regarding the potential clinical application of blood and salivary miRNAs as a novel, non-invasive, and accurate diagnostic tool for OSCC.

Furthermore, in this thesis, we have also analyzed the potential of saliva and blood for detecting genetic biomarkers in head and neck cancer (HNC). On the one hand, one of the objectives included in this part was the characterization of salivary DNA (***Integrity and Quantity of Salivary Cell-Free DNA as a Potential Molecular Biomarker for Oral Cavity Cancer Diagnosis: A Preliminary Study***). To carry out this purpose we investigated the concentration and integrity of cell-free DNA fragments in saliva (scfDNA) from oral cavity squamous cell carcinoma (OCSCC) patients and healthy individuals in order to explore their value as diagnostic biomarkers. A total of 34 saliva samples from 19 OCSCC patients and 15 healthy controls were analyzed. Total concentration of scfDNA was determined using a fluorometric method and quantitative real-time PCR (qPCR). Thus, to evaluate the scfDNA quantity and integrity, qPCR targeting Arthobacter luteus (*ALU*) sequences at three amplicons of different lengths (60 base pairs (bp), 115 bp, and 247 bp, respectively) was carried out. The scfDNA integrity indexes (ALU115/ALU60 and ALU247/ALU60) were calculated as the ratio between the absolute concentration of the longer amplicons 115 bp and 247 bp and the total scfDNA amount (amplicon 60 bp). Total scfDNA concentration (ALU60) was higher in OCSCC than in healthy donors, but this trend was not statistically significant. However, medians of scfDNA integrity indexes, ALU115/ALU60 and ALU247/ALU60, were significantly higher in OCSCC, showing AUC values to discriminate patients and controls of 0.8211 and 0.7018, respectively. These preliminary results suggest that scfDNA integrity indexes (ALU115/ALU60 and ALU247/ALU60) have potential to identify the presence of OCSCC.

On the other hand, we explored the feasibility to detect tumor somatic mutations in plasma and saliva DNA from head and neck squamous cell carcinoma (HNSCC) patients ***(Somatic Mutations in Tumor and***

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Plasma of Locoregional and/or Metastatic Head and Neck Cancer using a Next-Generation Sequencing Panel). The main objective of this work was to evaluate whether a targeted next-generation sequencing (NGS) panel (TruSight Tumor 170-TST170), previously used for tissue characterization, could be suitable for detecting somatic mutations in both tumor and plasma cell-free DNA (cfDNA) from locoregional recurrent and/or metastatic HNSCC patients. After applying the TST170 panel 24 gene variants were found in tissue, of which 14/24 were detected in plasma cfDNA, showing an overall concordance rate of 58.63%. Importantly, 39.13% of variants (9) identified in plasma were not identified in tumor tissue suggesting that plasma analyses can provide additional information. In addition, a set of somatic mutations previously described in HNSCC were characterized in the tumor, plasma, and saliva DNA by digital droplet PCR (ddPCR). Overall, the results obtained in this study showed the potential clinical utility of the TST170 panel for detecting gene variants in plasma cfDNA from locoregional recurrent and/or metastatic HNC patients and the possibility of monitoring somatic mutations in plasma or saliva by ddPCR.

Finally, the last objectives of this thesis were focused on the DNA methylation in HNC. Since aberrant DNA methylation is considered an early epigenetic event in cancer, the detection of DNA methylation in saliva has emerged as a potential method for the early diagnosis of HNC. In this context, to provide comprehensive evidence about the role of salivary DNA methylation in HNC we developed two meta-analyses addressed both to analyze the potential of salivary DNA methylation as a diagnostic biomarker and the impact of salivary DNA methylation on HNC risk. In the diagnostic meta-analysis ***(Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-Analysis)*** we systematically evaluated the overall diagnostic accuracy of salivary DNA methylation for detecting HNC. To carried out this objective, electronic databases were searched included PubMed EMBASE, Web of Science, LILACS, and the Cochrane Library. Then, we applied the meta-analytic methods previously described in the meta-analysis of diagnostic accuracy of blood and salivary miRNAs in OSCC. A total of 84 study units from 18

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articles involving 4,758 HNC patients and 3,605 healthy individuals were included. The pooled sensitivity and specificity of salivary DNA methylation were 0.39 and 0.87, respectively, while PLR and NLR were 3.68 and 0.63, respectively. The AUC was 0.81 and the dOR was 8.34. Although the pooled results of this study demonstrated a low sensitivity but a high specificity for salivary DNA methylation markers, the diagnostic accuracy measurement indicated a "good" capacity for discriminating HNC. Interestingly, a subgroup analysis based on tumor anatomic location revealed a better diagnostic accuracy of salivary DNA methylation markers in oral cancer (AUC = 0.88) and oropharyngeal cancer (AUC = 0.87). Moreover, the combination of methylated genes showed a greater diagnostic accuracy (AUC, 0.92 and dOR, 36.97) compared to individual gene analysis (AUC, 0.77 and dOR, 6.02). Therefore, these findings provide evidence regarding the potential clinical application of salivary DNA methylation for HNC diagnosis. Besides, with other meta-analysis (***Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-Analysis***) we evaluated the overall and specific impact of salivary gene promoter methylation on HNC risk. The methodological quality was assessed using the Newcastle–Ottawa scale (NOS). ORs and 95% CIs were calculated to evaluate the strength of the association, and Egger's and Begg's tests were applied to detect publication bias. This meta-analysis based on a total of 4,453 patients and 3,233 controls revealed a significant association between salivary DNA promoter hypermethylation and HNC with an OR of 8.34 (95% CI, 6.10–11.39; $p < 0.01$). Interestingly, the subgroup analysis based on tumor anatomic location revealed that this association was higher in oral (OR: 13.07, 95% CI, 8.19–20.88) and oropharyngeal cancer (OR: 13.26, 95% CI, 3.17–5.42). Furthermore, a significant association was found between the methylation of specific tumor-related genes and the risk of HNC development: *p16* (OR: 3.75; 95% CI, 2.51–5.60), *MGMT* (OR: 5.72; 95% CI, 3.00–10.91), *DAPK* (OR: 5.34; 95% CI, 2.18–13.10), *TIMP3* (OR: 3.42; 95% CI, 1.99–5.88), and *RASSF1A* (OR: 7.69; 95% CI, 3.88–15.23). Overall, these findings provide precise evidence on the association between salivary DNA promoter hypermethylation and HNC risk indicating that detection of promoter

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DNA methylation in saliva represents a potential biomarker for predicting HNC risk. Therefore, these studies provide comprehensive evidence regarding the potential clinical application of salivary DNA methylation markers in HNC. Taking this into account, we developed a study whose objective was to characterize the DNA methylome of oral tongue squamous cell carcinoma (OTSCC) and identify novel tumor-specific methylation markers in saliva for early tumor detection ***(Genome-Wide DNA Methylation Profiling of Oral Tongue Squamous Cell Carcinoma)***. For this aim, we performed a genome-wide DNA methylation analysis analyzing over 850,000 cytosine-guanine dinucleotide (CpG) sites on 12 oral tongue tissue samples including 6 OTSCC and 6 adjacent normal tissues. Differential methylation analysis in specific promoter regions including CpG islands (CpGIs) and CpG shores was performed comparing tumor and normal samples. Venn diagrams were used to identify methylation-regulated genes by analyzing gene expression data from The Cancer Genome Atlas (TCGA)-OTSCC samples. Next, a list of differentially hypermethylated genes identified in tumor tissue were compared against methylome data from TCGA-OTSCC. Further, the methylation levels of tumor-specific hypermethylated genes associated with a downregulated expression were evaluated in saliva samples. After bioinformatic preprocessing and analysis, a total of 25,890 CpGs (20,505 hypomethylated and 5,385 hypermethylated) were differentially methylated (DMCpGs) between OTSCC and normal adjacent tissue. At the CpG context, the majority of the hypermethylated CpG sites (48%) were in the CpGIs, while hypomethylated CpG sites (86%) were primarily enriched at the open sea region. By analyzing DMCpGs overlapping promoter region a total of 1,000 tumor-specific DMCpGs (714 hypermethylated and 286 hypomethylated) spanning 807 genes were identified. Based on gene expression TCGA-OTSCC data, 40 hypermethylated genes were linked with a downregulated expression, and 33 hypomethylated genes with an upregulated expression. The methylation pattern of 11 genes (*A2BP1, ACSS3, ALDH1A2, ANK1, CA3, GABRB3, GFRA1, HS3ST1, NDRG2, TTYH1,* and *PDE4B*) that showed specific hypermethylation in at least two CpGs was validated in TCGA-OTSCC methylation data,

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suggesting that these tumor-specific hypermethylated CpGs are frequent alterations in OTSCC and could be early events with an important role in carcinogenesis. Of them, six genes (*A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1,* and *PDE4B*) showed in saliva the same tumor-specific hypermethylated CpG markers. Furthermore, the site-specific CpG markers of these genes analyzed in saliva were able to distinguish OTSCC patients from healthy controls with high AUC, sensitivity, and specificity values. Overall, this is the first and largest genome-wide DNA methylation study on OTSCC samples that identifies a group of novel tumor-specific DNA methylation markers with potential for discriminating OTSCC from healthy individuals. The detection in saliva of these site-specific methylation markers in *A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1,* and *PDE4B* genes could represent a promising strategy for screening and early detection of OTSCC. Further investigations are required to elucidate the biological function of these genes in OTSCC carcinogenesis and their clinical translation.

In summary, the present thesis shows the potential clinical value of different saliva and blood genetic/epigenetic biomarkers in CRC and HNC. Actually, we demonstrated the value of saliva as liquid biopsy both in local and distant tumors to the oral cavity, opening new horizons in the salivary research field. Besides, we focused our work on different types of markers developing a multi-omic approach, including both genetic and epigenetic alterations, to identify non-invasive biomarkers. In fact, the application of multi-omic panels will improve the sensitivity and specificity of biomarkers and have a positive impact to perform more precise oncology in both tumors.

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RESUMEN DE LA TESIS

Esta tesis de doctorado destaca el valor potencial de diferentes biomarcadores analizados en biopsias líquidas, como saliva y sangre, en cáncer colorrectal (CRC, del inglés *colorectal cancer*) y cáncer de cabeza y cuello (HNC, del inglés *head and neck cancer*), lo que supone un paso hacia adelante en la medicina de precisión. Durante la carcinogénesis, los tumores liberan distintos componentes o elementos tumorales (p. ej., células, proteínas, vesículas y ácidos nucleicos) en los fluidos corporales que podrían usarse como biomarcadores para el diagnóstico, el pronóstico y la selección de terapia. Por tanto, las biopsias líquidas constituyen una estrategia novedosa y mínimamente invasiva para la identificación y seguimiento de biomarcadores tumorales que podrían proporcionar información dinámica relevante sobre la evolución molecular del tumor.

Entre los distintos tipos de biopsia líquida, la saliva es considerada como un "espejo del cuerpo" debido a que su composición varía en diferentes estados fisiológicos y patológicos. Además, la saliva presenta numerosas ventajas como medio de diagnóstico porque su recolección es simple, fácil, no invasiva y rentable. Esto la convierte en un biofluido atractivo para la detección, el diagnóstico y el seguimiento de enfermedades como el cáncer. Los continuos avances en el campo de las ómicas salivales, que investigan el proteoma, el transcriptoma, el metaboloma, el genoma, el epigenoma y el microbioma, han permitido la identificación de diferentes clases de moléculas relacionadas con enfermedades malignas como prometedores biomarcadores. En esta línea, numerosos estudios se han centrado en la identificación de diferentes biomarcadores salivales en el cáncer oral, pero también en cánceres distantes a la cavidad oral demostrando el potencial de la saliva para detectar alteraciones moleculares derivadas del tumor. En este contexto, el primer objetivo de la presente tesis (***Salivary Biomarkers for Cancer Diagnosis: A Meta-analysis***) fue analizar la precisión diagnóstica de los biomarcadores salivales para la detección

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de tumores malignos no orales, con el objetivo de definir mejor el valor de la saliva como una biopsia líquida alternativa. Para evaluar sistemáticamente la precisión diagnóstica de los biomarcadores salivales para la detección de tumores no orales, llevamos a cabo una revisión sistemática y un metanálisis. Para ese estudio, se eligieron aquellos estudios que analizaban el potencial de diferentes biomarcadores salivales para el diagnóstico de tumores no orales. Las búsquedas se realizaron en diferentes bases de datos electrónicas, incluidas PubMed, Embase, LILACS y la Biblioteca Cochrane. Para evaluar la precisión global, calculamos el *odds ratio* (OR) de diagnóstico (dOR, del inglés *diagnostic odds ratio*), el área bajo la curva (AUC, del inglés *area under the curve*) de rendimiento diagnóstico (ROC, del inglés *receiver operating characteristic curve*), la sensibilidad, la especificidad, la razón de verosimilitud positiva (PLR, del inglés *positive likelihood ratio*) y la razón de verosimilitud negativa (NLR, del inglés *negative likelihood ratio*), utilizando un método aleatorio o modelo de efectos fijos. Se evaluaron la heterogeneidad y el sesgo de publicación. En este metanálisis se incluyeron 155 unidades de estudio de 29 artículos con 11 153 sujetos. La sensibilidad, especificidad, PLR, NLR, dOR y AUC globales fueron 0,76 (Intervalo de Confianza (IC) del 95%, 0,74–0,77), 0,76 (IC 95%, 0,75–0,77), 3,22 (IC 95%, 2,92–3,55), 0,31 (IC 95%, 0,28–0,34), 13,42 (IC 95%, 12,28–15,96) y 0,85 (IC 95%, 0,84–0,87), respectivamente. Los resultados basados en este enfoque analítico revelaron que los biomarcadores salivales pueden usarse potencialmente para el diagnóstico no invasivo de tumores malignos no orales.

Teniendo en cuenta el valor prometedor de la saliva para detectar el cáncer y sus ventajas como fluido diagnóstico, desarrollamos varios estudios para proporcionar más evidencia sobre la posible utilidad clínica de esta muestra fluida para el manejo del cáncer. En este sentido, el segundo objetivo de esta tesis (***A Novel Saliva-Based miRNA Signature for Colorectal Cancer Diagnosis***) fue caracterizar los microARNs (miRNAs, del inglés *microRNAs*) salivales en CRC con el fin de identificar marcadores no invasivos para el diagnóstico. Para la realización de este estudio diseñamos una fase de descubrimiento y una fase de validación de biomarcadores. En la fase de descubrimiento, se

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realizó el cribado de 754 miRNAs en muestras de saliva de 14 pacientes con CRC y 10 controles sanos. Además, parte de los miRNAs expresados diferenciales fueron validados por la reacción en cadena de la polimerasa con transcripción inversa en tiempo real (RT-qPCR, del inglés *reverse transcription-qPCR*) en 51 pacientes con CRC, 19 pacientes con adenomas y 37 controles sanos. Se realizaron curvas ROC y modelos de regresión logística para analizar el valor clínico de estos miRNAs. Veintidós miRNAs salivales aparecieron alterados de forma estadísticamente significativa en pacientes con CRC vs. individuos sanos ($p < 0.05$) en la fase de descubrimiento. De estos, se confirmó que cinco miRNAs (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p* y *miR-491-5p*) presentaban niveles de expresión significativamente más elevados en pacientes con CRC vs. el grupo sano ($p < 0,05$). Esta firma de cinco miRNAs mostró valor diagnóstico (72% de sensibilidad, 66,67% de especificidad y AUC de 0,754) para detectar el CRC, siendo incluso mayor en combinación con los niveles del antígeno carcinoembrionario (CEA). Después de la primera caracterización global de los miRNAs salivales en el CRC, identificamos un panel de 5 miRNAs como una herramienta prometedora para diagnosticar esta malignidad, lo que representa un enfoque novedoso para detectar alteraciones epigenéticas asociadas al cáncer mediante una estrategia no invasiva.

Aunque no existen modelos animales que demuestren específicamente el origen de los miRNAs salivales en el cáncer, la evidencia científica indica que los exosomas derivados del tumor podrían funcionar como lanzadera entre el tumor remoto y la cavidad oral, permitiendo la identificación de miRNAs asociados a la enfermedad. En comparación con los tumores remotos a la cavidad bucal, en los cánceres orales hay una liberación directa de miRNAs en la saliva debido al contacto directo con las lesiones del cancerosas. Además, los miRNAs también pueden liberarse al torrente sanguíneo como en los cánceres remotos y, por tanto, llegar a las glándulas salivales a través de los exosomas, que podrían modificar la composición de los miRNAs salivales. Por lo tanto, esta liberación directa tanto en sangre como en saliva representa una oportunidad para identificar miRNAs en muestras fluidas con potencial para el diagnóstico del cáncer oral. En este sentido, otro

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objetivo de la tesis (***miRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic Review and Meta-analysis***) fue evaluar sistemáticamente la precisión diagnóstica global de los miRNAs presentes en sangre y saliva para la detección del carcinoma oral de células escamosas (OSCC, del inglés *oral squamous cell carcinoma*). En este trabajo, realizamos un metanálisis completo basado en valores dados de sensibilidad y especificidad de los miRNAs detectados en biopsias líquidas de pacientes con OSCC. Los estudios elegibles fueron identificados mediante búsquedas a través de PubMed EMBASE, Web of Science, LILACS, Scopus y la Biblioteca Cochrane. La calidad de los estudios se evaluó con la herramienta de valoración de la evidencia para estudios de pruebas diagnósticas (QUADAS-2, del inglés *Quality Assessment of Diagnostic Accuracy Studies-2*), y la sensibilidad, especificidad, PLR, NLR, dOR y sus correspondientes IC del 95% se calcularon mediante un modelo de metanálisis bivariado de efectos aleatorios. Se realizaron análisis de meta-regresión y de subgrupos para evaluar la heterogeneidad. Veinticinco unidades de estudio de 16 artículos con 2562 sujetos fueron incluidas. La sensibilidad y la especificidad global de los miRNAs en sangre y saliva fueron de 0,78 (IC 95%, 0,76–0,80) y 0,82 (IC 95%, 0,79–0,84), respectivamente, y la PLR y la NLR fueron de 4,31 (IC 95%, 3,38–5,51) y 0,25 (IC 95%, 0,20–0,32), respectivamente. El AUC global fue de 0,91 (IC 95%, 0,88–0,93), con un dOR de 21,46 (IC 95%, 13,37–34,45). Estos hallazgos proporcionan más evidencia con respecto a la posible aplicación clínica de los miRNAs detectados en sangre y saliva como una herramienta de diagnóstico novedosa, no invasiva y precisa para OSCC.

Además, en esta tesis también hemos analizado el potencial de la saliva y la sangre para detectar biomarcadores genéticos en HNC. Por un lado, uno de los objetivos incluidos en esta parte fue la caracterización del ADN salival (***Integrity and Quantity of Salivary Cell-Free DNA as a Potential Molecular Biomarker for Oral Cavity Cancer Diagnosis: A Preliminary Study***). Para llevar a cabo este propósito, investigamos la concentración e integridad de los fragmentos de ADN libres de las células en saliva (scfDNA, del inglés *salivary cell-free DNA*) de pacientes con carcinoma de células escamosas de cavidad oral

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(OCSCC, del inglés *oral cavity squamous cell carcinoma*) e individuos sanos, con el fin de explorar su valor como biomarcadores de diagnóstico. Se analizaron un total de 34 muestras de saliva de 19 pacientes con OCSCC y 15 controles sanos. La concentración total de scfDNA se determinó mediante un método fluorimétrico y la reacción en cadena de la polimerasa en tiempo real (qPCR, del inglés *quantitative real-time PCR*). Por tanto, para evaluar la cantidad e integridad del scfDNA, se llevó a cabo qPCR dirigida a secuencias de Arthobacter luteus (ALU) con tres amplicones de diferentes longitudes: 60, 115 y 247 pares de bases (pb), respectivamente. Los índices de integridad de scfDNA (ALU115/ALU60 y ALU247/ALU60) se calcularon como la relación entre la concentración absoluta de los amplicones más largos de 115 pb y 247 pb y la cantidad total de scfDNA (amplicón de 60 pb). La concentración total de scfDNA (ALU60) fue mayor en OCSCC que en donantes sanos, pero esta tendencia no fue estadísticamente significativa. Sin embargo, las medianas de los índices de integridad del scfDNA, ALU115/ALU60 y ALU247/ALU60, fueron significativamente más altas en OCSCC, mostrando valores de AUC para discriminar pacientes y controles de 0,8211 y 0,7018, respectivamente. Estos resultados preliminares sugieren que los índices de integridad de scfDNA (ALU115/ALU60 y ALU247/ALU60) tienen potencial para identificar la presencia de OCSCC.

Por otro lado, exploramos la viabilidad de detectar mutaciones somáticas tumorales en el ADN del plasma y la saliva de pacientes con carcinoma de células escamosas de cabeza y cuello (HNSCC, del inglés *head and neck squamous cell carcinoma*) (***Somatic Mutations in Tumor and Plasma of Locoregional and/or Metastatic Head and Neck Cancer using a Next-Generation Sequencing Panel***). El objetivo principal de este trabajo fue evaluar si un panel de secuenciación dirigido (TruSight Tumor 170-TST170), previamente utilizado para la caracterización de tejidos, podría ser adecuado para detectar mutaciones somáticas tanto en el ADN libre de células (cfDNA) plasmático como en el tumor de pacientes con HNSCC locorregional recurrente y/o metastásico. Después de aplicar el panel TST170, se encontraron 24 variantes genéticas en tejido, de las cuales 14/24 se detectaron en cfDNA plasmático, mostrando una tasa de concordancia global del

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58,63%. Es importante destacar que el 39,13% de las variantes (9) identificadas en el plasma no se identificaron en el tejido tumoral, lo que sugiere que los análisis de plasma puedeN proporcionar información adicional. Además, un conjunto de mutaciones somáticas descritas previamente en HNSCC fueron caracterizadas en ADN de tumor, plasma y saliva mediante la tecnología de PCR digital de gotas (ddPCR, del inglés *droplet digital PCR*). En general, los resultados obtenidos en este estudio mostraron la potencial utilidad clínica del panel TST170 para detectar variantes genéticas en cfDNA plasmático de pacientes con HNSCC locorregional recurrente y/o metastásico y la posibilidad de monitorizar mutaciones somáticas en plasma o saliva mediante ddPCR.

Finalmente, los últimos objetivos de esta tesis se centraron en la metilación del ADN en HNC. Dado que la metilación aberrante del ADN se considera un evento epigenético temprano en el cáncer, la detección de la metilación del ADN en la saliva ha surgido como un método potencial para el diagnóstico temprano del HNC. En este contexto, para proporcionar evidencia sobre el papel de la metilación del ADN salival en el HNC, desarrollamos dos metanálisis dirigidos tanto a analizar el potencial de la metilación del ADN salival como un biomarcador de diagnóstico como el impacto de la metilación del ADN salival en el riesgo de HNC. En el metanálisis de diagnóstico (***Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-Analysis***) evaluamos sistemáticamente la precisión diagnóstica global de la metilación del ADN salival para detectar HNC. Para llevar a cabo este objetivo, se realizaron búsquedas bibliográficas en diferentes bases de datos electrónicas incluyendo PubMed EMBASE, Web of Science, LILACS, y la Biblioteca Cochrane. Luego, se aplicaron los métodos analíticos descritos previamente en el metanálisis de precisión diagnóstica de los miRNAs identificados en sangre y saliva en OCSCC. Se incluyeron un total de 84 unidades de estudio de 18 artículos en los que participaron 4758 pacientes con HNC y 3605 individuos sanos. La sensibilidad y la especificidad globales de la metilación del ADN salival fueron 0,39 y 0,87, respectivamente, mientras que PLR y NLR fueron 3,68 y 0,63, respectivamente. El AUC fue de 0,81 y el dOR fue de 8,34. Aunque los

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resultados globales de este estudio demostraron una baja sensibilidad, pero una alta especificidad para los marcadores de metilación del ADN salival, la medición de la precisión del diagnóstico indicó una "buena" capacidad para discriminar HNC. Interesantemente, el análisis de subgrupos basado en la localización anatómica del tumor reveló una mejor precisión diagnóstica de los marcadores de metilación del ADN salival en el cáncer oral (AUC, 0,88) y el cáncer de orofaringe (AUC, 0,87). Además, la combinación de genes metilados mostró una mayor precisión diagnóstica (AUC, 0,92 y dOR, 36,97) en comparación con el análisis de genes individuales (AUC, 0,77 y dOR, 6,02). Por lo tanto, estos hallazgos proporcionan evidencia con respecto a la posible aplicación clínica del análisis de metilación en ADN salival para el diagnóstico de HNC. Además, con otro metanálisis (***Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-Analysis***) evaluamos el impacto global y específico de la metilación en ADN salival de la región promotora del gen sobre el riesgo de HNC. La calidad metodológica se evaluó mediante la escala de Newcastle-Ottawa (NOS). Se calcularon los OR y los IC del 95% para evaluar la fuerza de la asociación, y se aplicaron las pruebas de Egger y Begg para detectar el sesgo de publicación. Este metanálisis basado en un total de 4453 pacientes y 3233 controles reveló una asociación significativa entre la hipermetilación en la región promotora del gen en ADN salival y el HNC con un OR de 8,34 (IC 95%, 6,10–11,39; $p < 0.01$). De nuevo, el análisis de subgrupos basado en la localización anatómica del tumor reveló que esta asociación era mayor en el cáncer oral (OR: 13,07, IC 95%, 8,19–20,88) y orofaríngeo (OR: 13,26, IC 95%, 3,17–5,42). Además, se encontró una asociación significativa entre el análisis de metilación de genes específicos relacionados con el tumor en ADN salival y el riesgo de desarrollo de HNC: *p16* (OR: 3,75; IC 95%, 2,51–5,60), *MGMT* (OR: 5,72; IC 95%, 3,00–10,91), *DAPK* (OR: 5,34; IC 95%, 2,18–13,10), *TIMP3* (OR: 3,42; IC 95%, 1,99–5,88) y *RASSF1A* (OR: 7,69; IC 95%, 3,88–15,23). En general, estos hallazgos proporcionan evidencia precisa sobre la asociación de la hipermetilación en la región promotora de los genes en ADN salival y el riesgo de HNC, lo que indica que la detección de la metilación en la región promotora del gen en ADN salival representa una

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potencial estrategia para predecir el riesgo de HNC. Por lo tanto, estos estudios proporcionan una mayor evidencia con respecto a la posible aplicación clínica de los marcadores de metilación en ADN salival en el HNC. Teniendo esto en cuenta, desarrollamos un estudio cuyo objetivo fue caracterizar el metiloma del ADN del carcinoma oral de lengua de células escamosas (OTSCC, del inglés *oral tongue squamous cell carcinoma*) e identificar nuevos marcadores de metilación específicos del tumor en la saliva para la detección temprana del mismo (***Genome-Wide DNA Methylation Profiling of Oral Tongue Squamous Cell Carcinoma***). Para este objetivo, realizamos un análisis de metilación del ADN de todo el genoma analizando más de 850 000 sitios de dinucleótidos citosina-guanina (CpG, del inglés *cytosine-guanine dinucleotide*) en 12 muestras de tejido de lengua, incluidos 6 OTSCC y 6 tejidos normales adyacentes. Se realizó un análisis de metilación diferencial en regiones promotoras específicas, incluidas las islas CpG (CpGIs, del inglés *CpG islands*) y CpG *shores* comparando muestras tumorales y normales. Se utilizaron diagramas de Venn para identificar genes regulados por metilación mediante el análisis de datos de expresión génica de muestras de OTSCC procedentes del Atlas del Genoma del Cáncer (TCGA, del inglés *The Cancer Genome Atlas*). A continuación, se comparó una lista de genes hipermetilados diferencialmente en el tejido tumoral con datos de metiloma de muestras de OTSCC del TCGA. Además, los niveles de metilación de un grupo de genes hipermetilados específicos del tumor y asociados con una regulación negativa de expresión fueron evaluados en muestras de saliva. Después del preprocesamiento y análisis bioinformático, se identificaron un total de 25 890 CpGs diferencialmente metiladas (DMCpGs, del inglés *differentially methylated CpGs*) (20 505 hipometilados y 5385 hipermetilados) entre OTSCC y el tejido adyacente normal. En el contexto de las CpGs, la mayoría de los sitios CpG hipermetilados (48%) estaban en las CpGIs, mientras que los sitios CpG hipometilados (86%) se enriquecieron principalmente en la región de *open sea*. Mediante el análisis de las DMCpGs que se superponen a la región promotora, se identificaron un total de 1000 DMCpGs específicas de tumor (714 hipermetiladas y 286 hipometiladas) que correspondían a 807 genes. Según los datos de expresión génica de las muestras de OTSCC del

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TCGA, 40 genes hipermetilados fueron asociados con una regulación negativa de expresión y 33 genes hipometilados con una regulación positiva de expresión. El patrón de metilación de 11 genes (*A2BP1, ACSS3, ALDH1A2, ANK1, CA3, GABRB3, GFRA1, HS3ST1, NDRG2, TTYH1* y *PDE4B*) que mostraban hipermetilación específica en al menos dos sitios de CpGs fue validado en los datos de metilación de OTSCC del TCGA, lo que sugiere que estas CpGs hipermetiladas específicas del tumor son alteraciones frecuentes en OTSCC y podrían ser eventos tempranos con un papel importante en la carcinogénesis. De estos, seis genes (*A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1* y *PDE4B*) presentaron en la saliva los mismos marcadores CpG hipermetilados específicos de tumor. Además, los marcadores CpG específicos de sitio de estos genes analizados en la saliva permitieron distinguir a los pacientes con OTSCC de los controles sanos con valores altos de AUC, sensibilidad y especificidad. En general, este es el primer y más grande estudio de metilación de ADN de todo el genoma en muestras de OTSCC que identifica un grupo de nuevos marcadores de metilación de ADN específicos de tumor con potencial para discriminar OTSCC de individuos sanos. La detección en la saliva de estos marcadores de metilación específicos de sitio en los genes *A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1* y *PDE4B* podría representar una estrategia prometedora para el cribado y la detección temprana de OTSCC. No obstante, se requieren más investigaciones para dilucidar la función biológica de estos genes en la carcinogénesis de OTSCC y su traslación clínica.

En resumen, la presente tesis muestra el valor clínico potencial de diferentes biomarcadores genéticos/epigenéticos detectados en saliva y sangre en CRC y HNC. De hecho, demostramos el valor de la saliva como biopsia líquida tanto en tumores locales como distantes a la cavidad oral, abriendo nuevos horizontes en el campo de la investigación salival. Además, enfocamos nuestro trabajo en diferentes tipos de marcadores desarrollando un enfoque multi-ómico, incluyendo alteraciones tanto genéticas como epigenéticas, para identificar biomarcadores no invasivos en CRC y HNC. De hecho, la aplicación de paneles multi-ómicos permitirá mejorar la sensibilidad y la especificidad de los biomarcadores y tendrá un impacto positivo en el desarrollo de una oncología más precisa en ambos tumores.

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RESUMO DA TESE

Esta tese doutoral destaca o valor potencial de diferentes biomarcadores analizados en biopsias líquidas, como a saliva e o sangue, no cancro colorrectal (CRC, do inglés *colorectal cancer*) e no cancro de cabeza e colo (HNC, do inglés *head and neck cancer*), o que supón un paso adiante na medicina de precisión. Durante a carcinoxénese, os tumores liberan distintos compoñentes ou elementos tumorais (por ex., células, proteínas, vesículas e ácidos nucleicos) nos fluídos corporais que poderían usarse como biomarcadores para o diagnóstico, o prognóstico e a selección de terapia. Por tanto, as biopsias líquidas constitúen unha estratexia novidosa e minimamente invasora para a identificación e seguimento de biomarcadores tumorais que poderían proporcionar información dinámica relevante sobre a evolución molecular do tumor.

Entre os distintos tipos de biopsia líquida, a saliva considérase un "espello do corpo" porque a súa composición varía en diferentes estados fisiolóxicos e patolóxicos. Ademais, a saliva ten numerosas vantaxes como ferramenta de diagnóstico porque a súa recollida é sinxela, non invasora e custo-efectiva. Todo isto fai que sexa un biofluído atractivo para a detección, o diagnóstico e o seguimento de enfermidades como o cancro. Os continuos avances no campo das ómicas da saliva, que investigan o proteoma, o transcriptoma, o metaboloma, o xenoma, o epixenoma e o microbioma, permitiron a identificación de diferentes clases de moléculas relacionadas con enfermidades malignas como prometedores biomarcadores. Nesta liña, numerosos estudos centráronse na identificación de diferentes biomarcadores salivares no cancro oral pero tamén en cancros distantes á cavidade bucal, demostrando o potencial da saliva para detectar alteracións moleculares derivadas do tumor. Neste contexto, o primeiro obxectivo desta tese ***(Salivary Biomarkers for Cancer Diagnostic: A Meta-analysis)*** foi analizar a precisión diagnóstica dos biomarcadores salivares para a detección de tumores malignos non orais, co obxectivo

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de definir mellor o valor da saliva como unha biopsia líquida alternativa. Para avaliar de forma sistemática a precisión diagnóstica dos biomarcadores salivares para a detección de tumores non orais, realizamos unha revisión sistemática e unha metanálise. Para levar a cabo este obxectivo, elixíronse aqueles estudos que analizaron o potencial de diferentes biomarcadores salivares para o diagnóstico de tumores non orais. Realizáronse buscas en diferentes bases de datos electrónicas, incluíndo PubMed, Embase, LILACS e a Biblioteca Cochrane. Para avaliar a precisión global, calculamos o *odds ratio* (OR) de diagnóstico (dOR, do inglés *diagnostic odds ratio*), a área baixo a curva (AUC, do inglés *area under the curve*) de rendemento diagnóstico (ROC, do inglés *receiver operating characteristic*), a sensibilidade, a especificidade, a razón de verosimilitude positiva (PLR, do inglés *positive likelihood ratio*) e a razón de verosimilitude negativa (NLR, do inglés *negative likelihood ratio*), utilizando un método aleatorio ou un modelo de efectos fixos. Ademais, valorouse a heteroxeneidade e o sesgo de publicación. Nesta metanálise incluíronse 155 unidades de estudo de 29 artigos con 11 153 suxeitos. A sensibilidade global, a especificidade, a PLR, a NLR, o dOR e o AUC foron de 0,76 (Intervalos de Confianza (IC) do 95 %, 0,74–0,77), 0,76 (IC 95 %, 0,75–0,77), 3,22 (IC 95 %, 2,92–3,55), 0,31 (IC 95%, 0,28–0,34), 13,42 (IC 95%, 12,28–15,96) e 0,85 (IC 95%, 0,84–0,87), respectivamente. Os resultados baseados neste enfoque analítico revelaron que os biomarcadores salivares poden utilizarse potencialmente para o diagnóstico non invasor de tumores malignos non orais.

Tendo en conta o valor prometedor da saliva na detección do cancro e as súas vantaxes como fluído diagnóstico, desenvolvemos varios estudos para proporcionar máis evidencias sobre a posible utilidade clínica desta mostra fluída para o manexo do cancro. Neste sentido, o segundo obxectivo desta tese ***(A Novel Saliva-Based miRNA Signature for Colorrectal Cancer Diagnosis)*** foi caracterizar os microARNs (miRNAs, do inglés *microRNAs*) salivares en CRC coa finalidade de identificar marcadores non invasores para o diagnóstico. Para levar a cabo este estudo, deseñamos unha fase de descubrimento e unha fase de validación de biomarcadores. Na fase de descubrimento, realizouse o

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cribado de 754 miRNAs en mostras de saliva de 14 pacientes con CRC e 10 controis sans. Ademais, parte dos miRNAs expresados diferencialmente validáronse mediante a reacción en cadea da polimerasa con transcrición inversa en tempo real (RT-qPCR, do inglés *reverse transcription-qPCR*) en 51 pacientes con CRC, 19 pacientes con adenomas e 37 controis sans. Ademais, realizáronse as curvas ROC e modelos de regresión loxística para analizar o valor clínico destes miRNAs. Un total de vinte e dous miRNAs salivares foron desregulados significativamente en pacientes con CRC vs. individuos sans ($p < 0,05$) na fase de descubrimento. Deles, cinco miRNAs (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p* e *miR-491-5p*) foron validados mostrando niveis de expresión significativamente máis altos en pacientes con CRC en comparación co grupo san ($p < 0,05$). Esta sinatura de cinco miRNAs mostrou valor diagnóstico (72% de sensibilidade, 66,67% de especificidade, e AUC de 0,754) para detectar CRC, sendo incluso maior en combinación cos niveis do antíxeno carcinoembrionario (CEA). Tras a primeira caracterización global dos miRNAs salivares en CRC, identificamos un panel de 5 miRNAs como unha ferramenta prometedora para diagnosticar este tumor, o que supón un novo enfoque para detectar alteracións epixenéticas asociadas ao cancro mediante unha estratexia non invasora.

Aínda que non existen modelos animais que demostren especificamente a orixe dos miRNAs salivares no cancro, a evidencia científica indica que os exosomas derivados do tumor poderían funcionar como una lanzadeira entre o tumor remoto e a cavidade oral, permitindo identificar miRNAs asociados á enfermidade. En comparación cos tumores remotos á cavidade oral, nos cancros orais hai unha liberación directa de miRNAs na saliva debido ao contacto directo da saliva coas lesións cancerosas. Ademais, os miRNAs tamén poden ser liberados ao torrente sanguíneo como nos cancros remotos e, así, chegar ás glándulas salivares a través de exosomas, que poderían modificar a composición dos miRNAs salivares. Polo tanto, esta liberación directa tanto no sangue como na saliva representa unha oportunidade para identificar miRNAs en mostras fluídas con potencial para o diagnóstico de cancro oral. Neste sentido, outro dos obxectivos da tese ***(miRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic***

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Review and Meta-analysis) foi avaliar de forma sistemática a precisión diagnóstica global dos miRNAs presentes en sangue e saliva para a detección do carcinoma oral de células escamosas (OSCC, do inglés *oral squamous cell carcinoma*). Neste traballo, realizamos unha metanálise completa baseada nos valores dados de sensibilidade e especificidade dos miRNAs detectados en biopsias líquidas de pacientes con OSCC. A identificación dos estudos elixidos realizouse mediante a busca en diferentes bases de datos electrónicas incluíndo PubMed, EMBASE, Web of Science, LILACS, Scopus e a Biblioteca Cochrane. Para analizar a calidade dos estudos empregouse a ferramenta de avaliación da evidencia para estudos de probas diagnósticas (QUADAS-2, do inglés *Quality Assessment of Diagnostic Accuracy Studies-2*), e a sensibilidade, a especificidade, a PLR, a NLR, o dOR e o seus correspondentes IC do 95% foron calculados mediante un modelo de metanálise bivariada de efectos aleatorios. Tamén se realizaron análises de subgrupos e metaregresión para avaliar a heteroxeneidade. Un total de vinte e cinco unidades de estudo pertencentes a 16 artigos con 2562 individuos foron incluídas na análise. A sensibilidade e a especificidade global dos miRNAs en sangue e saliva foron de 0,78 (IC 95%, 0,76–0,80) e 0,82 (IC 95%, 0,79–0,84), respectivamente, e a PLR e a NLR foron de 4,31 (IC 95%, 3,38–5,51) e 0,25 (IC 95%, 0,20–0,32), respectivamente. O AUC global foi de 0,91 (IC 95%, 0,88–0,93), e o dOR foi de 21,46 (IC 95%, 13,37–34,45). Estes achados proporcionan máis evidencia sobre a potencial aplicación clínica dos miRNAs detectados en sangue e saliva como unha ferramenta de diagnóstico novidosa, non invasora e precisa para o OSCC.

Ademais, nesta tese tamén se analizou o potencial da saliva e do sangue para detectar biomarcadores xenéticos no HNC. Por unha banda, un dos obxectivos incluídos nesta parte foi a caracterización do ADN salival ***(Integrity and Quantity of Salivary Cell-Free DNA as a Potential Molecular Biomarker for Oral Cavity Cancer Diagnostic: A Preliminary Study)***. Para lograr este propósito, investigamos a concentración e a integridade dos fragmentos de ADN libre de células na saliva (scfDNA, do inglés *salivary cell-free DNA*) en pacientes con carcinoma de células escamosas da cavidade oral (OCSCC, do inglés

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oral cavity squamous cell carcinoma) e en individuos sans, para explorar o seu valor como biomarcadores diagnósticos. Analizáronse un total de 34 mostras de saliva de 19 pacientes con OCSCC e 15 controis sans. A concentración total de scfDNA determinouse mediante un método fluorimétrico e a reacción en cadea da polimerasa en tempo real (qPCR, do inglés *real-time quantitative PCR*) Polo tanto, para avaliar a cantidade e integridade do scfDNA, realizáronse qPCRs dirixidas ás secuencias de *Arthobacter luteus* (*ALU*) con tres amplicóns de diferentes lonxitudes: 60, 115 e 247 pares de bases (pb), respectivamente. Os índices de integridade do scfDNA (ALU115/ALU60 e ALU247/ALU60) calculáronse como a relación entre a concentración absoluta dos amplicóns máis longos de 115 pb e 247 pb e a cantidade total de scfDNA (amplicón de 60 pb). A concentración total de scfDNA (ALU60) foi maior en OCSCC que en doantes sans, pero esta tendencia non foi estatisticamente significativa. Non obstante, as medianas dos índices de integridade do scfDNA, ALU115/ALU60 e ALU247/ALU60, foron significativamente máis altas en OCSCC, mostrando valores de AUC para discriminar pacientes e controis de 0,8211 e 0,7018, respectivamente. Estes resultados preliminares suxiren que os índices de integridade do scfDNA (ALU115/ALU60 e ALU247/ALU60) teñen potencial para identificar a presenza de OCSCC.

Por outra banda, exploramos a viabilidade de detectar mutacións somáticas tumorais no ADN de plasma e saliva de pacientes con carcinoma de células escamosas de cabeza e colo (HNSCC, do inglés *head and neck squampous cell carcinoma*) ***(Somatic Mutations in Tumor and Plasma of Locoregional and/or Metastatic Head and Neck Cancer using a Next-Generation Sequencing Panel)***. O obxectivo principal deste traballo foi avaliar se un panel de secuenciación dirixido (TruSight Tumor 170-TST170), utilizado anteriormente para a caracterización de tecido, podería ser adecuado para detectar mutacións somáticas tanto no ADN libre de células (cfDNA) plasmático como no tumor de pacientes con HNSCC locorrexional recorrente e/ou metastásico. Despois de aplicar o panel TST170, atopáronse 24 variantes xenéticas no tecido tumoral, das cales 14/24 foron detectadas no cfDNA plasmático, mostrando unha taxa de

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concordancia global do 58,63%. É importante destacar que o 39,13% das variantes (9) identificadas no plasma non foron identificadas no tecido tumoral, o que suxire que a análise do plasma pode proporcionar información adicional. Ademais, un conxunto de mutacións somáticas anteriormente descritas no HNSCC foron caracterizadas no ADN tumoral, no plasma e na saliva mediante a PCR dixital en gotas (ddPCR, do inglés *digital droplet PCR*). En xeral, os resultados obtidos neste estudo mostraron a potencial utilidade clínica do panel TST170 para detectar variantes xenéticas no cfDNA plasmático de pacientes con HNSCC locorrexional recorrente e/ou metastásico e a posibilidade de monitorizar as mutacións somáticas en plasma ou saliva mediante ddPCR.

Finalmente, os últimos obxectivos desta tese centráronse na metilación do ADN no HNC. Dado que a metilación aberrante do ADN considérase un evento epixenético precoz no cancro, a detección da metilación do ADN na saliva xurdiu como un método potencial para o diagnóstico precoz do HNC. Neste contexto, para proporcionar máis evidencia sobre o papel da metilación en ADN salival no HNC, desenvolvemos dúas metanálises destinadas tanto a analizar o potencial da metilación do ADN salival como biomarcador diagnóstico como o impacto da metilación do ADN salival no risco de HNC. Na metanálise diagnóstica ***(Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-Analysis)*** avaliamos sistematicamente a precisión diagnóstica global da metilación do ADN salival para detectar HNC. Para poder realizar este obxectivo, realizáronse buscas bibliográficas en diferentes bases de datos electrónicas incluíndo PubMed, EMBASE, Web of Science, LILACS, e a Biblioteca Cochrane. Logo, aplicáronse os métodos analíticos descritos anteriormente na metanálise de precisión diagnóstica dos miRNAs identificados en sangue e saliva en OSCC. Incluíronse un total de 84 unidades de estudo de 18 artigos nos que participaron 4758 pacientes con HNC e 3605 individuos sans. A sensibilidade e a especificidade global da metilación do ADN salival foron 0,39 e 0,87, respectivamente, mentres que a PLR e a NLR foron 3,68 e 0,63, respectivamente. O AUC foi de 0,81 e o dOR de 8,34. Aínda que os resultados xerais deste estudo demostraron unha baixa

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sensibilidade, pero unha alta especificidade para os marcadores de metilación do ADN salival, a medición da precisión diagnóstica indicaba unha "boa" capacidade para discriminar HNC. Interesantemente, a análise de subgrupos baseada na localización anatómica do tumor revelou unha mellor precisión diagnóstica para os marcadores de metilación do ADN salival no cancro oral (AUC, 0,88) e no cancro orofarínxeo (AUC, 0,87). Ademais, a combinación de xenes metilados mostrou unha maior precisión diagnóstica (AUC, 0,92 e dOR, 36,97) en comparación coa análise de xenes individuais (AUC, 0,77 e dOR, 6,02). Polo tanto, estes achados proporcionan máis evidencia sobre a posible aplicación clínica da análise de metilación do ADN salival para o diagnóstico de HNC. Ademais, con outra metanálise ***(Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-Analysis)*** avaliamos o impacto global e específico da metilación do ADN salival na rexión promotora do xen sobre o risco de HNC. A calidade metodolóxica dos estudos foi avaliada mediante a escala de Newcastle-Ottawa (NOS). Calculáronse os OR e os IC do 95% para avaliar a forza da asociación e aplicáronse as probas de Egger e Begg para detectar o sesgo de publicación. Esta metanálise baseada nun total de 4453 pacientes e 3233 controis revelou unha asociación significativa entre a hipermetilación na rexión promotora do xen no ADN salival e o HNC cun OR de 8,34 (IC 95%, 6,10–11,39; $p < 0,01$). De novo, a análise de subgrupos baseada na localización anatómica do tumor revelou que esta asociación era maior no cancro oral (OR: 13,07, IC 95%, 8,19–20,88) e orofarínxeo (OR: 13,26, IC 95%, 3,17–5.42). Ademais, atopouse unha asociación significativa entre a análise da metilación de xenes específicos relacionados co tumor no ADN salival e o risco de desenvolver HNC: *p16* (OR: 3,75; IC 95%, 2,51–5,60), *MGMT* (OR: 5,72; 95% IC, 3,00–10,91), *DAPK* (OR: 5,34; 95% IC, 2,18–13,10), TIMP3 (OR: 3,42; 95% IC, 1,99–5,88) e *RASSF1A* (OR: 7,69; 95% IC, 3,28–1,88). En xeral, estes achados proporcionan probas precisas sobre a asociación da hipermetilación na rexión promotora dos xenes no ADN salival e o risco de HNC, o que indica que a detección da metilación na rexión promotora do xene no ADN salival representa unha estratexia potencial para predicir o risco de HNC. Polo tanto, estes estudos

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proporcionan máis fundamentos sobre a posible aplicación clínica de marcadores de metilación do ADN salival no HNC. Tendo isto en conta, desenvolvemos un estudo cuxo obxectivo foi caracterizar o metiloma do ADN do carcinoma oral de células escamosas da lingua (OTSCC, do inglés *oral tongue squamous cell carcinoma*) e identificar novos marcadores de metilación do ADN específicos do tumor na saliva para a detección precoz do mesmo ***(Genome-Wide DNA Methylation Profiling of Oral Tongue Squamous Cell Carcinoma)***. Para este fin, realizamos unha análise de metilación do ADN en todo o xenoma analizando máis de 850 000 sitios de dinucleótidos citosina-guanina (CpG, do inglés *cytosine-guanine dinucleotide*) en 12 mostras de tecido da lingua, incluíndo 6 OTSCC e 6 tecidos normais adxacentes. Ademais, realizouse a análise de metilación diferencial en rexións promotoras específicas, incluíndo islas CpG (CpGIs, do inglés *CpG islands*) e CpG *shores*, comparando mostras de tecido tumoral e tecido adxacente normal. O estado de metilación dos xenes diferencialmente metilados na rexión promotora foron relacionados cos datos de expresión xénica de mostras de OTSCC do Atlas do Xenoma do Cáncer (TCGA, do inglés *The Cancer Genome Atlas*) mediante a aplicación de diagramas de Venn. Despois, comparouse unha lista de xenes hipermetilados diferencialmente no tecido tumoral cos datos de metiloma de mostras de OTSCC do TCGA. Ademais, os niveis de metilación dun grupo de xenes hipermetilados específicos do tumor e asociados cunha regulación negativa da expresión foron avaliados en mostras de saliva. Tras o preprocesamento e a análise bioinformática, identificáronse un total de 25 890 CpGs metiladas diferencialmente (DMCpGs, do inglés *differentially methylated CpGs*) (20 505 hipometilados e 5385 hipermetilados) entre o OTSCC e o tecido normal adxacente. No contexto das CpGs, a maioría dos sitios CpG hipermetilados (48%) estaban nas CpGIs, mentres que os sitios CpG hipometilados (86%) estaban enriquecidos principalmente na rexión de *open sea*. Ao analizar as DMCpGs que se solapan coa rexión promotora, identificáronse un total de 1000 DMCpGs específicos de tumor (714 hipermetilados e 286 hipometilados) correspondentes a 807 xenes. Segundo os datos de expresión xénica das mostras de OTSCC do TCGA 40 xenes hipermetilados foron asociados cunha regulación

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negativa da expresión e 33 xenes hipometilados cunha regulación positiva da expresión. O patrón de metilación de 11 xenes (*A2BP1, ACSS3, ALDH1A2, ANK1, CA3, GABRB3, GFRA1, HS3ST1, NDRG2, TTYH1* e *PDE4B*) que mostraron hipermetilación específica en polo menos dous sitios de CpGs validouse nos datos de metilación de OTSCC do TCGA, o que suxire que estas CpG hipermetiladas específicas do tumor son alteracións frecuentes no OTSCC e poderían ser eventos precoces cun papel importante na carcinoxénese. Deles, seis xenes (*A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1* e *PDE4B*) mostraron en saliva os mesmos marcadores tumorales hipermetilados específicos de sitio CpG. Ademais, os marcadores específicos de sitio CpG destes xenes analizados na saliva permitiron distinguir os pacientes con OTSCC dos controis sans con altos valores de AUC, de sensibilidade e de especificidade. En xeral, este é o primeiro e máis grande estudo de metilación do ADN de todo o xenoma en mostras de OTSCC que identifica un grupo de novos marcadores de metilación de ADN específicos do tumor con potencial para discriminar OTSCC dos individuos sans. A detección na saliva destes marcadores de metilación específicos do sitio nos xenes *A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1* e *PDE4B* podería representar unha estratexia prometedora para o cribado e a detección precoz do OTSCC. Non obstante, requírese máis investigación para dilucidar o papel biolóxico destes xenes na carcinoxénese do OTSCC e a súa traslación clínica.

En resumo, esta tese mostra o valor clínico potencial de diferentes biomarcadores xenéticos/epixenéticos detectados en saliva e sangue en CRC e HNC. De feito, demostramos o valor da saliva como biopsia líquida en tumores tanto locais como distantes á cavidade bucal, abrindo novos horizontes no campo da investigación salival. Ademais, centramos o noso traballo en diferentes tipos de marcadores desenvolvendo un enfoque multi-ómico, incluíndo alteracións xenéticas e epixenéticas, para identificar biomarcadores non invasores en CRC e HNC. De feito, a aplicación de paneles multi-ómicos mellorará a sensibilidade e a especificidade dos biomarcadores e terá un impacto positivo no desenrolo dunha oncoloxía más precisa en ambos tumores.

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PUBLISHED AND ACCEPTED

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   Ranking: Q1, D1; 7/92 (Category: Dentistry, Oral Surgery & Medicine)

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PENDING ACCEPTANCE

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and Plasma of Locoregional Recurrent and/or Metastatic Head and Neck Cancer Using a Next-Generation Sequencing Panel.

3. Rapado-González Ó, Costa-Fraga N, Bao-Caamano A, López-Cedrún JL, Álvarez-Rodríguez R, Muinelo-Romay L, López-López R, Díaz-Lagares A, Suárez-Cunqueiro MM. **Epigenome-Wide DNA Methylation Profiling in Oral Tongue Squamous Cell Carcinoma.**

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INTRODUCTION

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INTRODUCTION

Section 1.1. has been adapted/extracted from a published review entitled *"Liquid Biopsy in Oral Cancer".*

Section 1.1.2.1. has been adapted/extracted from a published review entitled *"Human Salivary microRNAs in Cancer".*

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Section 1.2.2. has been adapted/extracted from a published review entitled *"Cancer Salivary Biomarkers for Tumors Distant to the Oral Cavity"***.**

Section 1.2.3., 1.2.4., and 2.3.1.4. has been adapted/extracted from a published review entitled *"Human Salivary microRNAs in Cancer".*

Sections 2.1. and 2.3. have been adapted/extracted from a published review entitled *"Circulating microRNAs as Promising Biomarkers in Colorectal Cancer"*.

Section 3.3. have been adapted/extracted from a published review entitled *"Cell-Free microRNAs as Potential Oral Cancer Biomarkers: From Diagnosis to Therapy".*

Section 3.5. have been adapted/extracted from a published review entitled *"Saliva Gene Promoter Hypermethylation as a Biomarker in Oral Cancer".*

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1. LIQUID BIOPSIES

Cancer screening and early detection using non-invasive strategies have emerged as an important paradigm shift in precision medicine. Tissue biopsies are currently the *"gold standard"* for tumor detection and diagnosis, however, this invasive procedure is limited by the difficulties in access to tumor material that is not always feasible and high procedural costs [1]. Furthermore, tissues biopsies are temporally and spatially limited and often provide a brief "*snapshot*" of a single region of a heterogeneous tumor [2]. In fact, the molecular profile of cancer is highly dynamic during tumor evolution and treatment, but tissue biopsy cannot be generally used to provide dynamic information of the tumor genomic landscape [3,4]. To overcome these limitations liquid biopsies have emerged as a favorable alternative to traditional solid biopsy, providing an innovative tool in the field of precision oncology since they are minimally invasive and sampling can be repeated at multiple time points facilitating the monitoring of the disease [5,6]. Besides, one of the key advantages of studying liquid biopsies is that they can provide a personalized "*snapshot"* of both primary and metastatic tumors at successive time points, providing comprehensive and dynamic knowledge about the tumor evolution and helping clinicians in their therapeutic decision-making [6].

The term *"liquid biopsy"* was coined by Pantel & Panabiéres in 2010 for the analysis of circulating tumor cells (CTCs) in blood from cancer patients [7]. Nowadays, the term is widely used in oncology to refer to the sampling and analysis of tumor-derived material from different liquid biological sources, mainly blood, but also other body fluids such as saliva, urine, cerebrospinal fluid, ascites, or pleural effusions [4]. Several tumor-specific analytes can be detected in liquid biopsies comprising CTCs, circulating tumor DNA (ctDNA), circulating cell-free RNA (ccfRNA), extracellular vesicles (EVs), tumor-educated platelets, proteins, and metabolites [5], among others. Of note, the

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development of "*omics*" technologies has allowed the application of liquid biopsies for exploring the genome, the epigenome, the transcriptome, the proteome, and the metabolome, providing molecular knowledge about the features of the primary tumor and/or metastasis. In this context, liquid biopsies approaches have shown promising clinical applications in precision oncology including early cancer detection, prognostication, detection of minimal residual disease, real-time monitoring of treatment response, and the identification of resistance mechanisms [6].

1.1. BLOOD-BASED LIQUID BIOPSY

CTCs, ctDNA, ccfRNA, and EVs are the main circulating tumor-derived components that constitute the tumor circulome (Figure 1).

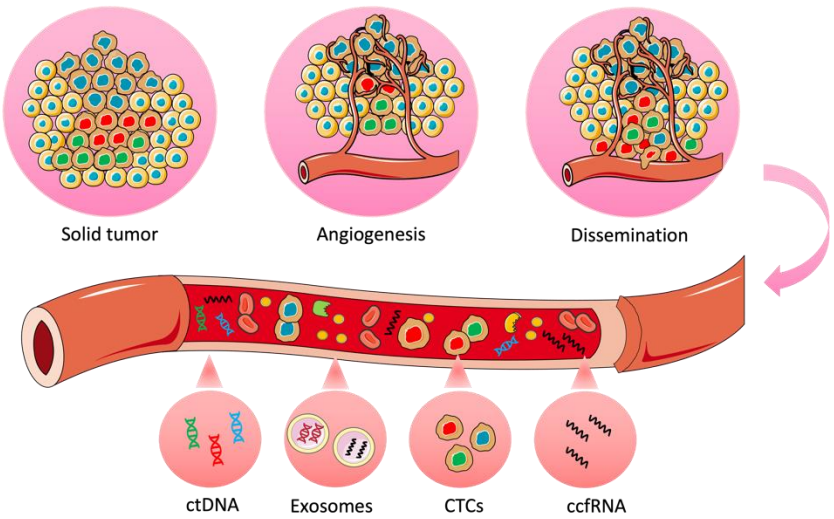

Figure 1. Schematic representation of blood-based liquid biopsy: CTCs (circulating tumor cells), ctDNA (circulating tumor DNA), ccfRNA (circulating cell-free RNA), and exosomes are released from tumor cells into the bloodstream.

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1.1.1. Circulating cell-free DNA

Mándel and Metais were the first to report the presence of cell-free DNA (cfDNA) in blood from healthy individuals in 1948 [8] and Leon et al. [9] were the first to detect cfDNA in serum from cancer patients in 1977. CfDNA comprises nuclear and/or mitochondrial DNA fragments released (both encapsulated in EVs and non-encapsulated) from the cells into physiological body fluids, such as blood, saliva, urine, or cerebrospinal fluid [10]. In healthy individuals, the majority of cfDNA released in plasma is derived from hematopoietic cells [11]. In cancer patients, cfDNA is a mixture of tumoral and non-tumoral DNA originating from healthy cells, cancer cells, and tumor microenvironmental cells [10]. The cfDNA shaded from cancer cells into the bloodstream is known as ctDNA and represents a small variable fraction of total cfDNA (usually <1%) [12]. The main mechanisms of ctDNA release include apoptosis, necrosis, and active secretion from EVs [13,14]. Most of cfDNA in plasma is thought to be released from apoptosis and showed a size distribution peak at ~167 base pairs (bp) which corresponds approximately to the DNA wrapped around a nucleosome (~147 bp) plus a linker fragment (~20 bp) as a result of cleavage by endonucleases at inter-nucleosomal DNA [15]. Longer cfDNA fragments have been also identified, suggesting other release mechanisms such as necrosis [13], phagocytosis of necrotic neoplastic cells by macrophages [12], or exosomes [16]. Furthermore, ctDNA has a short half-life of about 114 minutes due to the rapid clearance by lymphatic circulation via the kidneys, liver, and spleen [15,17].

Nowadays, ctDNA is being studied extensively since it is easy to analyze compared with other biomarkers and contains tumor-specific molecular alterations, including point mutations, chromosomal rearrangements, copy-number aberrations (CNAs), and specific DNA methylation patterns [18]. Currently, the technologies available for ctDNA analyses are based on the polymerase chain reaction (PCR) or next-generation sequencing (NGS). Highly sensitive PCR techniques such as BEAMing (beads, emulsions, amplification, and magnetics) and droplet digital PCR (ddPCR) allow the detection of ctDNA with limits of 0.001% and 0.01%, respectively. Despite the high sensitivity, rapidity, and relatively low costs, PCR-based assays are limited by low

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multiplexing capacity, allowing the analysis of only a restricted number of loci simultaneously. However, using NGS-based methods it is possible to detect novel genetic aberrations or multiple mutations simultaneously. Additionally, the non-targeted genome-wide analysis represents an opportunity to identify genomic alterations underlying therapy resistance and the identification of actionable mutations without prior knowledge of the genetic alterations of the tumor [2,4]. Therefore, the study of cfDNA provides quantitative data from the measurement of the mutant allele fraction (MAF) as well as qualitative information from the profiling of mutations, amplifications, deletions, and translocations present in the ctDNA. Regarding the clinical applications, plasma ctDNA testing has the potential to enhance cancer genotyping at early diagnosis, minimal residual disease detection, risk stratification, treatment monitoring, and the identification of resistance mechanisms [6,19].

1.1.2. Circulating cell-free RNA

The existence of circulating tumor RNA (ctRNA) was first described in 1996 by Stevens et al. [20] who detected tyrosinase messenger RNA (mRNA) in peripheral blood from melanoma patients. Different classes of ccfRNA can be detected like mRNA but also non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) or small nucleolar RNAs (snoRNAs), among others ncRNAs [21]. Overall, circulating RNAs show high stability in body fluids since they are protected from endogenous RNase activity by encapsulation in lipoprotein complexes (apoptotic bodies, microvesicles, or exosomes), binding to ribonucleoprotein complexes such as Argonaute (AGO)-2 or nucleophosmin 1, or binding to high-density lipoproteins [14,22]. Their detection and characterization can be performed with different techniques ranging from the analysis of single or small panels of RNAs such as reverse transcription-quantitative real-time PCR (RT-qPCR) or ddPCR to the genome-wide characterization of RNA using microarrays or RNA sequencing (RNA-seq) [4]. Accumulating evidence has shown the potential of liquid biopsies for detecting ncRNAs [23,24], gene fusion transcripts, and splice variants [25], as well as gene expression profiling [26].

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1.1.2.1 MicroRNAs

MiRNAs or miRs are small ncRNA molecules consisting of ~22 nucleotides (nt) that negatively regulate the gene expression at the post-transcriptional level by inducing mRNA degradation or inhibition of mRNA translation [27]. They constitute one of the most abundant class of gene-regulatory molecules, estimating that more than 60% of human protein-coding genes contain at least one conserved miRNA-binding site in addition to other non-conserved ones [28]. Diverse functions have been attributed to miRNAs including the regulation of fundamental cellular biological processes such as cell cycle regulation, differentiation, proliferation, and modulation of cell survival or apoptosis. Thus, the dysregulation of miRNAs displays an essential role in the pathogenesis of multiple human diseases, including cancer. Interestingly, a single miRNA can regulate the expression of multiple genes simultaneously and in turn, multiple miRNAs can regulate specific individual targets [29].

MiRNA biogenesis is a complex phenomenon where several transcriptional and post-transcriptional factors are involved. Despite their small size, miRNA production, maturation, and regulatory function require the action of a large number of proteins, including the two RNase III proteins, DROSHA and DICER, and AGO proteins [28]. They are generally transcribed by the RNA polymerase II, generating a long transcript called primary miRNA (pri-miRNA). This pri-miRNA contains a stem-loop hairpin structure, in which mature miRNA sequences are embedded. Typically, a pri-miRNA consists of a double-strand RNA (dsRNA) stem of 33–35 bp, a terminal loop, and single-stranded RNA segments at both the 5′ and 3′ sides. The processing of pri-miRNA to precursor miRNA (pre-miRNA) occurs within the nucleus by the Microprocessor complex formed by DROSHA and its essential cofactor DGCR8. DROSHA has two RNAse III domains (RIIIDa and RIIIDb) that cut specifically the dsRNA stem at 3´and 5´ releasing a small dsRNA harpin (including the terminal loop) of ~65 bp (pre-miRNA). Both DROSHA and DGCR8 contain dsRNA-binding domains that recognize pri-miRNA for efficient pri-miRNA processing. After pri-miRNA processing, pre-miRNA is exported to the cytoplasm

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by the protein exportin 5 (EXP5) and guanosine triphosphate (GTP)-binding nuclear protein RAN (RAN-GTP) forming the transport complex. After exportation to the cytoplasm through the nuclear pore complex, GTP is hydrolyzed, resulting in the disassembly of the complex and the release of the pre-miRNA into the cytoplasm. Then, DICER cleaves pre-miRNA near to the terminal loop at a distance of 21-25 nt from 3´end and 22 nt from 5´end yielding a small RNA duplex, named as mature miRNA. DICER recognizes the pre-miRNA by the PIWI–AGO–ZWILLE domain that contains two pockets: one interacts with the 5′-phosphorylated end and the other one interacts with the 3′ end of the pre-miRNA. Similarly, to DROSHA, DICER interacts with dsRNA-binding proteins such as TAR RNA-binding protein (TRBP) that modulates the efficiency of pre-miRNA processing and the length of mature miRNAs. The mature miRNA generated by DICER is then associated with AGO proteins to form the RNA-induced silencing complex (RISC). All human AGO proteins (AGO 1-4) are capable of inducing translational repression and decay of target mRNAs, but only AGO-2 can directly cleave mRNAs that are matched with miRNA. After loading RNA duplex onto AGO proteins, the "passenger" strand is removed whereas the "guide" strand is retained in the RISC complex resulting in the mature RISC which drives the specific silencing of the targeted mRNAs. The "guide strand" is determined during the AGO loading step based on the relative thermodynamic stability of the two ends of the small RNA duplex. Typically, the "guide" strand is the 5' strand of the pre-miRNA and the "passenger" is the 3' strand of the pre-miRNA. Due to strand selection is not a strict process, the "passenger" strand can be also selected and activates silencing of target mRNA [28] (Figure 2). The rapid growth in the miRNAs field had led to miRNA database (miRbase) that comprises 38,589 miRNA entries (1917 in humans) for which provides information on universal nomenclature, miRNA sequences, predicted target genes, and references [30].

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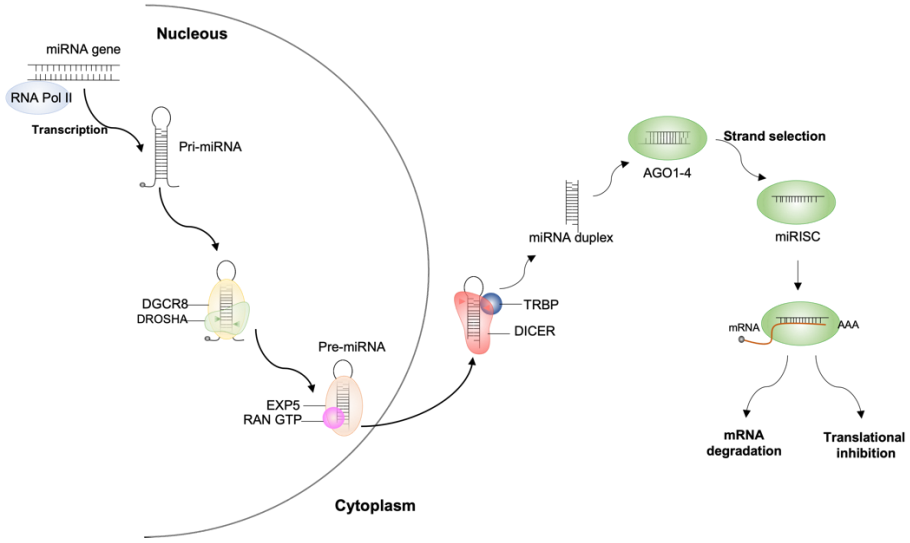

Figure 2. Schematic overview of canonical miRNA biogenesis pathway. miRNA gene is transcribed from non-coding intra-or intergenic regions of RNA transcripts by RNA polymerase II to generate the primary miRNA (pri-miRNA). The pri-miRNA is processed by DROSHA-DGCR8 Microprocessor complex to form the precursor-miRNA (pre-miRNA). Then, pre-miRNA is exported by exportin 5 (EXP5) with RAN-GTP (RAN-guanosine triphosphate) complex to the cytoplasm where it is processed into a mature miRNA by DICER (type III endoribonuclease miRNA). One of the strands of mature miRNA ("guide strand") gets incorporated into RNA-induced silencing complex (RISC) in association with the AGO (Argonaute protein) family member. Binding of target mRNAs to miRISC (miRNA-induced silencing complex) is followed by translational suppression and/or mRNA degradation.

In cancer biology, miRNAs have been established as key molecular components in tumor growth, invasion, angiogenesis, and immune evasion [31]. Thus, the dysregulation of miRNAs plays an important role in the initiation and progression of cancer and, depending on their transcript targets, they act as "*oncomiRs*" if they silence tumor suppressor genes, or "*tumor-suppressive miRNAs"* if they target the expression of oncogenes [32,33]. In this sense, carcinogenesis is associated with aberrant expression of miRNA profiles ("miRNome") due to they are different in tumors vs. normal tissue. In fact, this expression can be specific to certain tumor types yielding specific miRNA signatures. In this line, several miRNA profiling studies have

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identified specific signatures with value for the tumor diagnosis, staging, prognosis, and prediction of response to therapy [34]. Biologically, miRNAs have been identified in twelve body fluids: amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, peritoneal fluid, plasma, pleural fluid, seminal fluid, tears, urine, and saliva [35]. There are multiple sources of circulating miRNAs including tumor cells, immune cells, and blood cells [36,37], and several proposals have been made on how miRNAs are released into circulation (*e.g.* packaged into microvesicles or exosomes, or in vesicle-free form, that is in association with protein complexes) [38].

Different mechanisms have been established as responsible for the miRNAs dysregulation in cancer. Genetic alterations such as chromosomal rearrangements, genomic amplifications, deletions, or point mutations have been established to play an important role in cancer initiation and progression through the aberrant expression of miRNAs located in these affected regions and, subsequently, by the dysregulation of their downstream mRNAs targets [39]. Besides, a substantial number of miRNA genes are subjected to epigenetic alterations such as DNA hypermethylation of tumor suppressor miRNAs, extensive genomic DNA hypomethylation, and alteration of histone modification patterns. Altogether, these events can be related to aberrant miRNA expression in cancer [40,41]. In oncogenesis, several mutations have been found in components of the miRNA biogenesis machinery as in DROSHA and DICER1, which have been characterized to affect the expression of several miRNAs in cancer [42]. In addition, at the transcriptional level, alterations in tumor suppressor or oncogenic factors that function as transcriptional activators or repressors have been described as modulators of miRNA levels. For example, the oncogene *MYC* regulates the transcriptional activation of *miR-17-92* cluster, an oncogenic miRNA over-expressed in several malignancies [43]. Another example of cancer-involved transcriptional regulation of miRNA genes is the tumor-suppressor protein *p53* that promotes the transcription of all *miR-34* family members [44]. Due to the important role of miRNAs in cancer biology, future work will uncover additional pathways that regulate the expression of individual miRNAs and miRNA clusters in tumorigenesis.

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On the other hand, since miRNAs modulate important cellular processes by regulating multiple targets, anticancer therapies based on miRNAs are now under investigation. For the treatment of cancer, there have been discovered different potential therapeutic strategies as the following: RNA interference (RNAi)-based therapy (sandwich and multiplex RNAi inhibition strategy), miRNA inhibition therapy, and miRNA mimetic agents [45]. Sandwich RNAi inhibition strategy focuses on the use of multiple agents to target one specific molecular defect whereas multiplex RNAi inhibition strategy targets multiple molecular defects accumulated in the same pathway of specific cancer [46]. In the second RNA therapeutic drugs, specific miRNAs such as anti-miR oligonucleotides, locked nucleic acid anti-miRNAs, antagomiRs, miRNA sponges, miRNA masks, and small molecule inhibitors of miRNAs (SMIRs) act upon the mature miRNA inhibiting the interaction of that miRNA with its target mRNA [34,47,48]. For a similar purpose, it has emerged the "SMIR-approach", a novel inhibitory-miRNA therapy based on SMIRs that act inhibiting miRNA biogenesis or impeding miRNA-target interaction [49]. Another therapeutic alternative is the "miRNA mimics", which goal is to recover the function of tumor-suppressive miRNAs using synthetic miRNA-like molecules [50]. Nevertheless, the study of miRNA-based therapies is still in its infancy and the side effects of these therapies need a more exhaustive evaluation. Research efforts should be addressed to investigate miRNA-based targeted therapies since they could represent a great advance to personalized medicine for cancer treatment [45].

1.1.3. Extracellular vesicles

EVs comprise a complex population of cell-derived membranous structures that are secreted from many cell types to the extracellular space under physiological and pathological conditions. Based on the biogenesis, size and contents of EVs can be classified in large-EVs (100 to >1000 nm) including apoptotic bodies, large oncosomes, and microvesicles or small-EVs (50 to 130 nm) including exosomes [51]. EVs have been detected in numerous biological fluids such as blood, saliva, urine, or cerebrospinal fluid and show high stability protecting their molecular cargo (proteins, lipids, mRNAs, ncRNAs, and single-

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stranded or double-stranded DNAs) from the protease and nuclease activity by limiting membrane [52,53]. In cancer, EVs have been recognized as critical mediators of intercellular communication between tumor cells and stromal cells, playing a pivotal role in tumor growth, invasiveness, and metastasis [54,55]. Therefore, the analysis of liquid biopsies focused on EVs and their molecular content have emerged as a potential source for detecting cancer biomarkers [56,57]. Overall, EVs can be isolated by different techniques based on physical or biological properties such as ultracentrifugation, density-gradient centrifugation, polymer-based precipitation, immune selection, and microfluidic isolation [58]. The study of EVs provides both quantitative data related to the number of EVs as well as qualitative information from the molecular characterization of EVs content. Regarding their clinical application in cancer, several studies have highlighted their potential utility for early detection, diagnosis, prognosis, and treatment of cancer patients [57].

1.2. SALIVA AS A NOVEL LIQUID BIOPSY

1.2.1. Salivary secretion and functions

Human saliva is a clear, slightly acidic (pH = 6.0–7.0) and heterogenous biofluid composed of secretions from the three major salivary glands (parotid, submandibular, and sublingual) as well as minor salivary glands (labial, buccal, lingual, and palatal). Salivary glands are exocrine glands that contain parenchyma and stromal components. The glandular histophysiological unit called sialone is composed of secretory end piece called adenomere and the ductal portions [59]. The acinar cells that formed each adenomere are responsible for secreting serous, mucous, or mixed saliva into the ductal lumen. Parotid gland acinar cells produce mainly serous saliva, submandibular, and sublingual gland acinar cells mixed serous and mucous saliva, and the minor salivary glands mainly mucous secretions [59]. The complex duct system of the parotid and submandibular salivary glands comprises intercalated, striated, and excretory ductal segments whereas the sublingual and minor glands lack striated ducts

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[60]. Therefore, saliva is secreted from acinar cells into the ductal system, moving through the intercalated duct, striated duct, and the excretory duct to be finally secreted into the oral cavity. The excretory ducts of the major salivary glands are of the *Stensen duct* in the parotid gland, the *Wharton duct* in the submandibular gland, and *Walther´s duct* in the sublingual gland [59]. Additionally, myoepithelial cells are arranged intimately around the acinar cells and could assist the secretion process by compressing these structures. The major salivary glands are enclosed by a connective tissue capsule that branches inside the gland forming septa, which subdivide the parenchyma in lobes, that comprises multiple lobules separated from each other by an interlobular septum. These septa provide rich support of blood vessels and nerves surrounding acini and ducts [60]. The process of salivary secretion is controlled by the autonomic system nervous and regulated by reflexes. The acinar, ductal, and myoepithelial cells and blood vessels are innerved both parasympathetic and sympathetic branches of the autonomic nervous system that regulates the secretion of salivary flow and protein, interacting synergistically. The parasympathetic activity produces large volumes of poor-protein saliva whereas the sympathetic activity produces small volumes of rich-protein saliva. The blood vessels increase up to-20 folds the blood flow to the glands under parasympathetic stimulation providing water for secretion whereas the sympathetic stimulation causes vasoconstriction [60]. The major types of neurotransmitters released by the autonomic nerves innervating salivary glands are acetylcholine and noradrenaline which provide cholinergic and adrenergic signaling responses, respectively [59]. Other non-adrenergic and non-cholinergic co-transmitters such as calcitonin gene-related peptide, neuropeptide Y, neurokinin A, neuronal nitric oxide synthase, pituitary adenylate cyclase-activating peptide, substance P or vasoactive intestinal peptide may be released from autonomic nerves inducing effects on blood flow and salivary secretion [59]. These neurotransmitters act upon the receptors located on the basolateral part of the cell membrane stimulating fluid secretion. The neurotransmitter acetylcholine acts on muscarinic receptors (M1 and M3), whereas the neurotransmitter noradrenaline acts on adrenergic receptors (α_1 and β_1) [60].

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The formation of saliva is considered to be a two-step process. Upon stimulation firstly, the acinus secretes plasma-like primary saliva. This process is started with the transepithelial movement of Cl^- in acinar cells and its neutralization by Na^+ movement through acinar tight junctions, leading to an osmotic gradient for NaCl that involves the movement of water via the water channels and across thigh junctions to the acinar lumen. Then, the ion composition of this primary saliva is modified in the salivary ducts by the action of different absorption and secretion pathways. Thus, during the transit of saliva through salivary ducts, Na^+ and Cl^- are reabsorbed while K^+ and HCO_3^- are secreted by the epithelial cells. However, the water content does not vary due to the low permeability of the salivary gland duct system. Due to NaCl absorption is greater than K^+ secretion, the resulting saliva excreted by the salivary glands is hypotonic, generally less than 200mOsm [61] (Figure 3). The salivary secretion from salivary glands is largely stimulated by the parasympathetic nervous system through releasing acetylcholine. The association of acetylcholine with the muscarinic receptors (predominantly M3) produces activation of G proteins and elevation of intracellular calcium through the phospholipase C/inositol trisphosphate pathway. This regulated elevation in cytosolic calcium activates the chloride and potassium channels resulting in a transepithelial osmotic gradient, that allows the movement of water via water channels to follow Na^+ and Cl^- into the acinar lumen to create the primary fluid secretion. The driving force for Cl^- uptake is provided by the Na^+, K^+ -ATPase, and the Na^+/HCO_3^- cotransporters [61].

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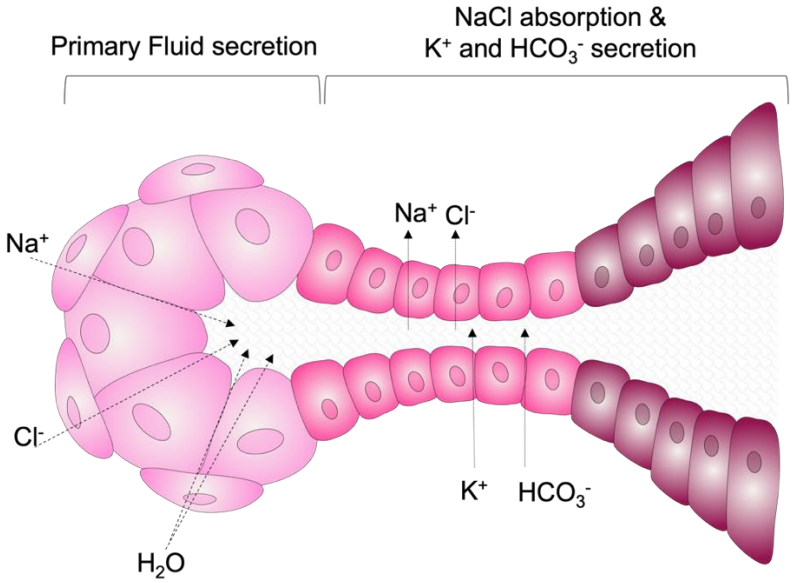

Figure 3. Saliva secretion model. Salivary secretion is a two-step process. First, acinar cells secrete a plasma-like fluid that contains Na^+, Cl^-, and H_2O. Secondly, in ductal cells, NaCl is reabsorbed and K^+ and $HCO3^-$ are secreted.

Salivary secretion is composed of water (99%), salivary proteins, and inorganic components, including also macromolecular elements from the blood. Most of proteins are secreted from salivary glands by exocytosis of protein storage granules in acinar cells. In addition to the organic constituents produced locally in the salivary glands, blood-based elements can enter into salivary ducts through different mechanisms of transport including transcellular (*e.g.* passive and active transport) or paracellular (*e.g.* extracellular ultrafiltration) routes [62] (Figure 4). Due to the capillaries surrounding the salivary glands are quite porous, small size molecules like steroid hormones can migrate mostly by passive diffusion from blood to saliva. Another way for the entry of molecules into saliva is the vesicular protein transport. Based on this mechanism, Immunoglobulin (Ig) A that is secreted by plasma cells located in the connective tissue of gland bound to a receptor (epithelial polymeric immunoglobulin receptor) on the basolateral surface of salivary acinar and ductal cells and it is endocytosed. The

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vesicle transports the IgA across the cell to the apical membrane, where the receptor is cleaved to release secretory IgA [59]. Ultrafiltration is another pathway of molecular transport. By this extracellular mechanism, molecules that are <1900 Da (*e.g.*, water, ions, catecholamines, or steroids) are transferred from the blood into saliva through the spaces between acinus and ductal cells. Additionally, serum components may also reach the saliva by crevicular fluid or directly from the oral mucosa, such as plasma albumin [63].

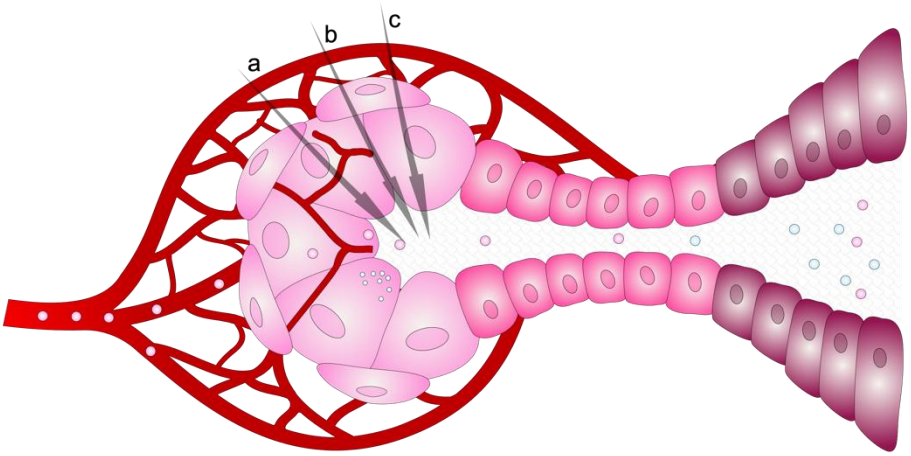

Figure 4. Mechanism of molecular transport from the blood into salivary gland ducts. The schematic representation shows the rich support of blood vessels in a major salivary gland allowing for the exchange of blood-based constituents. Blood-derived molecules enter salivary ducts via transcellular— (a) passive and (c) active transport—or paracellular—(b) extracellular ultrafiltration—routes. Then, alterations in the molecular composition of the blood may subsequently modify the output of salivary secretions, yielding saliva-based biomarkers of systemic diseases.

The combination of the glandular secretions from major and minor salivary glands forms the whole saliva. In addition to the salivary secretions, the whole saliva contains blood derivatives such as gingival crevicular fluid, serum exudate, or leukocytes; desquamated epithelial cells such as keratinocytes; nasal and bronchial secretions; food debris; as well as microbiota (bacterial, virus, and fungi) [59,64]. The daily salivary secretion ranges between 0.5 and 1.5 L at an average flow rate

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of 0.3–0.7 mL/min [62]. In addition, saliva secretion is under the influence of various factors such as reflex stimulation, circadian rhythms, level of hydration, and age that can influence the volume, flow, and saliva composition [60].

Saliva has numerous functions which are relevant for maintaining oral health and homeostasis. Salivary mucins contribute to mucosal lubrication reducing the friction between tissue surfaces and diminishing mechanical wear. The continuous flow of saliva cleanses the teeth and oral mucosa from bacteria and food particles. Importantly, saliva contains buffer ions, particularly bicarbonate, that regulate salivary pH by neutralizing acids and protecting dental enamel against demineralization. Saliva plays a critical role in the formation of the acquired enamel pellicle that protects the tooth from direct access to acids and allows the adhesion of salivary proteins such as sthaterins which are responsible for the maturation and remineralization of enamel. Saliva also contains numerous proteins with antibacterial, antiviral, and antifungal properties. Furthermore, saliva is involved in taste and bolus formation, initiates the digestion process by the breakdown of starch with salivary amylase, and facilitates the mastication, swallowing, and speaking [59,65].

1.2.2. Saliva as a source of biomarkers

Saliva has been found to contain a wide collection of measurable biomarkers such as proteins, DNA, mRNA, miRNA, metabolites, and microbiota that can be used for biomarker development and personalized medicine in oral and systemic diseases. Saliva is called the "*mirror of the body*" [66], as it is considered an ultra-filtrate of the blood [67] and because its composition changes under different pathological conditions. Advances in the field of saliva have allowed the study of the different salivary "*omics*" constituents including the proteome, the transcriptome, the metabolome, the microbiome, the genome, and the epigenome highlighting the potential of saliva for clinical and translational applications [68]. The vast amount of salivaomic data from high-throughput technologies has led to Salivaomics Knowledge Base (SKB), a data repository, management system and Web resource

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supporting human salivary proteomics, transcriptomics, miRNA, metabolomics, and microbiome research [69].

The attractiveness of saliva for diagnosis is based on the fact that it can be obtained easily, repeatedly, and non-invasively. Moreover, storage is simple and no special equipment is needed for sample collection, making it very cost-effective [70]. Multiple molecules isolated from saliva can be used as biomarkers for diagnosis, prognosis, drug monitoring, and pharmacogenetic studies [71]. Currently, six major diagnostic alphabets (Figure 5) have been characterized in saliva: proteome, transcriptome (miRNA and mRNA), metabolome, microbiome, genome, and epigenome [68].

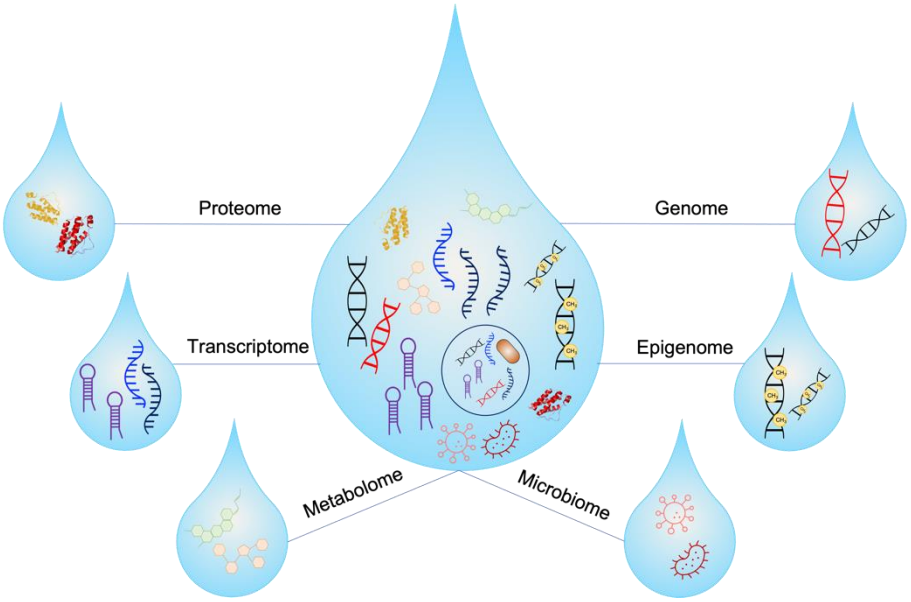

Figure 5. The components of saliva-omics. The different salivary "omics" constituents comprise the proteome, the transcriptome, the metabolome, the microbiome, the genome, and the epigenome.

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1.2.2.1. Proteome

The salivary proteome is considered the first diagnostic alphabet. Nowadays, more than 3000 different proteins have been identified in saliva [72]. The use of several proteomic techniques such as high-throughput mass-spectrometry proteomic technology and two-dimensional polyacrylamide gel electrophoresis have allowed deciphering the salivary proteome [73,74], leading to the generation of the Saliva Proteome Knowledge (SPKB) and the Salivary Proteome Wiki (SPK). The first catalog of the human salivary proteome was defined in 2008 and comprised 1166 proteins, out of which 914 were identified in the parotid and 917 in submandibular and sublingual saliva [75]. Interestingly, 30% of salivary proteins are found in blood [76], which evidence the potential of saliva for detecting biomarkers associated with local and distant diseases. Thus, numerous studies have reported discriminatory protein profiles for periodontal disease [77], diabetes [78], or cancer [79,80]. Actually, a proteomic study of saliva performed by Xiao et al. [81] found that three proteins (HP, AZGP1, and human calprotectin) were significantly higher in lung cancer patients in comparison with healthy controls. A regression model including the three proteins yielded 88.5% sensitivity and 92.3% specificity with an area under the receiver operating characteristic (ROC) curve (AUC) of 0.90. In the context of breast cancer, Streckfus et al. [82–84] found significantly higher c-*erb*B-2 and CA15-3 in the saliva and serum in the cancer group as compared to the non-cancer groups. In addition, Bigler et al. [85] evaluated the usefulness of c-*erb*B-2 for monitoring breast cancer and found significant differences in pre- and post-therapy salivary concentrations of c-*erb*B-2, suggesting its potential for tracking treatment and guiding therapy.

1.2.2.2. Transcriptome

Salivary transcriptome represents the second diagnostic alphabet in saliva. Global profiling of the salivary transcriptome by microarrays led to the generation of a reference database of over 3000 RNA species, out of which 185 transcripts (named normal salivary core transcripts) were consistently presented in healthy individuals [86]. The

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stability of salivary mRNA could be explained by the identification of AU-rich elements in their 3´unstranslated region [87]. Also, as previously commented, mRNA has been detected inside salivary exosomes that protect RNA against ribonucleases action [88]. The use of different transcriptomic techniques, such as microarray analysis, RT-qPCR, and RNA-seq analysis have allowed defining the salivary transcriptome. Spielman et al. [89] performed the first characterization of salivary transcriptome by RNA-seq using cell-free saliva (supernatant) and whole saliva. More than 90% of the uniquely mapped genes were coding (*i.e.,* mRNAs), and the remaining percentage corresponded to ncRNAs. However, ordering the genes by reads per kilobase per million criteria showed that 98 of the 100 most highly expressed genes encoded for non-coding sequences, predominantly small nuclear RNAs (snRNAs). Furthermore, cell-free saliva showed a lower fraction of microbial RNA compared to the whole saliva which enhanced the sensitivity for human gene transcription detection. Numerous studies have reported the detection of salivary mRNAs biomarkers both in local and systemic diseases [90–92]. For instance, Zhang et al. [93] found salivary transcriptomic biomarkers associated with resectable pancreatic cancer. The authors used a logistic regression model that included four mRNAs (*KRAS*, *MBD3L2*, *ACRV1*, and *DPM1*) with 90% sensitivity and 95% specificity.

NcRNAs comprising miRNAs have emerged as potential biomarkers due to their role in carcinogenesis and their high stability in body fluids, including saliva [94]. Actually, salivary miRNAs represent the third diagnostic alphabet. MiRNA profiling of 314 out of 708 human miRNA revealed that miRNAs can be detected in whole saliva and cell-free saliva [95]. Numerous studies have shown that salivary miRNAs are frequently dysregulated in several diseases [96,97], including cancer [98,99]. In a recent pilot study, Humeau et al. [98], found that four miRNAs (*miR-21, miR-23a, miR-23b,* and *miR-29c*) were significantly expressed in the saliva of patients with pancreatic cancer as compared to the control group, showing 71.4%, 85.7%, 85.7%, and 57% sensitivity, respectively, and 100% specificity. In the context of esophageal cancer, Xie et al. [100] reported salivary expression levels of *miR-10b, miR-144, miR-21,* and *miR-451*

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significantly higher in cancer patients than in control subjects, showing their potential use as diagnostic biomarkers. More recently, piwi-interacting RNAs (piRNAs), circular RNAs (circRNAs), and other ncRNAs have also been described as a new landscape of salivary RNAs [101].

1.2.2.3. Metabolome

Saliva contains hundreds of small-molecule metabolites that can provide important information into pathophysiological mechanisms of different diseases, which potentially enables biomarker discovery. The salivary metabolome is considered the four diagnostic alphabet. The application of different techniques including nuclear magnetic resonance and mass spectrometry combined with different separation systems, such as gas chromatography, liquid chromatography, and capillary electrophoresis has allowed the analysis of salivary metabolome [102]. Sugimoto et al. [103] characterized the salivary metabolome in healthy controls and patients with oral, pancreatic and breast cancer, and periodontal disease, using capillary electrophoresis time-of-flight mass spectrometry. Interestingly, three metabolites (taurine, piperidine, and a peak at 120.0801 m/z) were specific for oral cancer and eight metabolites (leucine with isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, and aspartic acid) were specific for pancreatic cancer. Suma et al. [104] evaluated ferric reducing antioxidant power (FRAP) and protein thiol levels as possible markers of oxidative stress and predictors of prognosis in brain tumors saliva. On one hand, the mean salivary FRAP values were significantly lower among patients with benign and malignant brain tumors. On the other hand, the mean protein thiol values were significantly higher among patients with benign and malignant brain tumors. However, no significant differences in salivary FRAP or protein thiols were observed between the benign and malignant tumor groups, although these two markers appear to be good indicators of oxidative stress status in the saliva of patients with brain tumors. Additionally, numerous studies have also demonstrated the potential of salivary metabolites for detecting local and systemic disease [105–108].

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1.2.2.4. Microbiome

The human oral cavity harbors an abundant and diverse oral microflora that plays an important role in health and disease conditions, acting as a potential source of microbial biomarkers. The salivary microbiome is considered the five diagnostic alphabet. The Human Oral Microbiome Database (HOMD) characterized the microbiota from different anatomic regions from healthy individuals and a total of 687 microbial species were identified (WWW.HOMD.ORG). The development of sequencing-based approaches such as whole metagenome shotgun sequencing or 16S ribosomal RNA metabarcoding have been used to provide insights in the oral microbiome [109,110]. In this line, more than 19,000 phylotypes have been identified in saliva contributing to the knowledge of oral microbiota diversity [111]. Numerous studies have explored the salivary microbiota alterations in different systemic diseases, such as periodontal disease [112] or cancer [113,114]. For example, Farrell et al. [113] found two microbial (*Neisseria elongata* and *Streptococcus mitis*) species that showed significant differences between the patients with pancreatic cancer and the healthy controls. A regression model including these microbial biomarkers yielded 96.4% sensitivity and 82.1% specificity with an AUC = 0.90.

1.2.2.5. Genome and epigenome

Saliva is considered a source of high-quality DNA for genomic and epigenomic studies [115,116]. Thus, saliva has been established as an important source of genomic DNA for the detection of germline mutations in cancer-related genes [117]. Advances in NGS-based technologies have revolutionized the field of genomics, making possible the detection of somatic alterations analyzing the primary tumor, blood and saliva, and opening new horizons in the field of salivary biomarkers [118]. It is important to clarify that in the whole saliva, including liquid and cellular components, coexist (in addition to bacterial DNA) two different sources of human DNA: the cellular DNA and the cfDNA (Figure 6). Salivary cellular DNA refers to genomic and mitochondrial DNA, from non-malignant and malignant cells, which are released into saliva. On the other hand, the term salivary cell-free

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DNA (scfDNA) should be used for referring to DNA fragments located in the non-cellular fraction of the saliva and originated from non-tumor cells, malignant cells, and tumor microenvironmental cells. We hypothesize that the scfDNA may be composed of circulating cell-free DNA (ccfDNA) from blood and non-ccfDNA released from local and proximal cells into the oral cavity (local source) as a result of multiple processes such as apoptosis, necrosis, and active release associated with EVs transport [119]. In addition, as it was demonstrated in plasma from head and neck cancer (HNC) [120], cell-free mitochondrial DNA could be detected in saliva, although it has not been yet reported. The ccfDNA derived from the tumor (ctDNA) normally represents a small population of the ccfDNA and, as we previously mentioned, it is characterized by harboring tumor-specific somatic mutations [121]. It is important to remark that in the oral cavity and proximal to oral cavity tumors, such as pharyngeal tumors, there is a direct release of tumoral DNA into saliva. Then, we coined for the first time the term "*salivary tumor DNA: stDNA*" [122], which is part of scfDNA that involves in HNC two different origins: ctDNA and non-ctDNA (local source). The ctDNA present in blood from oral and non-oral tumors could travel to the salivary glands through circulation and then, it could be secreted into saliva named "*salivary ctDNA*". Although the mechanism underlying the existence of ctDNA in saliva remains unclear, evidence has shown that tumor-derived exosomes can communicate with salivary glands cells transferring their cancer-derived products, which may alter the composition of the exosomes released by salivary glands [123,124]. Interestingly, some studies have reported the detection of ctDNA in saliva from patients with remote tumors to the oral cavity [125,126].

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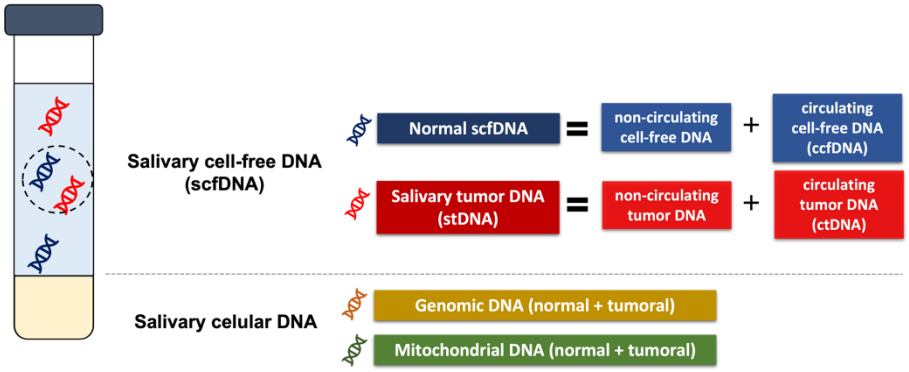

Figure 6. Schematic representation showing the composition of cellular and cell-free salivary DNA.

In addition, focused on salivary epigenetic analyses numerous studies have demonstrated the potential of salivary cellular DNA for exploring DNA methylation profiles in local and systemic diseases, including cancer [127–130]. Interestingly, saliva was also used to study the DNA methylation changes with age in order to predict the risk of age-related diseases [131].

1.2.3. The origin of salivary biomarkers in cancer

Saliva is a proximal body fluid in the oral cavity and, therefore, is intuitively sound for the detection of oral diseases. Currently, there are several theories and studies (including pre-clinical models) supporting why saliva is termed as "*liquid biopsy*", useful for detection of other systemic diseases as well. Some studies hypothesize that molecules from the primary tumor can be carried in the bloodstream and appear altered in saliva from cancer patients compared to healthy individuals (or patients with benign diseases). Supporting this hypothesis Gao et al. [132] used induced tumor-bearing mice models of melanoma and lung cancer and defined a salivary transcriptome profile associated with each tumor type. Six transcription factors (*Runx1, Mlxipl, Trim30, Egr1, Tbx1*, and *Nr1d1*) were significantly altered in salivary gland tissue of melanoma-bearing mice that can be responsible for the 83% of upregulated and the 63% of downregulated expression of salivary

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mRNAs. Moreover, the data analysis of nerve growth factor (NGF)/Erg1 signal pathway suggests that mediators such as NGF released by distant tumors are transferred through circulation to the salivary gland, where can stimulate transcription factors such as Egr1 and alter salivary mRNA expression and protein profile. Lau et al. [133], by using a pancreatic tumor-bearing mice model, revealed the basic mechanisms underlying the origin of cancer salivary biomarkers through the hypothesis that exosome-like vesicles carry, drive, and deliver tumor markers into the saliva. First, a tumor-specific salivary transcriptomic profile based on seven biomarkers was established upon the development of a pancreatic mice model. Then, after the inhibition of exosomes' release at the tumor source, a disruption of the pancreatic cancer-specific salivary transcriptomic panel was observed and only one out of the seven biomarkers were significantly upregulated in the saliva of mice bearing pancreatic tumors compared with the saliva of healthy mice. Their data suggest that tumor-derived exosomes are involved in the connectivity between the pancreatic tumors and the oral cavity. Yet, the lack of certainty and detailed mechanistic insights showing how salivary biomarkers can reflect disease states elsewhere in the body has compromised the scientific acceptance of this emerging field in clinics, justifying the need for more research focused to clarify the saliva composition in distant tumors.

1.2.4. Salivary biomarkers as point-of-care systems

Despite we are far from fully understanding the fundamental biology of salivary biomarkers, some achievements have been made aiming at developing a device for specific salivary biomarkers' detection. In particular, the Oral Fluid NanoSensor Test (OFNASET) is a prototype nanotechnology point-of-care sensor that has the ability to detect multiplexed analytes in saliva for oral cancer detection, through the combination of mRNA and protein electrochemical detection [134]. In addition, a device able to detect *EGFR* mutation at DNA level by using patients' saliva for lung cancer diagnosis, was developed in order to reduce the current invasive procedures and unnecessary biopsies [126]. More recently, another device for oral cancer detection was published based on a novel optical microfluidic biosensor with highly

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sensitive organic photodetectors for absorbance-based detection of salivary protein biomarkers [135]. The results of measuring IL-8 and IL-1E protein levels were in agreement with those provided by two commercial assays of ELISA thereby offering an attractive and cost-effective tool for diagnostic or screening purposes at the point of care. Several biosensors allowed the detection of multiple analytes including DNA, RNA, and proteins for both oral and systemic diseases [136], including the unique study that developed a biosensor system to detect salivary miRNAs for prostate cancer detection [137]. Continuous research on the field is needed to translate salivary diagnostics into routine clinical practice to improve cancer diagnosis and therefore, reduce patients' mortality.

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2. BIOMARKERS FOR COLORECTAL CANCER MANAGEMENT

2.1. EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most commonly occurring cancer in men and the second in women and represents the second leading cause of cancer-related deaths worldwide [138]. Although CRC incidence and mortality rates vary widely worldwide, its burden is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030 [139]. The risk of CRC development is associated with numerous factors, including age, family history and inflammatory bowel diseases, diet or lifestyle, and only a small number of cases are due to underlying genetic disorders [140]. Hereditary CRC syndromes such as Lynch syndrome, familiar adenomatous polyposis, MUTYH-associated polyposis, Peutz-Jeghers syndrome, juvenile polyposis, and Cowden/PTEN hamartoma syndrome represent only between 2% and 5% of all CRCs [141]. Colorectal carcinogenesis is associated with three important genetic alterations (chromosomal, microsatellite, and epigenetic instabilities) which play a key role in the two morphologic multistep pathways: the traditional model adenoma-carcinoma and the serrated neoplasia [142,143]. Importantly, 60–70% of diagnosed cases in non-screened symptomatic patients are detected in the advanced stage of the disease [144].

2.2. CURRENT SCREENING TOOLS

Nowadays, several CRC screening modalities (Figure 7) are available ranging from non-invasive to invasive tests characterized by specific advantages and disadvantages.

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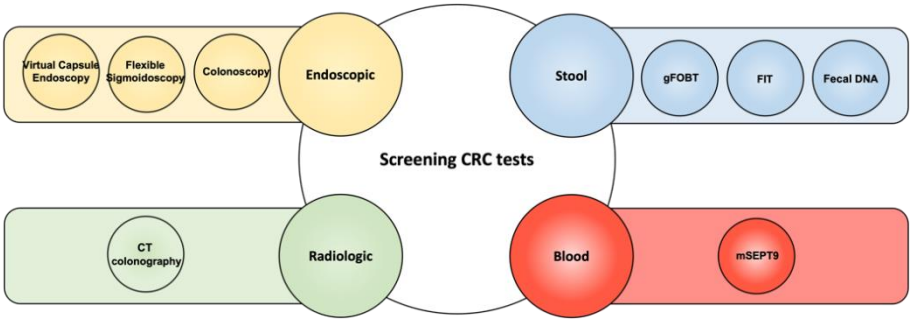

Figure 7: Screening tests for CRC (colorectal cancer) detection. *Abbreviations:* gFOBT, guaiac fecal occult blood testing; FIT, fecal immunochemical testing; CT, computed tomography; mSEPT9, methylated *Septin9*.

2.2.1. Stool-based tests

The guaiac fecal occult blood testing (gFOBT) and fecal immunochemical testing (FIT) are the two more common stool-based tests for CRC screening. The gFOBT detects the presence of blood in feces through the pseudo-peroxidase activity of hemoglobin. Although this test is simple and inexpensive, it has numerous disadvantages. It requires the collection of three samples on three different days for each screening round, it is not able to distinguish the source of bleeding and dietary restrictions are necessary due to is not specific for human hemoglobin [144]. Furthermore, gFOBT is limited by its relatively poor sensitivity to detect advanced colorectal adenomas (19.8%) and CRC (54.2%) [145].

FIT represents an improved version of gFOBT and is based on an antibody to detect human globin, therefore, it does not cross react with dietary meats. FIT is insensitive to upper gastrointestinal bleeding and only requires one stool sample [146]. Importantly, FIT has shown high diagnostic accuracy for CRC detection (93% sensitivity and 91% specificity) however, the accuracy for advanced neoplasia is moderate (48% sensitivity and 93% specificity) [147]. In 2014, Cologuard® was approved by the U.S. Food and Drug Administration (USFDA) for CRC screening. Cologuard® is a multitarget stool DNA test based on the detection of DNA targets (*KRAS* mutations and *NDRG4* and *BMP3* hypermethylation) plus FIT. Cologuard® showed greater sensitivity for

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detecting CRC (92.3% vs. 73.8%) and advanced adenomas (42.4% vs. 23.8%) compared with the FIT, respectively, whereas the specificity was lower (87% and 94%, respectively) [148]. In addition, FIT and colonoscopy have shown to be more effective and less costly than the stool DNA test, hindering its application for screening population programs [149].

Overall, stool-based tests show a low sensitivity to detect preneoplastic lesions and a high rate of false positives compared to colonoscopy [150].

2.2.2. Endoscopic tests

Flexible sigmoidoscopy allows a visual examination of the distal colon providing at the same time screening the opportunity for biopsy taking and/or polyp removal. In contrast with colonoscopy, flexible sigmoidoscopy involves limited bowel preparation, lower complication rates, and inferior costs [144]. However, this endoscopic technique has been largely replaced by colonoscopy due to its limitation to explore the proximal colon [150].

Colonoscopy is considered the "*gold standard"* for CRC screening since it provides a complete and direct examination of the entire colon, allowing to make biopsy and polyp removal. Colonoscopy can be used as a primary screening test or as a diagnostic test after a positive result of another primary screening approach [144]. Despite its high sensitivity and specificity, colonoscopy can miss around 10% of CRC and advanced adenomas due to insufficient bowel cleansing, lesions located hidden behind *folds* in the colon, or insufficient examination by the endoscopist. Additionally, colonoscopy is an invasiveness procedure that requires bowel preparation, time-consuming, expensive, and painful resulting in low adherence rates for CRC screening [151].

Video capsule endoscopy (VCE) has emerged as a promising alternative to colonoscopy because it can provide a non-invasive inspection of the colonic mucosa. It involves the ingestion of a large pill-sized camera that captures images of intestinal mucosa during its transit through the intestine. This procedure requires an adequate bowel

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preparation and the sensitivity for detected serrated polyps is limited [152]. Importantly, VCE is not approved by the USFDA for average-risk CRC screening but this procedure may be used for patients with incomplete colonoscopy or patients who are not candidates for colonoscopy [150].

2.2.3. Radiological tests

Computed tomographic (CT) colonography or virtual colonoscopy has been developed for visualization of the entire colorectum by computer-generated images of the colon constructed with data obtained from an abdominal CT scan [151]. This technique avoids the risk associated with intubation and sedation, however, bowel cleansing and CO_2 insufflation via a rectal balloon catheter are required. Despite this procedure is less invasive than other techniques, it is associated with other disadvantages such as radiation exposure, the low detection rate of precancerous adenomas, and high costs. Furthermore, does not eliminate the need to perform colonoscopy if lesions are detected [144,150].

2.2.4. Blood-based test

The methylated *Septin9* (m*SEPT9*) DNA assay was approved by the USFDA in 2016 as the first blood-based test (Epi proColon®) for CRC screening. Despite being approved for CRC screening, this blood-based test presents a lower sensitivity for CRC detection compared with other screening modalities. The m*SEPT9* analysis showed 63% sensitivity and 94.5% specificity for detecting CRC whereas its sensitivity for precancerous lesions was minimal (17.1%) [153], so it may have limited practical utility.

2.3. MICRORNAS AS BIOMARKERS

As was previously described, miRNAs have been detected in various biological fluids including serum, plasma, urine, tears, saliva, seminal fluid, cerebrospinal fluid, extracellular fluid, and others [35]. They

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show remarkable stability because they are protected against endogenous RNases by different mechanisms such as miRNA modifications, miRNAs encapsulation by EVs or miRNA-binding proteins as AGO-2 or high-density lipoproteins [154]. Three major pathways could explain the presence of miRNAs in body fluids: (1) passive leakage of cellular miRNAs into the circulation due to cell apoptosis and tissue damage, (2) active selective secretion as microvesicles-free miRNAs (cell-free miRNAs), and (3) active secretion via binding to cell-derived microvesicles and exosomes [155]. Therefore, the expression profile of circulating miRNAs may reflect the miRNA profile of primary tumor or metastatic lesions, facilitating the detection of the tumor at different stages in a non-invasive manner. This approach represents a unique opportunity to discover novel biomarkers involved in CRC carcinogenesis. Considering the potential of miRNAs as valuable CRC biomarkers, scientific evidence has highlighted the potential of circulating miRNAs for diagnosis, prognosis, and therapy prediction in CRC patients (Figure 8).

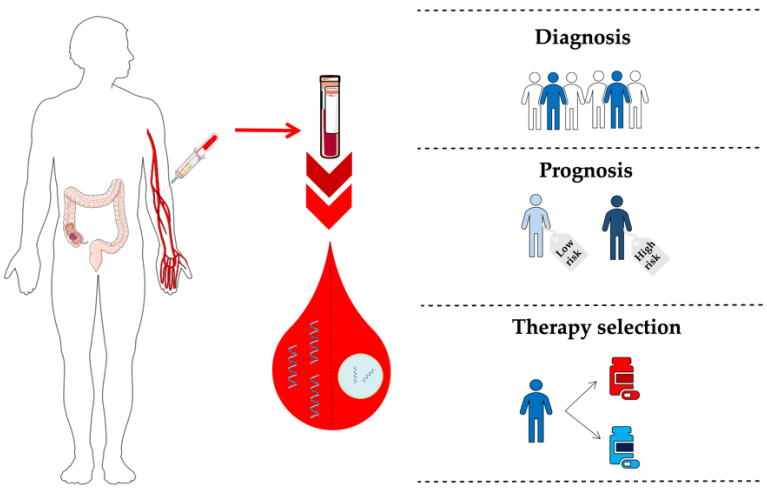

Figure 8. Clinical applications of circulating miRNAs (microRNAs) in CRC (colorectal cancer).

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2.3.1. MicroRNAs as diagnostic biomarkers

The first report that assessed the use of circulating miRNAs as a non-invasive diagnostic test for CRC was performed in 2009 [156]. Since then, several researchers have focused their work on the diagnostic value of circulating miRNAs for CRC detection [157,158]. Furthermore, ongoing research is aimed to identify specific miRNA profiles for early and advanced CRC stages, and even determine if there is a correlation with the Tumor-Node-Metastasis (TNM) staging system. The discovery of miRNA signatures involved in each step of the disease may increase the diagnostic accuracy in CRC. Therefore, circulating miRNAs are potential non-invasive biomarkers for CRC diagnosis (Figure 9).

2.3.1.1. Cell-free miRNAs in early stages

Detection of precancerous lesions and early-stage CRC (I–II) is essential to reduce mortality. Over the last few years, several studies [159–162] have evaluated the potential of circulating miRNAs as diagnostic biomarkers in CRC. Huang et al. [159] were the first to evaluate the diagnostic value of plasma miRNAs for early CRC detection. They found significantly elevated expression of *miR-29a* and *miR-92a* in 37 patients with advanced adenomas in comparison to healthy controls. ROC curve analysis yielded an AUC of 0.769 for *miR-29a* with 62.2% sensitivity and 84.7% specificity, and an AUC of 0.749 for *miR-92a* with 64.9% sensitivity and 81.4% specificity. Importantly, when both miRNAs were combined, an increase of the AUC value was observed. Further research has revealed different miRNAs as potential biomarkers for early CRC diagnosis in serum and plasma. Wang et al. [160] profiled 86 differentially expressed miRNAs in CRC patients and healthy controls, choosing 17 novel miRNAs with fold changes >5 for further validation. Plasma expression levels of *miR-601* and *miR-760* were significantly decreased in CRC compared to controls, showing at early stages a remarkedly decreased expression in respect to advanced adenomas and healthy controls. In addition, *miR-601* and *miR-760* expression was significantly reduced in advanced adenomas compared to healthy controls and increased compared to CRC. Interestingly, the

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combination of these biomarkers and carcinoembryonic antigen (CEA) yielded an AUC of 0.805 and 80.4% sensitivity and 65.5% specificity, indicating their potential diagnostic value for early CRC. Later, Kanaan et al. [163] reported a panel of eight upregulated cell-free miRNAs (*miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532,* and *miR-652*) that differentiated advanced adenomas from healthy controls with 88% sensitivity and 64% specificity. Moreover, 5-miRNA panel (*miR-331, miR-15b, miR-21, miR-142-3p,* and *miR-339-3p*) distinguished patients with advanced adenomas from CRC with an AUC of 0.856 and 91% sensitivity and 69% specificity. These findings suggest the existence of different miRNA profiles in the early steps of CRC carcinogenesis. In another study [161], *miR-21, miR-29a,* and *miR-125b* showed significantly higher serum expression levels in patients with early colorectal neoplasia compared to healthy controls, showing AUC values of 0.706 for *miR-21,* 0.741 for *miR-29a* and 0.806 for *miR-125b.* Also, *miR-21, miR-29a,* and *miR-125b* could discriminate patients with advanced neoplasia from non-advanced neoplasia and healthy controls showing AUC values that ranged from 0.690 to 0.731. Interestingly, the combination of these three miRNAs improved the discriminatory power for both early (AUC = 0.826) and advanced (AUC = 0.759) colorectal neoplasms. These three miRNAs were significantly increased in patients with tubular adenomas and high-grade intraepithelial neoplasms while *miR-29a* and *miR-125b* were also significantly elevated in tubule-villous adenomas and small colorectal neoplasms (≤5 mm).

In addition to these studies, massive characterization of cell-free miRNAs between CRC patients and healthy individuals provided the researchers alternative miRNAs panels for early CRC detection. Thus, Hofsli et al. [164] identified a model of 21 miRNAs that showed the same expression profile at early and advanced CRC stages. This serum-based miRNA model discriminated early CRC from healthy controls with 90% specificity and 87.5% sensitivity. Wang et al. [162] also identified in plasma a 3-miRNA panel (*miR-7, miR-409-3p,* and *miR-93*) with high diagnostic accuracy for discriminating early CRC from healthy individuals (82% sensitivity and 89% specificity, AUC = 0.892). Using NGS, Vychytilova-Faltejskova et al. [165] found 54

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miRNAs significantly dysregulated in the serum of 144 CRC patients and 96 healthy controls. Of these, 4 miRNAs (*miR-23a-3p, miR-27a-3p, miR-142-5p,* and *miR-376c-3p*) were validated as diagnosis biomarkers demonstrating a high discriminatory power to detect early CRC (81% sensitivity and specificity, AUC = 0.877). More recently, a plasma 6-miRNA (*miR-19a, miR-19b, miR-15b, miR-29a, miR-335,* and *miR-18a*) panel was validated for early CRC detection in a multicenter study including 96 CRC, 101 advanced adenomas, and 100 healthy individuals. This miRNA signature demonstrated 95% sensitivity and 90% specificity for advanced adenomas and 94% sensitivity and 87% specificity for early CRC [166]. Furthermore, other miRNAs, such as *miR-506, miR-4316, miR-182,* and *miR-30a-5p* have also been evaluated as potential markers for early CRC detection [167–169].

Overall, all these studies combining different panels of cell-free miRNAs, reach high accuracy to detect the presence of CRC at early stages with rates of sensitivity and specificity above 80%, making evident the great potential of these molecules as screening biomarkers.

2.3.1.2. Cell-free microRNAs in advanced stages

As previously mentioned, miRNAs have been involved in the advance of CRC, promoting invasion, migration, and disease progression through epithelial to mesenchymal transition (EMT) activation and other mechanisms behind the tumor spread [32]. Accordingly, researchers have examined the expression levels of circulating miRNAs in advanced stages and their diagnostic value for discriminating patients with lymph nodes and/or distant metastasis. In 2011, Cheng et al. [170] analyzed 3 miRNAs (*miR-21, miR-92a,* and *miR-141*) in the plasma of CRC patients, finding significantly higher levels of *miR-141* in stage IV CRC patients compared to healthy individuals. Plasma *miR-141* was significantly correlated with tumor stages and upregulated in distant metastasis compared to non-distant metastasis. Thus, ROC curve analysis showed that *miR-141* discriminated stage IV from stages I–II with 66.7% sensitivity and 80.8% specificity, stage IV from stage III with 66.7% sensitivity and

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89.7% specificity, and stage IV from stages I–III with 66.7% sensitivity and 84% specificity. In addition, the combination of *miR-141* and CEA could detect additional metastatic CRC patients who were not detected using one of both biomarkers independently. These results indicate that plasmatic *miR-141* levels represent a non-invasive biomarker for detecting metastatic CRC patients. In this line, Wang and Gu evaluated the expression levels of *miR-29a*, *miR-17-3p*, and *miR-92a* in serum of 38 metastatic CRC patients compared to 36 non-metastatic CRC cases. They found that serum *miR-29a* was significantly upregulated in patients with liver metastasis, showing 75% sensitivity and specificity (AUC of 0.803) for discriminating the metastatic from non-metastatic. They also observed a significant positive correlation between serum and tissue *miR-29a* expression levels in metastatic CRC patients, being its levels associated with advanced T stages [171]. Other studies have described upregulated levels of *miR-126, miR-141, miR-21,* and *miR-200* in CRC patients with liver affectation [172,173]. Some of these miRNAs were associated with tumor metastasis through EMT regulation targeting *ZEB1* and *ZEB2* transcription factors [174]. Serum *miR-885-5p* high levels were also significantly associated with high TNM stage, lymph node, and distant metastasis [175]. Recently, also upregulated serum levels of *miR-103* were associated with lymph node metastasis and advanced tumor stage, reflecting its involvement in the progression of CRC [176]. Other circulating miRNAs expression patterns have been identified as potential biomarkers in advanced CRC stages. Giráldez et al. [33] reported a plasma 3-miRNA panel (*miR-19a, miR-19b,* and *miR-15b*) with high discriminatory power to detect advanced stages from healthy individuals with 76.19% sensitivity and 77.36% specificity (AUC = 0.81). In a similar study, Brunet-Vega et al. [177] analyzed 11 upregulated tissue miRNAs in serum samples from 30 stage III CRC patients and 26 healthy individuals, finding only significantly increased expression of *miR-18a* and *miR-29a* in the patients' cohort. These results suggest the potential role of these miRNAs in the development of lymph node metastasis. Another miRNA analysis performed in 187 CRC patients and 47 healthy controls revealed significantly increased *miR-141, let-7f-2, miR-628-5p, miR-203,* and *miR-200b* expression levels in advanced stages

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compared to early stages and healthy individuals. In addition, *miR-15b, miR-526, miR-96, miR-148a,* and *miR-22* were also significantly upregulated in advanced stages compared to healthy individuals. Logistic regression analysis showed that plasma *miR-203* differentiated advanced stages from early stages with 74.7% sensitivity and 71.4% specificity and, again, miR-141 distinguished stage IV from stages I–III with 80.0% sensitivity and 86.1% specificity [178]. Finally, downregulation of specific miRNAs was also described in advanced disease. Thus, serum levels of *miR-139-3p* were significantly downregulated in advanced stages CRC patients with respect to controls, showing 97.2% sensitivity and 97.8% specificity to identify patients and demonstrating higher diagnostic accuracy compared to blood CEA levels [179].

2.3.1.3. Exosomal microRNAs

Nowadays, the specific miRNAs contained in exosomes have emerged as promising diagnostic biomarkers in cancer. Thus, Ogata-Kawata et al. [180] identified 16 miRNAs significantly upregulated in serum exosomes from CRC patients compared to healthy controls as well as in colon cancer cell lines using a microarray-based approach. From those, serum exosomal *let-7a, miR-1224-5p, miR-1229, miR-1246, miR-150, miR-21, miR-223,* and *miR-23a* showed significantly lower levels after surgical resection of the primary tumor, demonstrating the tumoral origin of these exosomal miRNAs. According to ROC curve analysis, *miR-1246* and *miR-23a* showed sensitivities of 95.5% and 92% respectively, reflecting the high diagnostic power of both miRNAs. Notably, stage I patients showed significantly increased levels of seven miRNAs (*let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223,* and *miR-23a*), suggesting a role of exosomal miRNAs in the early steps of CRC development. Wang et al. [181] analyzed nine plasmatic exosomal miRNAs from 50 early CRC patients and matched healthy individuals but only *miR-125-3p* and *miRNA-320c* were significantly upregulated in CRC compared to healthy individuals, showing AUC values of 0.6849 and 0.5982, respectively. Interestingly, an important increase of diagnostic power for early CRC stage was observed by combining CEA and *miR-125-3p*

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(AUC = 0.8552). These data support evidence about the possibility to combine exosomal miRNAs and traditional tumor markers as useful CRC screening methods. *MiR-21* has been also described as upregulated in plasmatic exosomes from CRC patients compared to healthy individuals. Moreover, *miR-21* was higher in advanced respect to early stages and also in patients with liver metastasis [182]. Another circulating exosomal profile study including 77 CRC patients and 20 healthy individuals identified 7 dysregulated miRNAs of which *miR-638, miR-5787, miR-8075, miR-6869-5p,* and *miR-548c-5p* were significantly downregulated, and *miR-486-5p* and *miR-3180-5p* were significantly upregulated. Among these, low expression of *miR-638* was associated with a higher risk of liver metastasis and advanced TNM stage. Furthermore, network analysis showed that *miR-638, miR-5787, miR-8075, miR-6869-5p,* and *miR-548c-5p* may regulate the glucose metabolism in CRC [183]. Recently, *miR-17-5p, miR-181a-5p, miR-18a-5p,* and *miR-18b-5p,* which were detected previously as cell-free miRNAs in plasma, were described as upregulated in plasmatic exosomes. Also, exosomal *miR-27a* and *miR-130a* showed higher levels in plasma from CRC patients compared to healthy individuals and adenoma patients, providing the combination of both miRNAs a high diagnostic accuracy (AUC = 0.801) [184].

Overall, these studies support the interest of analyzing the miRNAs exosome cargo in addition to cell-free miRNAs, however, further research should be performed to reveal the mechanisms through which tumor cells secrete exosomes containing specific miRNAs in order to improve their clinical application as biomarkers and potential therapeutic targets.

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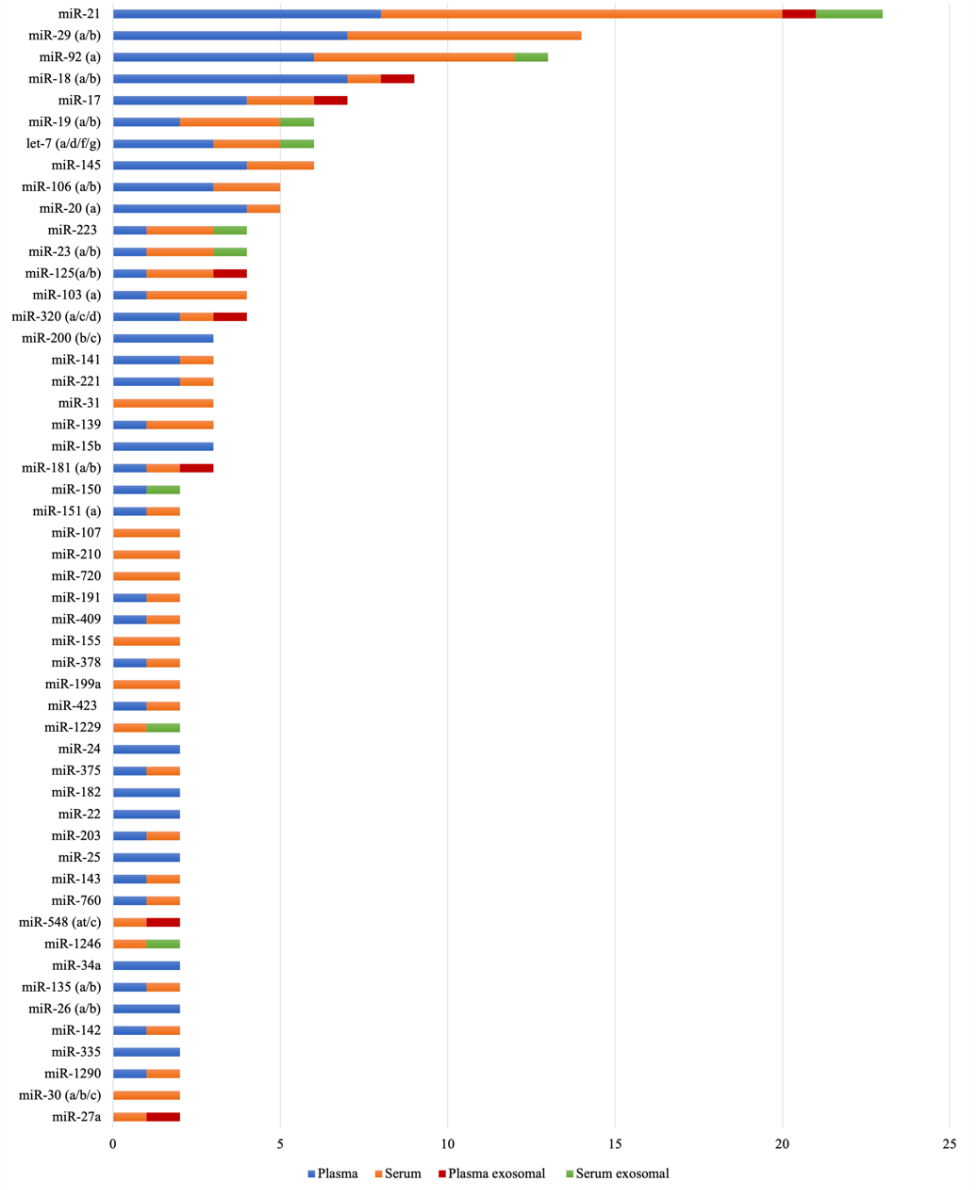

Figure 9. Summary list of circulating miRNAs (microRNAs) reported in two or more studies for CRC (colorectal cancer) diagnosis. These miRNAs were significantly deregulated in CRC compared to healthy individuals ($p < 0.05$).

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2.3.1.4. Salivary microRNAs

In 2017, Sazanov et al. [185] evaluated the expression levels of *miR-21* in plasma and saliva samples from CRC patients and healthy controls. In both body fluids, median levels of *miR-21* were significantly higher in the cancer group, but there were no differences between patients at stages II, III, and IV. In addition, *miR-21* levels in cancer and control groups were independent of gender and age. However, no significant individual correlation and linear regression of *miR-21* expression levels in the plasma and saliva were detected. Importantly, *miR-21* levels in plasma had 65% sensitivity and 85% specificity for discriminating CRC patients and controls, whereas in saliva *miR-21* had a sensitivity of 97% and specificity of 91%. The high sensitivity and specificity values for salivary *miR-21* reflected the potential use of this saliva-based test as a method of CRC diagnosis.

2.3.2. MicroRNAs as prognostic biomarkers

The first report that established a relationship between circulating miRNAs and prognosis in CRC was performed in 2010 [186]. Since then, several promising circulating miRNAs were associated with poor progression-free survival (PFS) and overall survival (OS) times in CRC patients. Some of the miRNAs that have demonstrated diagnostic potential were also identified as useful prognostic biomarkers, while other miRNAs have been exclusively associated with CRC prognosis (Figure 10).

2.3.2.1. Cell-free microRNAs

Pu et al. [186] analyzed plasmatic levels of *miR-21*, *miR-221*, and *miR-222* in 103 CRC patients and its correlation with *p53*, CEA, estrogen, and progesterone receptors status. In this study, factors known as histological grade, clinical stage, presence of metastasis, and high levels of *miR-221* were significantly correlated with poor OS. Interestingly, a significant correlation between plasma *miR-221* level and *p53* protein expression was found but the role of *miR-221* in the regulation of *p53* is nowadays unknown. Further, several works have

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found high levels of *miR-17-3p* [187], *miR-106a* [187], *miR-21* [188], *miR-92a* [189], *miR-1290* [190], *miR-210* [191], *miR-183* [192], *miR-885-5p* [175], *miR-592* [193], *miR-196b* [194], or *miR-155* [195] associated with shorter PFS or OS. More recently, serum *miR-203* has been proposed as another promising prognostic and metastasis-predictive biomarker in CRC. High circulating *miR-203* levels were associated with high TNM stage, presence of lymph node infiltration, distant and peritoneal metastasis, and a shorter OS. ROC curve analysis showed that *miR-203* could discriminate, with 62.5% sensitivity and 77.68% specificity, patients with poor prognosis. In addition, *miR-203* was an independent biomarker for predicting the formation of lymph node, liver, and peritoneal metastasis [196].

In addition, the prognostic impact of the *miR-200* family has been described in several studies focused on CRC [170,197–199]. Chen et al. [170] showed that high plasma *miR-141* levels were significantly associated with poor OS in metastatic CRC patients. Later, Toiyama et al. [198] analyzed the expression levels of *miR-200b*, *miR-200c*, *miR-141*, and *miR-429* in CRC serum samples, and they observed significantly elevated levels of *miR-200c* in stage IV tumors compared to stages I–III and healthy controls. Interestingly, high serum *miR-200c* levels were significantly associated with lymph node affectation and liver metastasis. Furthermore, multivariate logistic regression analysis showed that *miR-200c* was an independent prognostic marker for predicting shorter OS and tumor recurrence in stages II and III. In another study, Maierthaler et al. [197] found significantly increased levels of *miR-122, miR-141, miR-200a, miR-200b,* and *miR-203a* in metastatic compared to non-metastatic patients. According to univariate and multivariate analysis, patients with bad prognosis showed significantly higher levels of miR-122 in the metastatic CRC group, and significantly lower levels of miR-200c in the non-metastatic group. More recently, high levels of *miR-200c* and *miR-141* in plasma obtained from mesenteric vein were associated with shorter OS in CRC. By the contrary, these findings were not observed in blood from peripheral veins, which reflects a higher potential for detecting tumor-derived biomarkers from tumor-draining veins [200].

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It is noteworthy to point out that downregulated miRNAs have also been associated with an unfavorable prognosis in CRC. Low levels of *miR-30a-5p*, *miR-194*, *miR-29b*, and *miR-23b* were associated with shorter PFS and OS [169,201,202]. These miRNAs were significantly associated with a high TNM stage. In particular, *miR-23b* was significantly associated with tumor depth, distant metastasis, and tumor recurrence [202]. Nowadays, it is well known that the combination of various miRNAs with clinical data improves not only the ability to diagnose but also the prognosis. In fact, Vychytilova-Faltejskova et al. [165] designed a signature for the prediction of three-years OS consisting of three clinical factors (age, gender, and stage) and two miRNAs (*miR-23a-3p* and m*iR-376c-3p*). This model accurately predicted the outcome of more than 70% of CRC patients, even for each TNM stage independently. Recently, Vafaee et al. [203] developed a network based on an algorithm to discover robust miRNA signatures providing information about their biological role in CRC progression. By this approach, a plasma 11-miRNA signature was associated with several pathways related to CRC prognosis.

2.3.2.2. Exosomal microRNAs

Several circulating exosomal miRNAs have been also reported as potential prognosis biomarkers for CRC patients. Matsumura et al. [204] identified 6 serum exosomal miRNAs associated with liver CRC metastasis after a global microarray profiling in 124 CRC patients. In this study, upregulated expression of *miR-19a* and *miR-92a* was observed in CRC patients compared to healthy controls, with high levels of exosomal *miR-19a* significantly associated with higher tumor infiltration, lymph node affectation, the presence of liver metastasis, and therefore, with a higher risk of tumor recurrence and a worse overall prognosis. Liu et al. [205] validated as prognosis markers a total of 10 serum exosomal miRNAs identified by RNA-seq in 84 patients' samples with stages II and III CRC collected after tumor resection and before adjuvant therapy. They found out that *miR-4772-3p* allowed to discriminate recurrent from non-recurrent patients with 78.6% sensitivity and 77.1% specificity, which evidenced its potential as a prognostic biomarker for tumor recurrence. Interestingly, Tsukamoto et

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al. [182] evaluated the prognostic value of plasma exosomal *miR-21* according to TNM classification. Among CRC patients, high *miR-21* expression levels were significantly associated with a poor prognosis, determining worse disease-free survival (DFS) and OS in stages II–III CRC, while in stage IV only the OS was significantly worse in patients with high exosome-derived *miR-21*. Similarly, high serum exosomal *miR-6803-5p* levels were significantly and independently associated with shorter DFS and OS in CRC patients, mainly in patients with advanced stages and liver metastasis [206]. Other exosome-encapsulated miRNAs associated with a poor prognosis in CRC were *miR-548c-5p*, *miR-27a*, and *miR-130a* [184,207]. In particular, decreased expression levels of *miR-548c-5p* were significantly associated with a shorter OS, being this downregulation stronger in patients with liver metastasis and advanced tumor stages. Interestingly, potential *miR-548c-5p* target genes, such as *STAT3*, *PTPRO*, *IRF*, interleukins, and their receptors, implicated in CRC carcinogenesis and dissemination have been described providing valuable information regarding the role of this miRNA in the context of CRC [207]. Recently, Fu et al. [208] carried out an exosomal miRNA sequencing (miRNA-seq) on primary (SW480) and lymph node metastatic (SW620) CRC cell lines. Expression levels of 11 deregulated miRNAs (*miR-17, miR-19a, miR-20, miR-92a, miR-7, miR-181a, miR-375, miR-194, miR-30d, miR-192,* and *miR-146*) were further validated in serum exosomes from 29 CRC patients and 11 healthy individuals, finding significantly increased levels of *miR-17* and *miR-92a* in metastatic CRC compared to controls. In addition, circulating exosomal levels of both miRNAs were correlated with pathological stages and grades of CRC, suggesting their potential as prognostic biomarkers for early and metastatic CRC.

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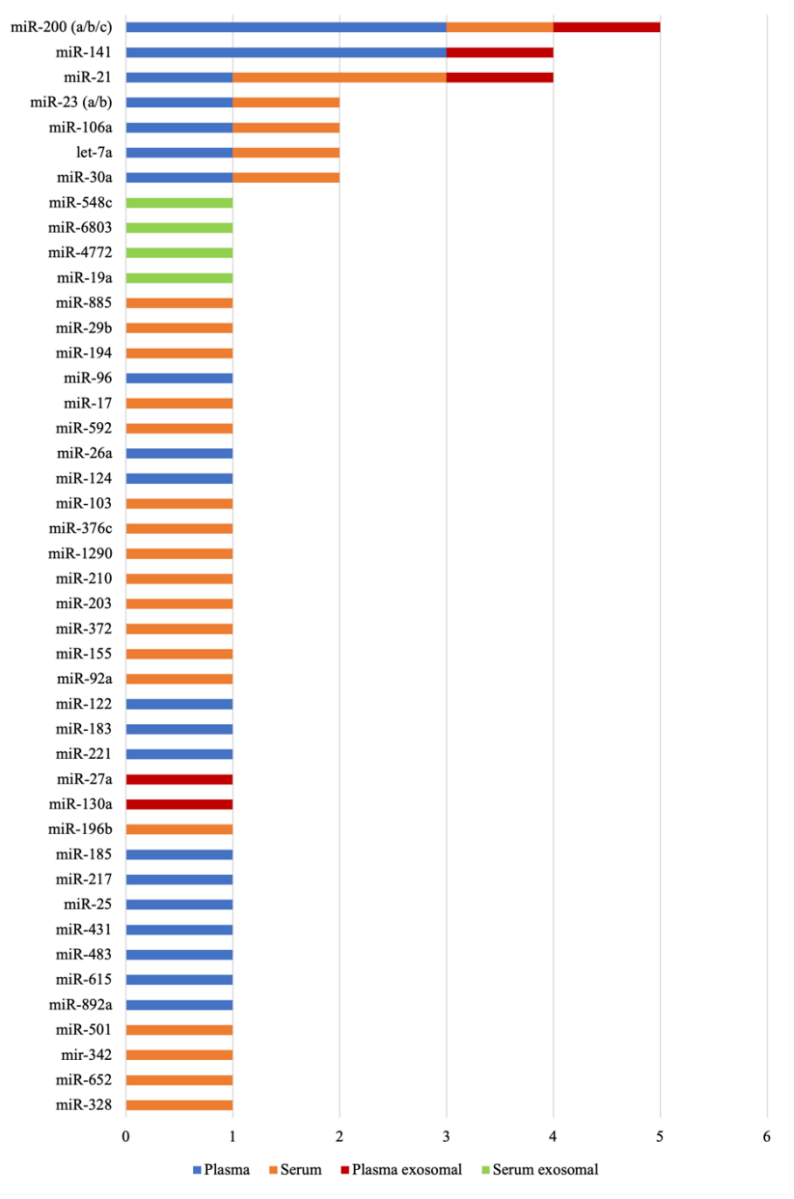

Figure 10. Summary list of circulating miRNAs (microRNAs) reported in one or more studies for CRC (colorectal cancer) prognosis. These miRNAs were significantly associated with survival ($p < 0.05$).

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3. BIOMARKERS FOR HEAD AND NECK CANCER MANAGEMENT

3.1. EPIDEMIOLOGY

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide, accounting for an estimated 890,000 new cases and 450,000 deaths in 2018 [209]. Although HNSCC incidence and mortality rates vary widely worldwide, its burden is expected to increase by 30% to more than 1.08 million new cases in 2030 [209]. HNSCC comprises a heterogeneous group of cancers arising from the epithelial cells of the mucosal linings of the oral cavity, oropharynx, larynx, and hypopharynx. Specifically, oral cavity cancer is the most common type of HNSCC with a total of 354,864 new cases and 177,384 deaths estimated in 2018 [138]. HNSCC is characterized by a multifactorial etiology and its development has been associated with several risk factors including tobacco smoking and alcohol consumption, exposition to environmental pollutants, and infection by viral agents including human papillomavirus (HPV). Currently, the prevalence of HPV-associated HNSCC, in particular, oropharyngeal squamous cell carcinoma (OPSCC) is increasing predominantly among younger people in North America and northern Europe [210]. HNSCCs are more frequent in men who have a 2-4-fold increased risk compared to women. The median age for diagnosis HPV-negative (HPV-ve) HNSCC is 66 years whereas for HPV-positive (HPV+ve) OPSCC is 53 years [209]. HPV+ve HNSCC represents a distinct molecular and clinical entity from HNSCC, underscoring the unique biology of this disease. Importantly, HPV+ve HNSCC shows better treatment outcomes and survival rates compared to HPV-ve HNSCC [209,211].

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3.2. CURRENT SCREENING TOOLS

Nowadays, there is no standard screening test for HNSCC detection. Due to the oral cavity is easily accessible, visual oral inspection represents the main screening modality for oral cancer detection. In addition to visual inspection, numerous adjunctive techniques have been developed for screening oral cancerous lesions or potentially malignant disorders including toluidine blue staining, oral brush cytology, or fluorescence imaging, however, these adjunctive modalities have failed to improve the sensitivity or specificity of screening beyond clinical visual oral inspection [212]. For HNSCCs located in the oropharynx, larynx, and hypopharynx, different endoscopic technologies could be applied for screening, however, these procedures are invasive and could require evaluation under anesthesia [213].

3.3. MIRNAS AS A BIOMARKERS

3.3.1. Origin of cell-free microRNAs in oral cancer

The release of miRNAs into the bloodstream and saliva is explained as a result of apoptotic and necrotic cell death and also by active cell secretion [22]. As we previously mentioned, circulating cell-free miRNAs are transported in the blood by different EVs (apoptotic bodies and exosomes) [214], whereas the majority of circulating miRNAs are co-fractionated with protein complexes such as AGO-2 [215]. In addition, oral tumors shed directly into saliva different markers, such as tumor DNA or miRNAs, which are secreted free or packed into EVs, such as exosomes [216]. Moreover, tumor-derived miRNAs harbored in exosomes could reach the salivary glands through the bloodstream and modify the saliva secretome as a result of tumor invasion (Figure 11).

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3.3.2. MiRNAs as diagnostic markers

Over the last 10 years, several researchers have investigated the clinical relevance of blood and saliva miRNAs as diagnostic markers in oral cancer. The first study that detected an altered expression of circulating miRNAs in oral cancer was performed by Wong et al. [217]. After a microarray expression analysis, overexpressed *miR-184* levels were validated in a cohort of 20 paired tongue squamous cell carcinoma and normal tissues. As in tissue, plasma levels of *miR-184* were significantly higher in cancer patients. Importantly, plasma *miR-184* levels were significantly decreased after tumor surgery which indicated a correlation between plasma and tissue *miR-184* levels. In addition, a functional assay revealed that *miR-184* enhanced the proliferation of tumor tongue cells and hindered the apoptotic process [217]. However, other authors [218,219] observed downregulated expression of *miR-184* in tissue from oral squamous cell carcinoma (OSCC). Interestingly, *miR-184* expression levels were higher at the surgical tumoral margin than at extra margin, indicating its potential for identifying minimal residual disease [218].

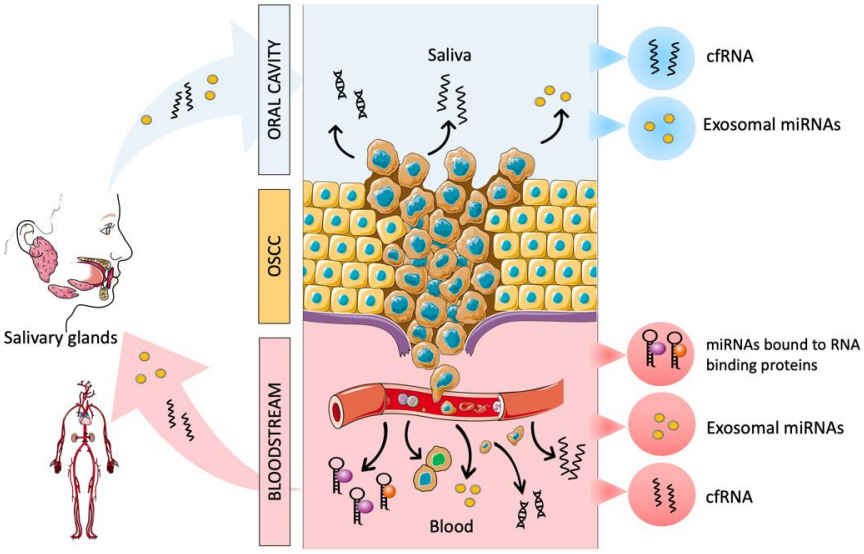

Figure 11. Schematic representation of cell-free miRNAs release into the bloodstream and saliva in oral tumors. *Abbreviations*: cfRNA, cell-free RNA; OSCC, oral squamous cell carcinoma

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Other studies have described the potential oncogenic role of different miRNAs in oral cancer. Lin et al. [220] found significantly overexpressed *miR-24* plasma levels in oral cancer patients as compared with healthy controls, yielding an AUC of 0.82 with 70% sensitivity and 82% specificity. Interestingly, upregulated circulating *miR-24* was also detected in tumoral oral tissue [220,221], showing a positive correlation with the clinical stage [221]. Moreover, it has been demonstrated that *miR-24* promotes the proliferation, migration, and invasion of tongue squamous cell carcinoma by targeting *FBXW7* [221]. Dysregulated circulating *miR-24* has been reported in other cancer locations, such as breast [222], colorectal [223], and lung [224] cancers. Other miRNAs have been described as oncogenic miRNAs in oral cancer, such as *miR-10b* [225] which was abnormally expressed in plasma of 54 oral cancer and seven patients with precancerous lesions as compared with 36 healthy controls. ROC analysis revealed a high discriminatory power for differentiating cancer (AUC = 0.932) and precancer (AUC = 0.967) patients with respect to healthy controls. In addition, OSCC xenograft mice models presented significantly increased expression of *miR-10* in plasma, which suggests its potential role in tumor formation. *In vitro* assays indicated that *miR-10b* was more strongly associated with cell invasion and migration than tumor growth, although the regulatory pathway requires further research [226]. Importantly, circulating *miR-10b* has also been involved in other tumors, *e.g.,* breast [227], pancreatic [228], and lung cancers [229]. Upregulated expression levels of circulating *miR-146a* were found in 51 oral cancer patients as compared with 12 healthy controls, differentiating cancer patients from controls with 79% sensitivity and 92% specificity (AUC = 0.86). Notably, after surgical tumor resection, circulating *miR-146a* levels were found significantly decreased. In addition, results based on the xenograft model revealed that high plasma levels of *miR-146a* were associated with a higher tumor burden and the development of neck metastasis. Furthermore, this study showed a relationship between increased *miR-146a* expression and tumorigenesis and metastasis by the regulation of *IRAK1*, *TRAF6*, and *NUMB* gene expression [230].

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Since early diagnosis is the key factor for improving the survival and quality of life of oral cancer patients, several studies have focused on identifying circulating miRNAs as potential biomarkers for early detection. Lu et al. [231] reported upregulated levels of *miR-196a* and *miR-196b* in plasma of oral cancer and patients with precancer lesions with respect to healthy controls. ROC analysis indicated that circulating *miR-196a* and *miR-196b* could differentiate oral cancer patients from healthy individuals with AUC values of 0.864 and 0.960, respectively, and individuals with precancer lesions from healthy ones with AUC values of 0.760 and 0.840, respectively. Importantly, a logistic regression model was performed to validate the discriminatory power of both miRNAs between the two diseases groups and the control group with the following results: oral cancer vs. healthy controls presented 87.8% sensitivity and 92.5% specificity (AUC = 0.963), precancer vs. healthy controls showed 68.8% sensitivity and 84.9% specificity (AUC = 0.845), and disease group (oral cancer and precancer) vs. healthy controls presented 90.6% sensitivity and 84.9% specificity (AUC = 0.950). In addition, a positive correlation was found between *miR-196a* and *miR-196b* plasma expression levels, indicating that both miRNAs were upregulated in parallel during oral carcinogenesis. On the basis of these findings, circulating *miR-196a* and *miR-196b* seem to be important in early oral cancer stages, and, therefore, the combination of both miRNAs could be useful for early oral cancer detection. Previously, Liu et al. [232] evaluated the expression levels of *miR-196a* and *miR-196b* in tissue and plasma samples from oral cancer patients, finding significantly upregulated *miR-196a* levels in comparison with healthy controls. It is important to mention that the human *miR-196* family (*miR-196a1*, *miR-196a2*, and *miR-196b*) is known to play a critical role in cancer development and pathogenesis by targeting several genes including *HOXB8*, *HMGA2*, and *Annexin A1* [233]. Recently, Chang et al. [234] validated the assessment of expression levels of *miR-222-3p*, *miR-150-5p*, and *miR-423-5p* in OSCC and oral leukoplakia patients, as a diagnostic tool. Interestingly, functional enrichment analysis revealed the involvement of *miR-222-3p*, *miR-423-5p*, and *miR-150-5p* in different cancer-related pathways, such as Wnt, PI3K-Akt, MAPK, and Ras signaling pathway. These findings

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suggest the potential role of plasmatic miRNAs in oral carcinogenesis, as well as their high diagnostic value for early oral cancer detection. *MiR-221*/*miR-222* cluster has been shown to promote oral carcinogenesis by downregulation of *PTEN* [235]. Unlike the above study by Chang et al. [234], Roy et al. [236] found that expression levels of *miR-423* were significantly downregulated in oral cancer tissue and can act as a tumor suppressor. Evidence shows a dual role of *miR-423* in cancer acting as an oncogene [237,238] or a tumor suppressor [239,240] in different malignancies. Moreover, dysregulated expression levels of *miR-150* have been reported in cancer, representing a potential diagnostic biomarker for acute myeloid leukemia [241], cholangiocarcinoma [242], and CRC [243].

Over the last few years, other circulating tumoral miRNAs have been highlighted as non-invasive biomarkers for oral cancer detection. In 2016, Tachibana et al. [244] identified 20 dysregulated miRNAs in plasma samples from gingival squamous cell carcinoma patients by profiling of 1211 human miRNAs. Plasma *miR-223* was significantly upregulated in cancer samples as compared with controls, showing 67.7% sensitivity and 61.3% specificity. However, *miR-223* expression levels were downregulated in oral cancer tissues with respect to non-cancerous tissues, suggesting that *miR-223* could be released into the bloodstream mainly from adjacent normal tissues as a biological defense mechanism to inhibit tumor growth. Functional analysis revealed that *miR-223* inhibited cell proliferation and induced apoptosis in oral cancer cell lines, and also observed downregulated expression of *STMN1* and *IGF1R*. Xu et al. [245] reported significantly upregulated serum *miR-483-5p* expression in 101 oral cancer patients with respect to 103 healthy controls. ROC analysis indicated that serum *miR-483-5p* could differentiate oral cancer from controls with 85% sensitivity and 74% specificity (AUC = 0.85). Interestingly, high serum *miR-483-5p* expression was positively correlated with lymph node metastasis and the TNM-based staging system, allowing differentiation of late and early stages with 66% sensitivity and 78% specificity (AUC = 0.75). In addition, *miR-483-5p* was significantly upregulated in oral tumor tissues, suggesting that oral tumor cells could be shedding high levels of this miRNA into the

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blood during oral carcinogenesis. Similarly, Sun et al. [246] found upregulated plasma levels of *miR-200b-3p* in OSCC with respect to healthy controls, being these levels higher in grade II-III than grade I tumors. A significant decrease in this miRNA was observed after surgical removal of the tumor, reflecting its tumoral origin. The expression levels of *miR-200b-3p* were significantly upregulated in paired normal adjacent tissues as compared with tumoral tissue, suggesting that tumor cells can reduce the potential antitumorigenic behavior of this miRNA by releasing *miR-200b-3p* into the blood. The high sensitivity and specificity (90% and 88.75%, respectively) of *miR-200b-3p* indicates the diagnostic potential of this circulating biomarker for oral cancer detection. Plasma *miR-187-5p* has also been associated with oral carcinogenesis. Liu et al. [247] examined the expression of *miR-187-5p* in plasma samples of 63 OSCC patients and 23 healthy controls finding significantly upregulated levels in oral cancer. Importantly, *miR-187-5p* could act as a potential oncogene promoting proliferation, migration, and anchorage-independent colony formation of oral cancer cells. Recently, Lu et al. [248] selected a set of five miRNAs (*miR-99a-5p*, *miR-138-5p*, *miR-375-3p*, *miR-21-5p*, and *miR-31-5p*) based on previous miRNA profiling studies and analyzed their expression levels in tissue and serum samples. Although only *miR-31-5p* was upregulated in tissue and serum samples, the serum expression levels of *miR-99a-5p*, *miR-138-5p*, and *miR-375-3p* were significantly related to the clinical stage. On the basis of ROC analysis, serum *miR-31-5p* could discriminate oral cancer from healthy controls with an AUC value of 0.661, whereas the model including the five serum miRNAs showed an AUC of 0.776, with 76.8% sensitivity and 73.6% specificity. These findings indicate the diagnostic potential of circulating *miR-31-5p* as an independent biomarker for oral cancer detection. Altered expression of circulating *miR-31* has been reported in oral cancer patients previously by Liu et al. [249]. In their study, *miR-31* expression was significantly upregulated in plasma from cancer patients as compared with healthy controls, showing an AUC value of 0.82 [249]. Besides, circulating *miR-21* which is dysregulated in many cancer types was also significantly overexpressed in oral cancer [250–252].

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Because the origin of extracellular miRNAs remains unclear, other large-scale miRNA profiling studies have been performed to identify novel miRNA expression patterns in tumoral tissue and the bloodstream. Rabinowits et al. [253] compared the miRNAs expression profile of benign and malignant tongue tissue and plasma from five tongue squamous cell carcinoma patients by microarrays. Out of the 359 miRNAs detected, 16 miRNAs (nine upregulated and seven downregulated) were differentially expressed between cancer and non-cancer tissue. As in the tumor, *miR-19a*, *miR-27b*, *miR-20a*, *miR-28-3p*, *miR-200c*, *miR-151-3p*, *miR-223*, and *miR-20b* were upregulated in plasma, when their levels were studied in free and exosomes associated RNA. On the other hand, *miR-512-3p* was only associated with exosomes. However, out of the seven tumor downregulated miRNAs, four miRNAs (*miR-370*, *miR-139-5p*, *miR-let-7e*, and *miR30c*) were expressed in plasma (in free RNA and exosomes fraction), one miRNA (*miR-516-3p*) was not detected, and two miRNAs (*miR-22* and *miR-145-3p*) were only associated with exosomes. Schneider et al. [254] compared the miRNA profile in tissue and serum samples of five OSCC patients by RNA-seq. After bioinformatic analysis, a total of 255 miRNAs were identified in tissue (cancer and noncancer) and 381 miRNAs were detected in serum; 214 miRNAs were present in both tissue and plasma. Importantly, out of the 48 miRNAs were found to be significantly dysregulated in cancer tissue as compared with normal tissue, and 30 were also detected in serum. Recently, Pedersen et al. [255] reported a thorough description of the miRNome in both OSCC and normal oral mucosa. Using miRNA-seq data of oral cancer tissues, expression levels of a set of miRNAs were analyzed in plasma samples from 55 oral cancer patients and 18 healthy controls. Only four miRNAs (*miR-30a-5p*, *miR-370-3p*, *miR-144-5p*, and *miR-769-5p*) were significantly upregulated in plasma from cancer patients as compared with healthy controls. Interestingly, a model generated by the combination of plasma *miR-30a-5p* and *miR-370-3p* yielded an AUC value of one, indicating its high discriminatory power for oral cancer detection. However, these predictive miRNAs were different between plasma and tissue. The authors considered that the

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discordance between tissue and plasma miRNAs could be the result of the systemic response to oral cancer.

On the other hand, salivary miRNAs have been described in head and neck tumors as potential clinical biomarkers. Park et al. [95] carried out one of the first studies that aimed to determine miRNA profile in whole and supernatant saliva samples from healthy controls and profiled a total of 314 miRNAs. Although whole and supernatant saliva showed similar profiles, a major heterogeneity was present in the whole saliva, which could be due to the presence of miRNAs derived from desquamated oral squamous cells. In addition, the same authors compared the expression profile of four miRNAs (*miR-142-3p, miR-93, miR-125a*, and *miR-200a*) in a cohort of 50 OSCC patients and 50 healthy controls, observing significantly decreased expression of *miR-125a* and *miR-200a* in supernatant saliva of cancer patients. The downregulation of the *miR-200* family, a group of tumor suppressor miRNAs, promotes EMT of oral tumor cells through the upregulation of *ZEB1* and *ZEB2* genes and increases vimentin expression [256]. Other miRNAs have been identified as regulators in the development and progression of oral cancer [257–259]. Thus, Liu et al. [260] explored the potential of *miR-31* as a clinical biomarker for OSCC, and they found upregulated *miR-31* in cancer patients as compared with healthy controls, both in saliva and plasma. Moreover, a significant decrease in salivary and plasma *miR-31* levels was observed after tumor resection, which suggests that *miR-31* was released from the tumor into the bloodstream and later into saliva. Importantly, a significant positive correlation between saliva and plasma *miR-31* levels was observed. However, *miR-31* expression levels were significantly higher in saliva with respect to plasma, indicating major local secretion of *miR-31* from OSCC cells, probably as a consequence of the direct contact between saliva and the oral tumor. These results revealed the potential application of salivary *miR-31* as a sensitive biomarker for detecting oral cancer. Epithelial *miR-31* upregulation and epithelial dysplasia were described as independent factors for oral potentially malignant disorders. Significant *miR-31* upregulation was also detected in saliva samples of oral potentially malignant disorders (hyperkeratosis epithelial hyperplasia, or dysplasia) patients as compared with healthy

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controls (AUC = 0.76), although no association was found with epithelial dysplasia. These data evidenced the potential role of *miR-31* in the early steps of oral cancer development [261]. Other miRNAs, such as *miR-21* and *miR-184*, showed significantly increased levels in saliva from OSCC and oral potentially malignant disorders, while salivary *miR-145* showed a significant decrease. However, only *miR-184* has value to discriminate between OSCC and oral potentially malignant disorders with dysplasia [262].

Advances in high-throughput screening techniques have allowed researchers to characterize salivary miRNome. Momen-Heravi et al. [263] analyzed a total of 734 human miRNAs by using NanoString nCounter and identified 13 significantly dysregulated miRNAs in saliva samples of OSCC patients compared with healthy controls, including 11 downregulated miRNAs (*miR-136*, *miR-147*, *miR-1250*, *miR-148a*, *miR-632*, *miR-646*, *miR-668*, *miR-877*, *miR-503*, *miR-220a*, and *miR-323-5p*) and two upregulated miRNAs (*miR-24* and *miR-27b*). In addition, *miR-27b* and *miR-136* gave an AUC of 0.964 and 0.963, respectively, for differentiating cancer patients from healthy controls. Of note, *miR-27b* could play a tumor-suppressor role in oral cancer by targeting Frizzled7 and Wnt signaling pathways, which are involved in cell proliferation, differentiation, migration, and invasion of oral cancer cells [264]. Another study identified 419 significantly altered miRNAs in tongue squamous cell carcinoma saliva samples as compared with healthy controls. Furthermore, expression levels of two upregulated miRNAs (*miR-33a-3p* and *miR-198*), and downregulated *miR-139-5p* were selected for validating in a cohort of 25 cancer patients and 25 healthy controls, observing significant downregulation of *miR-139-5p* in tumor samples. Moreover, after tumor surgery, this study evaluated the expression levels of *miR-139-5p*, which reached levels comparable to those of healthy controls. According to ROC curve analysis, *miR-139-5p* differentiated cancer patients from healthy individuals with an AUC of 0.805 [265]. Similarly, Chen et al. [266] found upregulated expression of *miR-139-5p* in normal tissue as compared with tissue from tongue squamous cell carcinoma, which could suggest the origin of salivary *miR-139-5p*.

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Like plasma and serum, salivary EVs currently represent a strong source of biomarkers for oral tumors because of protection from enzyme degradation [123]. Thus, Gai et al. [267] provided the first characterization of the miRNA profile in salivary EVs from oral cancer patients. *MiR-320-3p* and *miR-517b-3p* were expressed only in salivary EVs from oral cancer patients while *miR-412-3p* and *miR-512-3* were significantly increased in cancer patients as compared with healthy controls, presenting AUC values of 0.847 and 0.871, respectively. Importantly, functional analysis revealed the involvement of these miRNAs in different pathways normally active in oral cancer, such as TGFβ and ErbB signaling pathways. These findings indicated that salivary EVs could harbor specific molecular biomarkers with potential for diagnosing oral cancer. Recently, another study focusing on EVs isolated from oral swirls developed an algorithm-based risk classification of five miRNAs (*miR-24-3p*, *miR-21-5p*, *miR-99a-5p*, *let-7c-5p*, and *miR-100-5p*) discriminating normal mucosa and oral cancer with an AUC of 0.867. In addition, this dysregulation score showed significant statistical differences between non-oral cancer (histological normal epithelia and oral potentially malignant disorders) from oral cancer. Therefore, this miRNA signature constitutes a promising non-invasive screening tool [268]. Other salivary miRNAs, such as *miR-31*, *miR-125a*, and *miR-27b* were significantly altered in oral potentially malignant disorders [269,270]. Overall, these findings indicate that some cell-free miRNAs represent biomarkers for potentially oral malignant lesions and oral cancer; however, individually, they do not necessarily identify the anatomic origin of the systemic disease, nor do they discriminate potential malignancy from cancer.

3.3.3. MicroRNAs as prognostic biomarkers

Several tissues and circulating miRNAs have been put forward as prognostic predictors in oral cancer [246,271]. To the best of our knowledge, the first study that evaluated prognostic markers in liquid biopsy was by Liu et al. [232], observing increased plasma *miR-196a* levels associated with poor DFS. Xu et al. [245] examined the levels of *miR-483-5p* in serum samples from 85 oral cancer patients who

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underwent chemotherapy after surgery. High serum *miR-483-5p* expression was significantly associated with shorter OS, poorly differentiated tumors, late-stages, and lymph node metastasis. On the other hand, serum *miR-9* was downregulated in oral cancer as compared with oral leukoplakia patients and healthy controls, showing a significant association with different clinicopathological variables, lymph node metastasis, and TNM stage. Furthermore, low circulating *miR-9* levels were significantly associated with poor DFS and OS [272]. In another study, a multivariate logistic regression analysis showed that *miR-200b-3p* was an independent prognostic predictor, together with old age and cancer grade [246]. Interestingly, Liu et al. [247] analyzed the levels of *miR-187-5p* in oral cancer patients before and after tumor resection, observing that postoperative patients with decreased *miR-187-5p* levels presented a better prognosis than those with increased expression. These results indicated that circulating *miR-187-5p* represents a potential biomarker for predicting tumor recurrence. In the same line, downregulated plasma levels of *miR-486-5p*, *miR-375*, and *miR-92b-3p* were significantly associated with oral cancer recurrence after surgery, indicating their potential for discriminating the risk of recurrence [273]. More recently, Chen et al. [274] analyzed the expression level of *miR-99a* in serum samples from oral cancer patients and observed significantly lower levels as compared with healthy individuals and these levels were associated with shorter OS and DFS. In contrast to these studies that only evaluated the prognostic value of a single circulating miRNA, Shi et al. [275] were the first to report a miRNA-signature for predicting survival in oral cancer. This two-miRNA signature (*miR-626* and *miR-5100*) was independently associated with shorter DFS and OS. Moreover, a prognostic model integrating TNM stage and miRNA signature improved prognostic ability as compared with TNM stage or miRNA signature alone.

Although some studies have reported the prognostic value of circulating miRNAs, it is also essential to establish markers with predictive power for selecting therapies. To date, only one study has described the potential predictive value of circulating miRNAs for evaluating therapeutic efficacy in oral cancer. In this study, Nakashima et al. [276] evaluated the prognostic value of *miR-1290* in oral cancer

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patients who underwent preoperative 5-fluorouracil–based chemoradiotherapy, observing that patients with low circulating *miR-1290* expression levels had shorter DFS and OS as compared with patients showing high circulating *miR-1290* expression levels. Interestingly, patients with low *miR-1290* levels showed lower pathological differentiation and poor response for preoperative chemoradiotherapy, which indicates that circulating *miR-1290* is associated with tumor characteristics and resistance to chemoradiotherapy. These findings provide evidence regarding the potential of circulating therapy-responsive miRNAs as non-invasive predictive and prognostic biomarkers for oral cancer patients undergoing chemoradiotherapy.

3.4. CELL-FREE DNA AS A BIOMARKER

Since ctDNA can reflect the genetic and epigenetic alterations present in the tumor, cfDNA-based liquid biopsies have emerged as an opportunity for the non-invasive management of HNSCC.

3.4.1. Cell-free DNA concentration

Several studies have reported higher cfDNA levels in HNC patients compared to non-cancer controls, indicating its potential value as a diagnostic biomarker [277,278]. Coulet et al. [279] reported low levels of plasma cfDNA using a fluorimetric method in a cohort of 117 HNC patients, of which 65% showed plasma DNA concentrations of <100 ng/mL and 35% of > 100 ng/mL. Moreover, no correlation was observed between plasma DNA levels and tumor stage, tumor location, and gender. Later, Shukla et al. [280] quantified by spectrophotometry blood and plasma cfDNA levels in 150 non-metastatic OSCC, 150 healthy individuals, 90 potentially malignant lesions, and 150 post-treatment OSCC patients. Blood cfDNA levels didn´t show differences among the groups whereas in plasma significant higher levels were observed in OSCC compared to potentially malignant lesion groups. By the contrary, Li et al. [281] reported significantly higher cfDNA levels in OSCC patients compared to healthy controls, founding a significant

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association between plasma cfDNA levels and tumor size, TNM stage, and lymphovascular invasion. The ROC curve analysis revealed an AUC of 0.69 for discriminating oral cancer using a cut-off 20.2 ng/mL. Other authors have also reported an increase of cfDNA concentration in advanced cancer stages indicating its value as a biomarker for tumor progression [282]. In another study, Mazurek et al. [283] quantified cfDNA by quantitative real-time PCR (qPCR) of *TERT* gene in 200 HNC patients and 15 age-matched healthy controls being these levels higher in HNC patients compared to healthy controls, although this trend was not significant. However, when the analysis was performed according to the tumor anatomic sites, oropharyngeal cancer patients showed significantly higher cfDNA levels compared to other head and neck locations. This could be explained by a greater release of cfDNA to the bloodstream as a consequence of the inflammation process associated to oropharyngeal tumors. Also, significantly higher cfDNA levels were observed in stage IV tumors and N2-3 nodal affectation. Despite the diagnostic implications, high levels of cfDNA are not specific to cancer since physiological as well as other pathological conditions [284–286] can affect the cfDNA concentration. Interestingly, several researchers have also evaluated the association of cfDNA concentration as a prognostic biomarker in the lung [287], colorectal [288], breast [289], or oral cancer [281].

3.4.2. Cell-free DNA and salivary DNA for detecting somatic mutations

Since ctDNA harbors specific tumor genomic alterations and its analysis can provide a comprehensive view of tumor heterogeneity [290], several studies have explored the feasibility of detecting tumor DNA in HNC patients analyzing cfDNA for improving their clinical management. Wang et al. [118] interrogated the presence of HPV16 DNA sequences and somatic mutations present in *TP53*, *PIK3CA*, *CDKN2A*, *FBXW7*, *HRAS*, and *NRAS* in the tumor, plasma and saliva samples from 93 HNSCC patients. Tumor DNA was detected in 76% and 87% of saliva (n = 93) and plasma (n = 47) samples, respectively. Moreover, when both fluids were tested the detection rate increased to 96% (45/47). Interestingly, the detection of tumor DNA in saliva was

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influenced by the anatomic tumor location and stage, showing a 100% sensitivity for early-stage oral cavity tumors and a relatively high fraction of mutant DNA (median 0.65%). However, plasma was a more sensitive predictor than saliva tumor DNA in the oropharynx (91% vs. 47%), hypopharynx (86% vs. 70%), and larynx (100% vs. 67%) tumors, and in advanced-stage disease (92% vs. 70%). Interestingly, plasma HPV DNA showed a sensitivity of 86% (21/30 cases) while in saliva the detection rate was 40%, indicating the potential of HPV cfDNA as a biomarker for the detection HNSCC. Similarly, Perdomo et al. [291] analyzed in 36 plasma samples a total of 65 mutations in 5 genes (*TP53*, *NOTCH1*, *CDKN2A*, *CASP8*, and *PTEN*) identified previously by tumor sequencing. Opposite to Wang et al. study [118], only 42% (15/36) of cases were positive for tumor DNA, which could be explained by cfDNA degradation related to prolonged storage of plasma samples used in this study (>10 years). Specifically, a total of 18 mutations (28%) in *TP53*, *CASP8*, *NOTCH1*, and *CDKN2A* were detected both in tumor tissue and plasma from HNC patients. By sequencing the entire coding region of the *TP53* gene in 37 III-IV stages HNC patients they identified 36 mutations in tumor, 3 in plasma, and 26 in oral rinses. However, only 4 missense *TP53* mutations (p.Gly244Cys, p.Arg288Trp, p.Glu286Gly, and p.Val173Leu) were concordant between tumor and oral rinse and one missense mutation (*TP53* p.Val173Leu) was detected in the tumor, plasma and oral rinse, which indicates a low concordance for *TP53* mutations among tumor and liquid biopsies samples. The mutations detected only in oral rinses could reflect the tumor heterogeneity and/or the genetic alterations in the squamous epithelial cells lining the oral cavity as a result of field cancerization mostly related to alcohol and drinking consumption. Interestingly, oral cavity and oropharynx tumors showed a higher proportion of *TP53* variants in oral rinses compared to larynx tumors, which indicates the low fraction of mutant alleles from distal HNSCC in oral rinses. Highly sensitive detection techniques such as ddPCR or ultrasensitive NGS have been applied to detect low levels of tumor-derived DNA in HNC [292,293]. In this line, van Ginkel et al. [292] reported relative quantification measurements of mutant copies down to 0.01% in plasma samples from HNSCC patients. Recently,

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Shanmugam et al. [293] developed a targeted NGS panel (ultradeep sequencing) based on 7 genes (*CASP8*, *PIK3CA*, *FAT1*, *CDKN2A*, *NOTCH1*, *HRAS*, and *TP53*) for the identification of somatic mutations at low frequencies in saliva from 121 OSCC patients. This targeted sequencing approach detected 278 variants at ≥4% MAF in 87.6% (106/121) of tumor samples, of which 48.6% were missense variants and 28.8% non-sense mutations. In addition, mutations were detected in 75.5% of I-II stage tumors and 97% of III-IV stage tumors, with *TP53* remaining as the most mutated gene followed by *FAT1*, *CDKN2A*, and *CASP8*. The sequencing of oral rinses showed 377 variants at ≥0.1% MAF in 95.86% (116/121) of patients, of which 45.35% were missense variants and 28.9% non-sense mutations. Like in the tumor, *TP53* was the most mutated gene, followed by *FAT1*, *CDKN2A*, and *NOTCH1*. An overall concordance of 93.4% was observed between primary tumor and oral rinse samples, increasing this frequency in advanced stages (97%) compared to early ones (87.5%). Moreover, saliva revealed somatic mutations that were not detected by previous tumor sequencing, which evidences the potential of salivary tumor DNA for reflecting the intratumor heterogeneity in oral cancer. In addition, Galot et al. [294] analyzed the feasibility to detect ctDNA in locoregional recurrent and/or metastatic HNSCC patients by targeted sequencing using a custom panel of 604 genes with a MAF ≥1%. They detected ctDNA in 51% of patients (20/39), increasing this probability in patients with metastatic disease (14/20) compared to locoregional recurrent (6/19) (70% vs. 30%). As occurs in Wang et al. study [118], *TP53* (50%) was the most frequently mutated gene present in the cfDNA from HPV-ve HNC patients, followed by *PIK3CA* (15%). The ctDNA detection of different mutations associated with head and neck carcinogenesis such as tyrosine kinase receptors, Notch/HH/Wnt pathway, PI3K pathway, MAPK pathway, chromatin regulation, and DNA repair pathways showing the potential value of liquid biopsy for detecting HNC. Interestingly, a low concordance rate was observed between ctDNA and tumor variants (19%) in a cohort of 18 HNSCC patients (8 locoregional recurrent and 10 metastatic), while in metastatic patients the rate of solid tumor variants identified in ctDNA was 42%. Moreover, 26% of the plasma variants were not detected in

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the matched tumor tissue, indicating the potential of liquid biopsy cfDNA for representing the tumor heterogeneity and increasing the detection of actionable mutations. In addition, a multivariate analysis showed that the metastatic status, MAF, and ctDNA quantity were associated with the likelihood of detecting tumor tissue variants in plasma [294].

As in other tumors [295,296], somatic alterations in ctDNA have been associated with therapy resistance and disease recurrence in HNC [297,298]. Braig et al. [297] detected *RAS* mutations in ctDNA during Cetuximab-based treatment in 46% of non-responder HNSCC patients, while no *RAS* mutations were identified in the responder group, indicating that acquisition of *RAS* mutant clones during treatment correlated significantly with clinical resistance to the *EGFR* blockade. Importantly, the early detection of *RAS* mutations during treatment was a predictor of disease progression demonstrating the potential of cfDNA for monitoring therapy response. Later, Egyud et al. [299] demonstrated the utility of ctDNA analyses for preceding local recurrence of oral cavity tumors after surgical resection using tumor-specific profiles. However, ctDNA was not detected in two patients with distant recurrence which suggests a different mutational profile at recurrence compared to the primary tumor, and therefore, a limited utility of monitoring ctDNA based on primary tumor mutations. Like it occurs in plasma, Wang et al. [118] evidenced the potential of salivary tumor DNA for predicting tumor recurrence with high specificity and before clinical manifestation. Khandelwal et al. [298] analyzed in cfDNA the mutational profile of responder and non-responder oropharyngeal cancer patients using a commercial gene panel with a limit of detection of 1% MAF. After sequencing the tumor and plasma samples of 22 oropharyngeal cancer patients (11 non-responders and 11 responders), they identified somatic mutations in 12 and 11 samples, respectively. *TP53, FBXW7,* and *PTEN* were the most frequently mutated genes in tumor samples while in plasma the most altered were *EGFR, TP53,* and *APC*. Importantly, cfDNA sequencing revealed the same somatic variants of *TP53, FBXW7,* and *CDKN2A* detected in tumor in 4 patients HPV-ve non-responders and one patient HPV+ve responder, which suggests the potential of cfDNA for predicting recurrence in HPV-ve

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patients. Also, the high incidence of *TP53* mutations detected in cfDNA was also observed in the tumor suggesting a frequent dysregulation of *p53* in HPV-ve patients. In addition, survival analysis showed that HPV-ve status and the detection of ctDNA were associated with a worse OS. Recently, Hilke et al. [300] carried out for the first time a longitudinal analysis of ctDNA dynamics by ultra-deep sequencing in 20 patients with locally advanced HNC who underwent radio-chemotherapy. They found a significant negative correlation between the ctDNA present in plasma and the course of the treatment, showing a median decrease of 1% (baseline sample) to 0.01% (6-12 weeks after treatment). The presence of minimal residual disease was also evaluated, observing recurrence in the 100% of cases with ctDNA detectable after treatment. Interestingly, circulating HPV DNA showed a similar dynamic that ctDNA during treatment monitoring, remaining undetectable after treatment, which suggests its potential for diagnosis, therapy monitoring and recurrence detection in HNC. In another longitudinal study [301], serial plasma and saliva samples from 11 non-metastatic OSCC patients were analyzed for detecting minimal residual disease. Targeted deep sequencing of plasma cfDNA and salivary DNA revealed the tumor recurrence in five patients approximately four months before clinical recurrence. Interestingly, saliva tumor DNA was detected with greater sensitivity than ctDNA, showing a better efficiency for monitoring locoregional recurrence than plasma. Furthermore, the concordance rate of pathogenic gene variants between salivary tumor DNA and tumor tissue DNA was 72.7% whereas between plasma cfDNA and tumor tissue DNA was 9.1%, evidencing the different composition of both body fluids regarding the tumor-derived DNA content.

3.4.3. Cell-free DNA for detecting DNA methylation

Nowadays, numerous studies [302–304] have focused on the analysis of cfDNA methylation patterns in cancer as markers for diagnosis and prognosis, however, in HNC data are still limited. An important epigenetic pathway for gene transcriptional inactivation is hypermethylation at the cytosine-guanine dinucleotides (CpG) islands (CpGIs) within the promoter regions [305,306]. The first study that

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analyzed aberrant DNA methylation in liquid biopsies in HNC was performed by Sanchez-Cespedes et al. [307]. This study evaluated promoter hypermethylation of 4 genes (*p16, MGMT, GSTP1*, and *DAPK*) in 95 HNSCC tumors, detecting aberrant methylation of *p16*, *MGMT*, and *DAPK* in 55% (52/95) of cases. Interestingly, 42% (21/50) of HNSCC patients displayed the same methylation changes in serum with frequencies of *p16*, *MGMT*, and *DAPK* promoter hypermethylation of 36% (8/26), 48% (14/29), and 18% (3/17), respectively. Of note, *GSTP1* was not methylated in any serum and tumors samples. As a control group, they analyzed serum samples from 25 HNSCC patients who did not show aberrant methylation in the primary tumor and serum samples. Moreover, no association was found between the presence of aberrant methylation in serum DNA with the clinicopathological variables including stage, tumor size, node involvement, or tumor location [307]. Wong et al. [308] analyzed *p16* and *p15* methylation by methylation specific PCR (MSP) in 73 primary HNSCC tumors and 7 normal control samples finding methylation of *p16* and *p15* in 49% (36/73) and 60% (44/73) of primary tumors. Moreover, the methylation of *p16* and *p15* was evaluated by quantitative MSP (qMSP) in plasma samples from 20 HNSCC and 24 healthy controls. Methylated *p16* in plasma was found in 65% (13/20) of HNSCC and 20% (4/20) of healthy controls whereas methylated p15 was found in 60% (12/20) of HNSCC and 50% (15/24) of healthy controls. The plasma mean concentration of methylated *p15* and *p16* was significantly higher in HNSCC patients compared to healthy controls. Interestingly, methylation of *p16* and *p15* was detected in normal mucosa from chronic smokers and drinkers, suggesting the potential role of both markers for screening high-risk populations and monitoring treatment response [308]. Aberrant promoter methylation of *p16* has been also reported in serum from OSCC. Nakahara et al. [309] examined the methylation of *p16* in tumor and serum of 17 OSCC patients and 8 healthy controls by qMSP. Hypermethylation of *p16* was detected in the 64.7% (6/11) of tumors, of which 54.5% were also methylated in serum DNA. All patients were non-methylated in serum two months post-treatment. However, DNA methylation was detected in serum of 3 out 4 patients that developed recurrence, showing the

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potential of cfDNA methylation analysis for detecting disease recurrence. In 2008, Carvalho et al. [310] evaluated the promoter hypermethylation of different tumor suppressor genes in serum and saliva from 211 HNC patients and 527 healthy controls by qMSP. Various combinations of hypermethylated *CCNA1, DCC, DAPK, TIMP3, MINT31, MGMT*, and *p16* genes in saliva showed a sensitivity for detecting HNC ranging 24% to 35.1% with a specificity ranging 90% to 97%. In serum, six genes (*CCND2, TIMP3, HIC1, PGP9.5, TGFBR2,* and *CDH1*) were significantly associated with HNSCC, showing the multiple gene combinations a sensitivity ranging 38.6% to 81% with a specificity ranging from 92.3% to 92.5%. Overall, in this study both saliva and serum gene panels showed high specificity but a low sensitivity, which represents a limitation for its application as a screening tool for HNC detection. Similarly, hypermethylation of *EDNRB* has been also detected in serum cfDNA in 10% of HNSCC patients (10/100) with a high degree of specificity (100%). Interestingly, in 9 of these 10 patients promoter *EDNRB* hypermethylation was also detected in tumor and saliva rinses, suggesting the ability of liquid biopsies for reflecting the tumor methylation alterations [311]. Later, Schöck et al. [312] described *SEPT9* and *SHOX2* DNA methylation levels significantly increased in plasma of HNC patients compared to matched healthy controls, showing the combination of both genes an AUC of 0.80. Moreover, *SHOX2* and *SEPT9* plasma hypermethylation was significantly correlated with tumor and nodal categories. Based on survival analysis, HNC patients with positive *SEPT9* and *SHOX2* methylation in plasma were associated with a higher risk of death. In addition, methylation was found in 47% of patients with locoregional recurrence or distant metastasis, of which 78% positive methylation could be detected in plasma up to 377 days earlier with respect to clinicopathological confirmation of the tumor progression. Although *SHOX2* and *SEPT9* hypermethylation has been involved in other cancers [313–315], these data demonstrated the clinical value of both biomarkers for diagnosis, molecular staging, risk stratification, and disease monitoring of HNSCC patients [312]. In another study, the same research group evaluated the methylation of *SEPT9* and *SHOX2* using three different

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algorithms (relative quantification, absolute quantification and quasi-digital PCR) in order to improve the clinical performance of methylated ccfDNA [316]. Machado-Lais et al. [317] quantified by ddPCR the methylation levels of *CCNA1*, *CDH8*, *DAPK*, and *TIMP3* in 52 tumor and 20 plasma samples from oropharyngeal cancer patients. They detected methylation in 71% (37/52) of tumor samples whereas in plasma methylation was detected in 73.3% (11/15) of cases, of which *CCNA1* was methylated in 77% (7/11). Besides, a positive concordance of 80% was found between the methylation levels in tumor tissue and plasma. ROC curve based on ctDNA methylation revealed 73.3% sensitivity and 100% specificity (AUC = 0.867) for discriminating oropharyngeal cancer patients from healthy controls. Moreover, *CCNA1* methylation increase in plasma after treatment was associated with disease recurrence, that suggests the potential of cfDNA methylation for disease monitoring. Recently, Misawa et al. [318] identified by genome-wide characterization 21 epigenetic marker genes that were further analyzed in tumor tissues from 252 HNC patients by qMSP. This verification phase revealed a significant association between the hypermethylation of *ATP2A1*, *CALML5*, *DNAJC5G*, *GNMT*, *GPT*, *LY6D*, *LYNX1*, *MAL*, *MGC16275*, and *MRGPRF* and the increase of recurrence in oropharyngeal cancer patients. Then, the validation analysis showed the concordance of *ATP2A1*, *CALML5*, *DNAJC5G*, *GNMT*, *GPT*, and *LY6D* methylation status between tumor and matched ctDNA, however, only *CALML5*, *DNAJC5G*, and *LY6D* allowed to discriminate HNC patients from healthy individuals. Additionally, serial ctDNA analysis during treatment demonstrated the clinical utility of these three markers (hypermethylated *CALML5*, *DNAJC5G*, and *LY6D*) for evaluating the treatment response in HPV associated oropharyngeal cancer.

3.4.4. Multimodal profiling for circulating tumor DNA detection

An advantage of cfDNA-based liquid biopsy is the possibility to analyze simultaneously different genetic alterations such as somatic mutations or epigenetic aberrations contributing to understand the molecular landscape of HNC. Recently, Burgener et al. [319] carried

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out a multimodal profiling of plasma cfDNA without prior molecular characterization of the solid tumors in 30 HNSCC patients and 20 risk-matched healthy individuals. On the one hand, Cancer Personalized Profiling by deep sequencing (CAPP-seq) of cfDNA and matched peripheral blood leukocytes genomic DNA revealed the presence of ctDNA in 20 patients and allowed to identify 43 somatic mutations with MAFs ranging from 0.14% to 4.83%. Somatic mutations of driver genes identified in cfDNA from HNSCC such as *TP53, PIK3CA, FAT1,* and *NOTCH1,* showed similar mutational frequency compared to whole-exome sequencing data from 279 HNSCC tumors from The Cancer Genome Atlas (TCGA) database. Also, mutations in non-driver cancer genes such as *GRIN1* and *MYC* were detected in plasma, increasing the sensitivity of ctDNA detection. On the other hand, differential methylation analysis by cell-free Methylated DNA inmunoPrecipitation and high-throughput sequencing (cfMeDIP-seq) revealed 941-ctDNA hypermethylated regions enriched for CpGIs and HNSCC specific methylation patterns, which indicates that many differentially hypermethylated regions derived from tumor hypermethylated regions. Interestingly, in HNSCC patients a decrease in ctDNA fragment length was associated with ctDNA abundance, being correlated the mutation- and methylation-based ctDNA abundance. The detection of ctDNA in baseline plasma samples by both mutation- and methylation-based methods showed to be a predictor of poor OS, however, the tumor stage was not associated with the survival, which demonstrates the prognostic value of multimodal cfDNA profiling in HNC. Additionally, cfDNA methylome profiling revealed five differential methylated regions with prognostic value independently of ctDNA abundance. Also, the measurement of ctDNA abundance by cfMeDIP-seq was applied for assessing the response after definitive treatment allowing to identify patients at high risk of disease recurrence. This evidences its clinical utility for monitoring tumors with few recurrent or clonal mutations in serial samples. In another study, Mes et al. [320] showed that combined ctDNA analysis of CNAs, HPV DNA, and somatic mutations increased the sensitivity for HNC diagnosis compared to single genetic analysis. They developed a workflow based on low-coverage whole-genome sequencing to detect CNAs and HPV DNA and deep targeted

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sequencing of 12 cancer driver genes (*AJUBA, CASP8, CDKN2A, FAT1, FBXW7, HRAS, KMT2D, NOTCH1, NSD1, PIK3CA, PTEN*, and *TP53*) in plasma samples from HNC patients. CNAs and somatic mutations were detected in cfDNA of 52% (14/27) and 67% (18/27) of plasma samples, respectively. Importantly, a positive correlation was found between TNM stage and the detection of CNAs or mutations in plasma, whereas location of primary tumor and HPV-status were not associated with the detection of ctDNA. Moreover, HPV DNA was detected in the 100% HPV+ve HNC patients. Importantly, the detection rate of tumor DNA in plasma was increased up to 78% by the combined analysis of CNAs, HPV, and somatic mutations. As somatic mutations can differ between primary tumor and recurrence, the monitoring based only on the presence of somatic mutations detected in the primary tumor would not represent the real underlying genetic alterations present during post-treatment follow-up. In this sense, the detection of CNAs and HPV-DNA in plasma represents a promising strategy for detecting HNC with high specificity (>95%) when the tumor mutation landscape is unknown.

3.5. SALIVA DNA METHYLATION AS BIOMARKER

The detection of DNA methylation in saliva has emerged as a potential method for the early diagnosis in HNC. Numerous studies have evaluated the methylation profile of oral exfoliated cells collected from saliva (*i.e.*, salivary oral rinses or whole saliva) [321–324]. Thus, the presence of salivary DNA promoter hypermethylation represents a potential clinical biomarker in oral cancer.

3.5.1. Saliva hypermethylation: an early event

Promoter hypermethylation can be found early in tumorigenesis involving loss of cell cycle control, altered function of transcription factors, altered receptor function, disruption of normal cell–cell and cell–substratum interaction, inactivation of signal transduction pathways, loss of apoptotic signals, and genetic instability [325]. The characterization of the epigenetic alterations underlying oral carcinoma

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progression (normal mucosa-basal cell hyperplasia-dysplasia-carcinoma in situ- invasive carcinoma) is key for understanding the dynamic of the epigenetic landscape during the progression from oral precancer to cancer. Changes in DNA methylation patterns could be useful for predicting the rate and likelihood of malignant transformation, thus representing a potential biomarker for high-risk lesions [326]. Several studies have investigated the methylation profile of various tumor suppressor and DNA repair genes (such as *DAPK*, *MGMT*, or *p16*) in oral precancer [327–329]. Two longitudinal studies reported that *p16* hypermethylation in precancerous lesions could be a predictor of malignant transformation [330,331]. In another study [332], *DAPK* promoter hypermethylation showed similar frequency in tissue and blood from oral precancer (19.5% vs. 20.9%, respectively) and oral cancer (46.9% vs. 52.2%, respectively). Importantly, a significant correlation was found between tissue and blood *DAPK* hypermethylation, demonstrating the potential of methylated genes as early biomarkers in liquid biopsies. Of note, this study also detected *DAPK* hypermethylation at a very low level in salivary oral rinse samples. Previously, López et al. [333] reported high methylation of *p16* and *MGMT* in salivary oral rinses of patients with homogeneous oral leukoplakia. Interestingly, oral leukoplakia patients with a previous history of one or more OSCC showed an increased *p14* promoter hypermethylation, suggesting that inactivation *p14* increases the risk of oral cancer transformation. In addition, *p16* promoter methylation was observed in 50% of saliva samples from patients with oral submucosal fibrosis. This percentage increased up to 93.3% when *p16* methylation was analyzed in buccal cells obtained by cytobrush. The fact that no hypermethylation was found in healthy controls suggests *p16* as an early indicator of carcinogenic activity [334]. Recently, Cheng et al. [335] evaluated the methylation levels of *ZNF582* and *PAX1* genes in salivary oral rinses from normal controls, oral potentially malignant disorders (hyperplasia/hyperkeratosis, mild dysplasia, moderate dysplasia, and severe dysplasia), and oral cancer. The positive methylation rates of both genes (*ZNF582* and *PAX1*) gradually increased in line with oral lesion severity, showing a marked increase from mild dysplasia to moderate dysplasia. Moreover, salivary

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methylated *ZNF582* and *PAX1* could discriminate moderate dysplasia and other several oral lesions from normal controls as well as hyperplasia/hyperkeratosis and mild dysplasia lesions. Overall, these data support the very early occurrence of promoter hypermethylation in the process of oral carcinogenesis and, therefore, a progression model based on salivary methylated genes could have great clinical value for predicting the risk of oral malignant transformation.

3.5.2. Saliva hypermethylation as diagnostic biomarker

The first study to evaluate promoter hypermethylation in salivary DNA from oral cancer patients was carried out in 2007. Viet et al. [336] analyzed the promoter hypermethylation of five genes (*APC*, *ECAD*, *MGMT*, *p15*, and *p16*) in oral cancer, oral dysplasia, and normal controls. Methylation of *p16* was detected in 35% of oral cancer/dysplasia patients whereas *MGMT* and *p15* methylation were detected in 29%, *APC* in 14%, and *ECAD* in 7%. A high level of agreement was found between matched tissue and salivary DNA samples, presenting a correlation of 87.5% for *p16* and *ECAD*, and 62.5% for *MGMT* and *p15*. In another study, this research group identified a gene classifier based on 41 gene loci of 34 genes (*ADCYAP1*, *AGTR1*, *BMP3*, *CEBPA*, *EPHA5*, *ERBB4*, *ESR1*, *ETV1*, *EYA4*, *FGF3*, *FGF8*, *FLT1*, *GARB3*, *GALR1*, *GAS7*, *HLF*,*IHH*,*IL11*,*INSR*, *IRAK3*, *KDR*, *NOTCH3*, *NTRK3*, *p16*, *PKD2*, *PTCH2*, *PXN*, *RASGRF1*, *TFPI2*, *TMEFF1*, *TNFSF10*, *TWIST1*, *WNT2*, and *WT1*) that were methylated both in preoperative saliva and tissue from OSCC patients but were not methylated in postoperative saliva or normal subject. Interestingly, various diagnostic panels were performed using combinations of 4 to 10 genes, showing sensitivity values ranging from 62% to 77% and specificity values ranging from 88% to 100% [323]. A larger genome-wide DNA methylation study comprising 27,578 CpG sites performed by Guerrero-Preston et al. [322] revealed 301 potential tumor suppressor genes significantly hypermethylated in OSCC vs. normal tissues, 92 genes hypermethylated in leukoplakia vs. normal mucosa, and 143 hypermethylated genes in tumor vs. leukoplakia tissue. Based on multiple selection criteria, a total of 8 genes (*EDNRB*, *HOXA9*, *GATA4*,

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NID2, *MCAM*, *KIF1A*, *DCC*, and *CALCA*) were selected for validation by qMSP in 24 oral cancer and 12 normal oral mucosal tissue samples from a discovery cohort. Differential methylation between cases and controls was observed for *EDNRB*, *HOXA9*, *GATA4*, *NID2*, *KIF1A*, and *DCC* genes. The validation of *HOXA9* and *NID2* in an independent cohort including 55 tumors and 37 normal tissues showed the high diagnostic accuracy of *HOXA9* (85% sensitivity and 97% specificity) and *NID2* (87% sensitivity and 95% specificity) for discriminating HNC patients, attaining an AUC value of 0.97 when both genes were combined. Furthermore, the promoter methylation status of *HOXA9* and *NID2* was evaluated in salivary oral rinses from 16 oral cancer, 16 oropharyngeal cancer, and 19 healthy controls. ROC curve analysis yielded 0.75 of AUC for *HOXA9* and 0.73 of AUC for *NID2* for discriminating oral cancer patients from normal controls. Moreover, the combined analysis of two genes (*HOXA9* + *NID2*) improved this discriminatory power (AUC of 0.77). However, both genes showed a decrease in sensitivity for detecting oropharyngeal cancer patients using saliva, which could be due to the different etiology of these tumors. The authors also suggested that saliva could have less contact with tumors located in the oropharynx than those in the oral cavity, thus reducing the number of tumoral cells in saliva collection. Later, Langevin et al. [337] identified and validated a methylation classifier based on 22 CpGIs using oral rinses from 154 oral and pharyngeal cancer patients and 72 healthy controls through the Infinium Human Methylation 450 Bead Array. This saliva-based methylation biomarker panel showed an AUC of 0.92, which indicated its high degree of accuracy for predicting oral and pharyngeal carcinoma.

Over the last few years, several DNA-methylation studies in saliva have investigated the promoter hypermethylation status of various genes which had previously been identified as methylated in oral cancer tissue such as *CDKN2A*, *MGMT*, *DAPK1*, *RASSF1A*, or *ECAD* (Figure 12). Nagata et al. [324] analyzed the promoter methylation of 13 genes in salivary oral rinses from 34 oral cancer patients and 24 healthy controls, finding significantly higher levels of methylation for 8 genes (*ECAD*, *MGMT*, *DAPK*, *RARβ*, *p16*, *TMEFF2*, *WIF-1*, and *FHIT*) in tumor vs. normal salivary samples. Besides, a frequent event in oral

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carcinogenesis is the dysregulation of the *p16/CDKN2A gene*, a well-recognized tumor suppressor gene in cancer involved in cell-cycle control [338]. Aberrant promoter hypermethylation of *CDKN2A* has been reported in several HNC studies [339–341]. A pilot study performed by Kusumoto et al. [342] analyzed the association of different epigenetic alterations including DNA methylation and histone modification with *CDKN2A* inactivation in salivary oral rinses from 10 oral cancer patients and 3 healthy controls. Both DNA promoter methylation and/or histone modification were detected in all oral cancer patients, while *CDKN2A* presented negative or low expression in four cases. These findings suggest the involvement of different epigenetic alterations in the regulation of *CDK2A* expression. In another study, Ferlazzo et al. [343] analyzed *p16* and *MGMT* promoter methylation in saliva from 58 oral cancer patients and 90 controls. The methylation of either the *p16* or *MGMT* promoter regions was significantly higher in oral cancer compared to controls (44.8% vs. 13.4%). In addition, the *p16* promoter was more commonly methylated in oral cancer patients with methylenetetrahydrofolate reductase (MTHFR) CT/AC or TT/AA genotype with respect to normal MTHFR genotype. A similar frequency was observed for *MGMT* methylation, although no significant differences were found. These results suggest that MTHFR polymorphisms may have an important role in oral cancer, however, further research is necessary to understand their influence on the gene-specific methylation process. The *MGMT* gene is related to DNA repair and aberrant promoter hypermethylation has been observed frequently in HNC [341,344]. Recently, Liyanage et al. [321] investigated promoter hypermethylation using a panel of 4 tumor suppressor genes in saliva from 54 oral and 34 oropharynx cancer patients and 60 healthy controls using MSP coupled with densitometry analysis. *RASSF1A*, *TIMP3*, and *PCQAP/MED15* showed significant promoter hypermethylation in saliva from oral and oropharynx cancer groups compared to the control group, but no significant methylation was observed for *p16*. Promoter hypermethylation of the *p16* and *RASSF1A* genes was significantly associated with advanced-stage and high-grade oral cancer tumors. In addition, a significant association was observed between *p16*, *RASSF1A*, and *TIMP3* hypermethylation and high-grade

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oropharynx tumors. Interestingly, *p16* and *RASSF1A* were significantly associated with alcohol and tobacco consumption, whereas the promoter hypermethylation of the four tumor suppressor genes was significantly associated with betel quid chewing. These results suggest that external factors such as tobacco and alcohol may modulate the DNA methylation mechanisms contributing to oral cancer development. Furthermore, the combination of these 4 methylated genes presented a 91.7% sensitivity and 92.3% specificity for oral cancer detection, and 99.8% sensitivity and 92.1% specificity for oropharynx cancer. These results reflect the high discriminatory power of this salivary promoter hypermethylation gene-panel for both malignancies. The *TIMP3* is an extracellular matrix-bound protein that regulates the activities of matrix metalloproteinases and suppresses cancer cell growth, angiogenesis, migration, and invasion. Promoter methylation of *TIMP3* has been shown to favor oral cancer metastasis [345]. *PCQAP/MED15* is a transcriptional coactivator mediator essential for the regulated expression of protein-coding genes which have been linked to transforming growth factor-β signaling in HNC [346]. *RASSF1A* is one of the most frequently hypermethylated tumor suppressor genes in human cancer that modulates multiple apoptotic and cell cycle checkpoint pathways [347]. In another study [348] investigating two tumor suppressor genes (*p16* and *RASSF1A*), salivary promoter methylation was significantly more frequent in oral cancer patients (44.2% and 23.3%, respectively) compared to healthy controls (10% and 2%, respectively). High specificity was observed when both genes were combined, whereas the sensitivity of methylation detection was 53.5%. Moreover, *p16* and *RASSF1A* methylation was found to be significantly associated with advanced clinical stages, poorly differentiated tumors, and severe cellular atypia.

Recent studies investigating novel methylated tumor-related genes have made use of publicly available methylation microarray data to identify methylation markers for oral cancer diagnosis. Using data from the Gene Expression Omnibus (GEO) repository, Puttipanyalears et al. [349] identified 27,578 CpG sites and found that site cg01009664 of the *TRH* gene showed the greatest methylation levels HNC cells in comparison with non-malignant cells. This *TRH* site-specific

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methylation was validated in 9 healthy controls and 9 oral cancer patients by pyrosequencing, yielding a methylation percentage of 7% ± 3.43% in healthy cells in contrast to 63% ± 19.81% in cancerous cells. Moreover, high *TRH* methylation was found by qPCR in salivary oral rinses and oral swabs from a discovery cohort comprising 23 oral cancer patients and 33 healthy controls. The validation in oral rinses from 42 oral cancer and 54 healthy controls, demonstrated the high discriminatory power of *TRH* methylation (AUC = 0.93). Methylation of *TRH* in oral rinses could also discriminate oropharyngeal cancer patients from healthy controls with 82.61% sensitivity and 92.59% specificity (AUC = 0.88). Importantly, this gene presented very similar diagnostic ability when both sample cohorts were considered together (86.15% sensitivity, 89.66% specificity, and AUC = 0.93), indicating the potential of this biomarker for oral cancer detection. A more recent study [350] addressed by the same research group selected another specific CpG site of the *NID2* gene, cg22881914. After initial validation, the methylation of *NID2* was evaluated in salivary oral rinses from 43 oral cancer patients, 40 smokers, and 50 healthy controls by qPCR. High salivary methylation of *NID2* was observed in oral cancer patients whereas no methylation was detected in smokers and healthy controls. In addition, sensitivity increased up to 90.91% when the *NID2* methylation was detected in oral swabs from matched oral cancer patients (n = 22), which could be explained by the larger number of epithelial cells from cancer lesions using oral swabs vs. salivary oral rinses.

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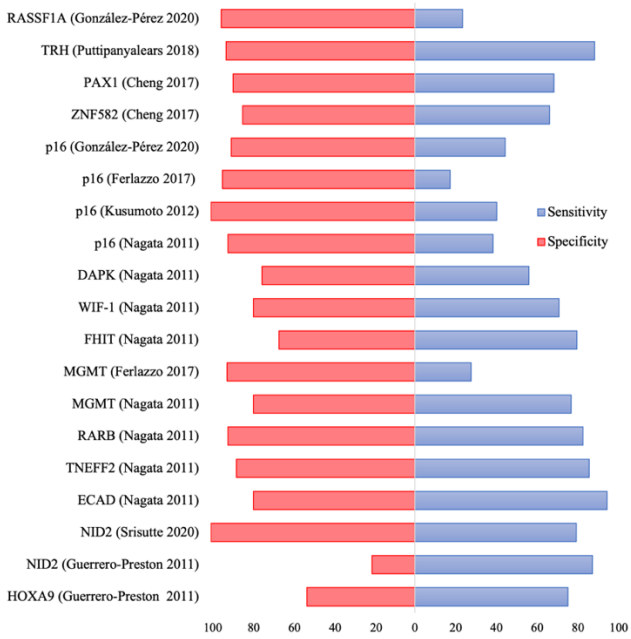

Figure 12. Reported sensitivities and specificities of DNA methylation biomarkers for the detection of oral cancer.

3.5.3. Saliva hypermethylation as prognostic biomarker

Numerous studies have demonstrated the prognostic value of gene promoter hypermethylation in several types of cancer [351–354]. However, few studies have evaluated DNA methylation markers in saliva for predicting prognosis in HNC [355,356]. Promoter hypermethylation of *TIMP3* gene in post-treatment salivary oral rinses has been reported as an independent prognostic factor for local recurrence in HNC [355]. Focusing on oral cancer, only one study [357] has described the prognostic potential of saliva DNA methylated targets. In this study, a total of 31,038 CpG loci were significantly associated with OS by data analysis of 88 oral cancer patients from the TCGA cohort. Afterward, an epigenome-wide array using oral rinses from 82 oral cancer patients (for replicating the putative survival-associated GpC loci) revealed 3,716 survival-associated CpG loci. Seven CpG loci belonging to *OPCML*, *ADCK4*, *ZFYVE26*, *GABBR1*,

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POLR3E, and *KIF11* genes were selected for validation by pyrosequencing in an independent cohort (n = 61). Of these, only the cg21022792 locus located in the body of *GABBR1* was validated as a survival-associated DNA methylation marker, which reflects the possible clinical utility of salivary DNA methylation markers for prognosis in oral cancer [357]. Although further research is required, DNA promoter hypermethylation has the potential to become a saliva-based prognostic marker in HNC.

Overall, the analysis of different tumor genetic and epigenetic biomarkers in liquid biopsies, such as blood and saliva have aroused a great interest in scientific community. Saliva has allowed the identification of different tumor-related biomarkers both in local and remote tumors to the oral cavity. Several studies have reported the potential diagnostic and prognostic value of salivary miRNAs in wide variety of malignancies, however, in CRC almost no evidence. Then, the characterization of salivary miRNA population could reveal novel miRNAs associated with CRC carcinogenesis, and therefore, the development of a non-invasive tool for CRC diagnosis. Also, numerous research efforts have been addressed on the identification of tumor biomarkers in saliva and plasma for improving HNC management. In this line, numerous studies have identified different cell-free miRNAs with an altered expression in oral cancer, suggesting its potential as promising diagnostic and prognostic biomarkers. Then, a comprehensive analysis of miRNAs detected in blood and saliva will allow to clarify the robustness and consistency of these biomarkers for diagnosing oral cancer. Nowadays, the characterization of the mutational landscape of HNC using fluid biopsies is gaining more and more attention due to its potential for identify and monitoring actionable mutations during tumor evolution in a non-invasive manner. In this context, although some studies have investigated the utility of ccfDNA and salivary total DNA for detecting and monitoring tumor somatic mutations in HNC using different methodological approaches, further research is required to provide more evidence about the potential clinical utility of saliva and plasma in the context of head and neck tumors. In addition to the genetic alterations, numerous studies have demonstrated the feasibility of detecting DNA methylation in saliva and

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blood from HNC patients. In particular, in saliva several genes have been associated with aberrant DNA methylation in HNC suggesting an important role of DNA methylation in head and neck carcinogenesis. Then, the analysis of the potential clinical impact of salivary DNA methylation in HNC is needed to provide more evidence about its value as biomarker. Since saliva is in direct contact with oral tumor lesions, different studies have evaluated the detection of salivary DNA methylation in oral cancer highlighting its promising value as diagnostic and prognostic biomarker. In this context, the characterization of the tumor methylation landscape in oral tongue squamous cell carcinoma (OTSCC) could reveal novel methylation markers associated with tumor carcinogenesis and with potential for early tumor detection.

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JUSTIFICATION, HYPOTHESIS, AND OBJECTIVES

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JUSTIFICATION, HYPOTHESIS, AND OBJECTIVES

Like the proverbial "*Elephant in the Room*", cancer is far too large to be safely ignored [1]. Cancer is a complex molecular disease that involves thousands of molecular alterations at multiple levels including genome, epigenome, transcriptome, proteome, and metabolome, representing a challenge when looking for clinical biomarkers for improving cancer management.

Nowadays, a large proportion of CRC and HNC cases are still diagnosed in advanced stages associated with poor prognosis and life expectancy. Around 50% of CRC patients present metastasis at diagnosis or develop it during disease course being the 5-year survival rate in these patients of <15% [2]. Although different CRC screening methods are available, none of them has been established as a well-accepted screening tool due to their low adherence rates, high cost, or low sensitivity [3]. In HNC, 60% of cases are diagnosed in advanced stages involving a high risk of locoregional recurrence and distant metastasis, with a 5-year survival rate of <50% [4]. Current standard detection methods for HNC include clinical examination supported by visual and tactile exploration, conventional medical imaging, and histopathological diagnosis, resulting in many patients being diagnosed at an advanced stage disease [5]. Taking into account this clinical context, research efforts are being addressed to the identification of novel biomarkers that improve CRC and HNC diagnosis.

Scientific evidence has demonstrated the high clinical value of liquid biopsies in oncology field. The possibility to detect and characterize tumor-derived material including CTCs, cfDNA, cell-free RNA (predominantly miRNAs), proteins, EVs, and tumor-derived platelets from fluid biopsies have emerged as a novel approach for non-invasive cancer diagnosis [6]. Importantly, liquid biopsies can reflect the molecular landscape of solid tumors and provide information regarding

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molecular cancer heterogeneity [7]. There is evidence regarding the potential of blood biomarkers for early cancer screening, therapy selection, prediction of clinical prognosis, tumor progression monitoring, and detecting the disease recurrence [8]. In addition to blood, numerous investigations have highlighted the potential of saliva as a non-invasive biopsy with application in local and systemic diseases [9]. Given that fluid biopsies have the potential for reflecting the molecular tumoral profile we hypothesized that associated with the tumor carcinogenesis and progression in both saliva and blood appear different genetic and epigenetic alterations (changes in miRNAs expression, somatic mutations, DNA methylation, etc.) related to tumor development that can be exploited for CRC and HNC management. Specifically, the hypothesis (H) are the following:

H.1. Salivary "omics" constituents could be used as potential biomarkers for cancer diagnosis.
H.2. Saliva presents miRNAs that could be used clinically to discriminate CRC from healthy individuals, constituting a new clinical tool for the diagnosis of this disease in its different stages.
H.3. In head and neck carcinogenesis different molecular genetic (somatic mutations and DNA integrity) and epigenetic (miRNAs and DNA methylation) alterations could be identified as potential biomarkers using fluid samples.
H.3.1. Saliva and blood present miRNAs that could be used as potential biomarkers for oral cancer diagnosis.
H.3.2. The length of scfDNA could be used as a potential diagnostic biomarker for oral cavity cancer due to during tumor necrosis large DNA fragments could be released into saliva.
H.3.3. Plasma ctDNA and salivary tumor DNA could reflect the mutational profile detected in tumor tissue in advanced HNC patients.
H.3.4. Salivary DNA methylation could be used as a potential biomarker in HNC.
H.3.4.1. Salivary DNA methylation could be used as a potential biomarker for HNC diagnosis.
H.3.4.2. Salivary DNA methylation could be used as a potential biomarker for predicting HNC cancer risk.

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H.3.4.3. Saliva presents tumor-specific DNA methylation markers that could be used clinically for the early detection of OTSCC.

Therefore, the general goal of this thesis is to explore the utility of combined analysis of blood and saliva samples for improving CRC and HNC diagnosis. Specifically, the objectives of the thesis are:

Objective 1. To evaluate, using a meta-analytic approach, the diagnostic value of saliva as a liquid biopsy in cancer distant to the oral cavity based on the fact that different biomolecules have been identified in saliva from cancer patients and salivary biomarkers can improve cancer detection.

The results corresponding to this objective are presented in Chapter 1 "*Salivary Biomarkers for Cancer Diagnosis: A Meta-analysis".*

Objective 2. To characterize the miRNAs profile present in saliva of CRC patients to identify new potential diagnostic biomarkers based on the fact that miRNAs have high stability on body fluids and saliva contains molecular components associated with local and systemic disease.

The results corresponding to this objective are presented in Chapter 2 *"A Novel Saliva-Based miRNA Signature for Colorectal Cancer Diagnosis*".

Objective 3. To evaluate the potential of different liquid biopsy approaches for improving HNC management, since saliva and plasma are biological sources of genetic and epigenetic biomarkers which harbor pathological molecular signatures reflecting the disease status.

Objective 3.1. To evaluate, using a meta-analytic approach, the diagnostic value of miRNAs in liquid biopsies as biomarkers for oral cancer detection.

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The results corresponding to this objective are presented in Chapter 3.1. *"MiRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic Review and Meta-analysis".*

Objective 3.2. To explore the potential of the scfDNA as a novel strategy to improve oral cancer diagnosis.

The results corresponding to this objective are presented in Chapter 3.2.*"Integrity and Quantity of Salivary Cell-Free DNA as a Potential Molecular Biomarker for Oral Cavity Cancer Diagnosis".*

Objective 3.3. To explore the value of plasma and saliva to detect somatic mutations from the advanced HNC.

The results corresponding to this objective are presented in Chapter 3.3. "*Somatic Mutations in Tumor and Plasma of Locoregional Recurrent and/or Metastatic Head and Neck Cancer using a Next-Generation Sequencing Panel".*

Objective 3.4. To explore the potential of salivary DNA methylation for HNC.

Objective 3.4.1. To evaluate the diagnostic value of salivary DNA hypermethylation as a biomarker for HNC diagnosis.

The results corresponding to this objective are presented in Chapter 3.4.1. *"Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-Analysis".*

Objective 3.4.2. To evaluate the potential value of salivary DNA hypermethylation as a biomarker for predicting HNC risk.

The results corresponding to this objective are presented in Chapter 3.4.2. *"Salivary DNA Methylation as an Epigenetic*

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Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-Analysis".

Objective 3.4.3. To characterize the tumor methylation landscape of OTSCC and to identify new potential diagnostic biomarkers in saliva for early detection of OTSCC.

The results corresponding to this objective are presented in Chapter 3.4.3. *"Genome-Wide DNA Methylation Profiling of Oral Tongue Squamous Cell Carcinoma".*

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CHAPTER 1

Salivary Biomarkers for Cancer Diagnosis: A Meta-analysis

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OBJECTIVE 1

SALIVARY BIOMARKERS FOR CANCER DIAGNOSIS: A META-ANALYSIS*

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ABSTRACT

Background: Saliva represents a promising non-invasive source of novel biomarkers for diagnosis and prognosis cancer. This meta-analysis evaluates the diagnostic value of salivary biomarkers for detection of malignant non-oral tumors to better define the value of saliva as an alternative liquid biopsy.

Materials and methods: We performed a systematic review and meta-analysis. PubMed, Embase, LILACS and the Cochrane Library were searched to identify articles that examined the potential of salivary biomarkers for the diagnosis of malignant non-oral tumors. To assess the overall accuracy, we calculated the diagnostic odds ratio (dOR), area under hierarchical summary receiver operating characteristic (AUC) curve, sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR) using a random- or fixed-effects model. Heterogeneity and publication bias were assessed. Statistical tests were two-sided.

Results: One hundred fifty-five study units from 29 articles with 11,153 subjects were included. The pooled sensitivity, specificity, PLR, NLR, dOR and AUC were 0.76 (95% confidence intervals (CI), 0.74–0.77), 0.76 (95% CI, 0.75–0.77), 3.22 (95% CI, 2.92–3.55), 0.31 (95% CI, 0.28–0.34), 13.42 (95% CI, 12.28–15.96) and 0.85 (95% CI, 0.84–0.87), respectively.

Conclusion: Salivary biomarkers may be potentially used for non-invasive diagnosis of malignant non-oral tumors.

Keywords

Saliva; salivary biomarkers; saliva-omics; cancer; diagnosis; liquid biopsy; meta-analysis.

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1. INTRODUCTION

Nowadays cancer represents one of the leading causes of morbidity and mortality worldwide, being the second leading cause of death globally. In 2015, cancer was responsible for 8.8 million of deaths and in the next two decades the number of new cases is expected to rise by about 70%. It is widely known that early detection of cancer benefits the patient, increasing the opportunity of an efficient treatment [1], therefore it is critical to promote non-invasive strategies which allow an early cancer diagnosis [2].

During the last years, liquid biopsy approaches have aroused great interest for early cancer detection, prediction of recurrent disease and assessment of therapeutic resistance mechanisms [3]. Liquid biopsy represents an attractive, novel and minimally invasive source of tumor derived biomarkers (cells, proteins, vesicles, and nucleic acids) shed into the bloodstream and other body fluids, such as saliva or urine [4]. The clinical application of liquid biopsy holds a promising approach to develop personalized medicine strategies the opportunity to determine the molecular landscape of the primary malignancy and metastases to perform real-time cancer management [5]. Importantly, the implementation of liquid biopsy routinely in the clinical practice will help to have a better picture of the tumoral heterogeneity, at the moment of diagnosis and during the evolutive course of tumor. Furthermore, circulating biomarkers will be crucial to diagnostic cancer before any clinical symptoms or/and radiological findings and to detect and track minimal residual disease [6].

Recently, accumulating evidence has demonstrated the diagnostic and prognostic value of saliva as promising novel and revolutionary liquid biopsy [7]. Saliva is a complex body fluid that contains a wide collection of proteins as well as DNA, mRNA, microRNAs (miRNA/miR), metabolites and microbiota [8]. As diagnostic approach, saliva offers many biochemical advantages over blood and tissue as non-invasive, easy storage, cost-effectiveness collection and, besides, dynamic availability for monitoring with less discomfort to the patient [9]. Saliva-based molecular diagnostics reflects the physiological conditions of the body, and therefore, offers the opportunity to monitor

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oral and systemic health and disease [10]. Ongoing advances in saliva research have allowed to the scientific community coined the term "saliva-omics" [11]. Salivary based approaches have been developed for the discovery of potential biomarkers into the six saliva-omics which englobe the genome, the microbiome, the epigenome, the transcriptome, the proteome and the metabolome [12]. Alterations in salivary concentrations of these molecules can be applied for the diagnosis, individual risk assessment, prognosis and monitoring of disease. To this regard a large number of studies provided different salivary biomarkers in head and neck cancers [13] and also in cancers distant to the oral cavity [12,14].

In spite of, the great interest of saliva, to the best of our knowledge, this is the first meta-analysis that evaluates the diagnostic value of salivary biomarkers for malignant non-oral tumors detection. We summarized the scientific evidence and carried out a systematic review and meta-analysis evaluating the impact of salivary biomarkers for the diagnostic of malignant non-oral tumors.

2. MATERIAL AND METHODS

2.1. Search strategy

A comprehensive literature search was carried out up through March 2018, including PubMed, EMBASE, LILACS and the Cochrane Library databases, without language restrictions. The keywords used for searching retrieval were free-style words and Medical Subjects Headings (MeSH): (saliva OR salivary) AND (cancer OR carcinoma OR tumour) AND (marker OR biomarker) AND (diagnosis OR diagnostic) AND (sensitivity OR specificity OR ROC curve). Free-text search was additionally performed. The references cited in eligible articles were also hand-checked to identify additional studies that could be inadvertently omitted during the electronic database search.

2.2. Study selection

Two authors (ORG and MMSQ) independently assessed the literature extracted by the search strategy. These authors selected

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articles that appeared to meet the inclusion criteria based on their titles and abstracts. The eligibility criteria for inclusion were as follows: (1) diagnostic studies using salivary biomarkers for malignant non-oral tumors; (2) subjects included cancer patients and non-cancer controls and (3) enough data available for generating in each study unit a two-by-two (2×2) table which contains true positive (TP), false positive (FP), true negative (TN) and false negative (FN) values. The exclusion criteria were the following: (1) reviews, letters, personal opinions, book chapters, case reports, conference abstracts and meetings; (2) duplicate publications; and (3) those experimental *in vitro* and *in vivo* studies that reported associations between saliva and cancer. Then, full articles in which salivary biomarkers were used as potential diagnostic markers for malignant non-oral tumors were independently evaluated. Disagreements between the two authors were discussed until a consensus was reached.

We defined a "study unit" (or dataset) as the analysis of the relation between salivary biomarkers and each anatomic tumor location. As different salivary biomarkers may be reported in the same manuscript, a single publication could then report more than one study unit.

2.3. Data extraction and quality assessment

Data extraction was performed by two researchers (ORG and MMSQ); who independently screened and retrieved data from each eligible study. The following data were collected for each study: name of first author, year of publication, anatomic tumor location, country, ethnicity, study design, number of cases and controls, type of biomarkers (proteomic, metabolomic, epigenomic, microbiomic, transcriptomic and genomic), fraction of saliva (whole, supernatant and pellet), type of salivary sample (stimulated or unstimulated) and candidate biomarkers selected for the analysis. If the required data were incomplete, attempts were made to contact the authors to get the missing information.

The quality assessment of the selected studies was separately conducted by three independent investigators (LMR, ORG and MMSQ) using the Quality Assessment of Diagnostic Accuracy Studies 2

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checklist (QUADAS-2) [15] as recommended by the Healthcare Research and Quality Agency. When discrepancies existed among investigators, an agreement was reached after discussion. The QUADAS-2 is a revised tool developed to assess the quality of studies of diagnostic tests which analyses four key domains: (1) patient selection, (2) index test, (3) reference test and (4) flow and times. Each domain was evaluated in terms of its risk of bias and besides, the first three domains were also evaluated in terms of applicability concerns. Risk of bias and concern of applicability for each domain were considered as "low", "high" or "unclear" [15]. If the response to the risk of bias and applicability questions were "low" risk or "low" concern, one point for each item was given. The articles were grouped based on their score into high (6 and 7 points), moderate (4 and 5 points) and low (0–3 points) quality categories.

2.4. Data synthesis and statistical analyses

The data analysis was performed using free R software (v.3.4.4; https://www.r-project.org), Reviewing Manager ver. 5.3 (Cochrane Collaboration, Oxford, UK), MetaDiSc software (v.1.4) [16] and STATA (v.14.0; https://www.stata.com). Raw data were used to calculate sensitivity and specificity of each study unit. To assess the overall accuracy, we calculated diagnostic odds ratio (DOR), area under hierarchical summary receiver operating characteristic (AUC), sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR) using the DerSimonian-Laird's estimator for random-effects or Mantel-Haenszel for fixed-effects. Interpretation of AUC values were as follows: >0.97 "excellent", 0.93–0.96 "very good", 0.75–0.92 "good" and 0.5–0.75 "reasonable" [17]. According to Grimes et al. [18] PLR in the range of 2–5, 5–10 and >10 represents small, moderate and large increases in the probability of disease when the test is positive and NLR in the range of 0.2–0.5, 0.2–0.1 and <0.1 represents small, moderate and large decreases in the probability of disease when the test is negative.

To assess the impact of the heterogeneity in the meta-analysis Higgins's I^2 test was used. This statistic represents the proportion of variability that is due to heterogeneity rather than to sampling error

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[19]. According to the I^2 test statistic the heterogeneity could be low (I^2 <50%) or high (I^2 >50%). If heterogeneity was high, the possible sources of heterogeneity were explored using Moses-Shapiro-Littenberg regression [19] and subgroup analyses. Publication bias was investigated using Deek´s funnel plot asymmetry test [20]. All statistical tests were two-sided. A *p* value less than .05 was considered statistically significant.

3. RESULTS

The systematic search identified a total of 751 studies (Supplementary Figure S1). After evaluating the titles and abstracts, a total of 38 articles were selected to a full-text review, of which nine were excluded according to the exclusion criteria. Finally, 29 eligible articles were used for the meta-analysis according to the inclusion criteria [21–48].

3.1. Study characteristics

The main characteristics of 29 articles are shown in Table 1. In total, 155 study units were included in the present meta-analysis. We identified eight studies with 71 study units on breast cancer, six studies with 29 study units on pancreatic cancer, four studies with 11 study units on oesophageal cancer, four studies with five study units on gastric cancer, three studies with 27 study units on lung cancer, three studies with 11 study units on ovarian cancer and only one study on colorectal cancer. Sample sizes ranged from 7–111 cancer patients and from 4–101 non-cancer controls. The articles were published between 1990 and 2018 and conducted in five countries: 17 in China, eight in USA, two in Japan, one in Russia and one in France. Twenty-seven articles were written in English and two articles in Chinese. All articles evaluated salivary biomarkers in adults and some studies appraised both saliva and blood. According to saliva-omics classification, 11 were proteomic studies, nine epigenomic studies, four transcriptomic studies, two metabolomic studies, two microbiome studies and two genomic studies. One of the studies included two complementary approaches (both transcriptomic and proteomic biomarkers) [46]. The saliva

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sample type was unstimulated in 19 studies and stimulated in eight studies, while no data regarding the saliva characteristics were available for two studies. Finally, eight studies evaluated the diagnostic value of a single biomarker whereas 21 studies analyzed several biomarkers.

3.2. Quality of included studies

There were five, 22 and two articles with good, moderate and low quality, respectively. Four studies presented a low risk of bias in three of the four domains and eight studies had a low risk in two of the four domains. Twelve studies did not show high risk of bias, eight studies were considered of high risk in two of the four domains and seven studies had high risk in one of the four domains. According to the applicability component the majority of, the studies were adequate with respect to the patient selection (93.10%), index test (96.55%) and reference standard (96.55%; Supplementary Figure S2). There were 20.69% ($N = 66/319$) sub-questions with "unclear" response. The most "unclear" responses were observed in the patient selection domain ($N = 24$, 36.36%) and in the reference standard domain ($N = 39$, 59.09%) of the risk of bias assessment. Most authors did not report clearly if a consecutive or a random patients' recruitment was performed and did not report a detailed description of the interval between index test and reference standard, and about the application of reference standard to the patients.

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Table 1. Main characteristics of the 29 studies included in meta-analysis.

Study	Cancer type	Country	Ethnicity	Sample size		Type of biomarker	Saliva specimen	Biomarkers
				Cancer	Non-cancer			
Chen et al., 1990	OC	China	NA	16	92	Proteomic	Supernatant	CA-125
Streckfus et al., 2000	BC	USA	NA	30	101	Proteomic	Whole	c-*erb*B-2
Brooks et al., 2008	BC	USA	Caucasian (including hispanic), black and asian	49	49	Proteomic	Supernatant	VEGF, EGF, CEA
Wu et al., 2009	GC	China	Asian	23	18	Proteomic	NA	1472.78Da, 2936.49Da, 6556.81Da, 7081.17Da
Zhang et al., 2010	PC	USA	Caucasian, african-american, asian, hispanic	30	60	Transcriptomic	Supernatant	KRAS, MBD3L2, ACRV1, CDKL3
Zhang et al., 2010	BC	USA	Caucasian, african-american, asian, hispanic, other	30	63	Transcriptomic and proteomic	Supernatant	CSTA, TPT1, IGF2BP1, GRM1, GRIK1, H6PD, MDM4, S100A8, CA6
Farrell et al., 2012	PC	USA	Caucasian, african-american, asian, hispanic	28	55	Microbiome	Pellet sample	*N elongate, S mitis, G adiacens*
Xie et al., 2012	OEC	China	Asian	32	16	Epigenomic	Supernatant	miR-21
Zhang et al., 2012	LC	USA	Caucasian and others	32	64	Transcriptomic	Supernatant	BRAF, CCNI, EGFR, FGF19, FRS2, GREB1, LZTS1
Xiao et al., 2012	LC	USA	Caucasian and others	26	26	Proteomic	Supernatant	calprotectin, AZGP1, HP
Lee et al., 2012	OC	China	Asian	21	35	Transcriptomic	Supernatant	H3F3A, SRGN, B2M, BASP1, AGPAT1, IL1B, IER3
Xie et al., 2013	OEC	China	Asian	39	19	Epigenomic	Whole/Supernatant	miR-10b, miR-144, miR-451, miR-21
Wu et al., 2013	OEC	China	Asian	67	50	Epigenomic	Whole/Supernatant	miR-144
Humeau et al., 2015	PC	France	NA	7	10	Epigenomic	Supernatant	miR-21, miR-23a, miR-23b, miR-29c, miR-216, miR-210, let-7c
Xie et al., 2015	PC	China	Asian	40	60	Epigenomic	Supernatant	miR-3679-5p, miR-940
Ye et al., 2014	OEC	China	Asian	100	50	Epigenomic	Supernatant	miR-21
Cheng et al., 2015	BC	China	Asian	27	28	Proteomic	Supernatant	SFAA: Phe, Trp, Met, Pro, Thr, Asp, Ser, Cit, Orn His, Gln, Leu, Val, Glu, Lys
Wood et al., 2015	BC	USA	NA	16	16	Proteomic	Whole	LRP
Yan et al., 2015	LC	China	NA	41	15	Microbiome	NA	*Capnocitophaga, Veillonela, Neisseria*
Machida et al., 2016	PC	Japan	NA	12	13	Epigenomic	Whole	miR-4644, miR-1246
Sazanov et al., 2017	CRC	Russia	NA	34	34	Epigenomic	Supernatant	miR-21
Takayama et al., 2016	BC	Japan	Asian	111	61	Metabolomic	Supernatant	Polyamines: SPM, N1-Ac-SPD, N8-Ac-SPD, Ac-PUT, CAD, Ac-SPM, DAc-SPM, DAc-SPD, PUT SPD, ORN, DAP
Xie et al., 2016	PC	China	Asian	55	75	Epigenomic	NA	lncRNAs: HOTAIR, PVT1
Xiao et al., 2016	GC	China	Asian	20	20	Proteomic	NA	CSTB, TPI1, DMBT1
Zhong et al., 2016	BC	China	Asian	30	25	Metabolomic	Supernatant	LysoPC(18:2), Palmitic amide, Phytosphingosine, LysoPC(18:1), PS(14:1/16:1), LysoPC(16:0), Acetylphenylalanine, Propionylcholine, LysoPC(22:6), MG(0:0/14:0/0:0), LysoPE(18:2/0:0), PC(18:1/16:0), Phenylalanine, Citrulline, Histidine, N-Acetylneuraminic acid, PE(22:0/20:4), 4-Hydroxyphenylpyruvic acid
Shu et al., 2017	GC	China	NA	23	54	Proteomic	Whole	Lectins: model GC (VVA and SBA), VVA
Xu et al., 2017	GC	China	NA	63	31	Genomic	NA	PPP2CA, PTGS2, ROCK1, SKP1, SLK
Li et al., 2018	OC	China	NA	26	92	Genomic	Supernatant	IEX-1
Liu et al., 2018	BC	China	NA	66	61	Proteomic	Supernatant	Lectins: model BC (BS-I, NPA, PNA, PTL-II and MAL-I)

Abbreviations: OC, ovarian cancer; BC, breast cancer; GC, gastric cancer; PC, pancreatic cancer; OEC, oesophageal cancer; LC, lung cancer; CRC, colorectal cancer; SFAA, salivary free amino acids; lncRNAs, long-non-coding RNAS; NA, not available.

3.4. Diagnostic accuracy

The total sample size for this meta-analysis was 11,153 adult subjects (cancer patients and non-cancer controls), including 155 study units. The diagnostic accuracy (sensitivity, specificity and 95% confidence intervals (CI) of each study unit included in this meta-analysis is shown in Figures 1–6. Biomarkers sensitivity and specificity varied widely from 31 to 100% and 34 to 100%, respectively. The overall diagnostic accuracy showed 0.76 (95% CI, 0.74–0.77) of sensitivity and 0.76 (95% CI, 0.75–0.77) of specificity. The overall dOR was 13.42 (95% CI, 12.28–15.96) and the pooled PLR and NLR were 3.22 (95% CI, 2.92–3.55) and 0.31 (95% CI, 0.28–0.34), respectively (Table 2). In addition to diagnostic accuracy, the summary receiver operating characteristic (SROC) curve was plotted, and the AUC was 0.85 (95% CI, 0.84–0.87), indicating "good" accuracy of the salivary biomarkers in malignant non-oral tumors (Figure 7).

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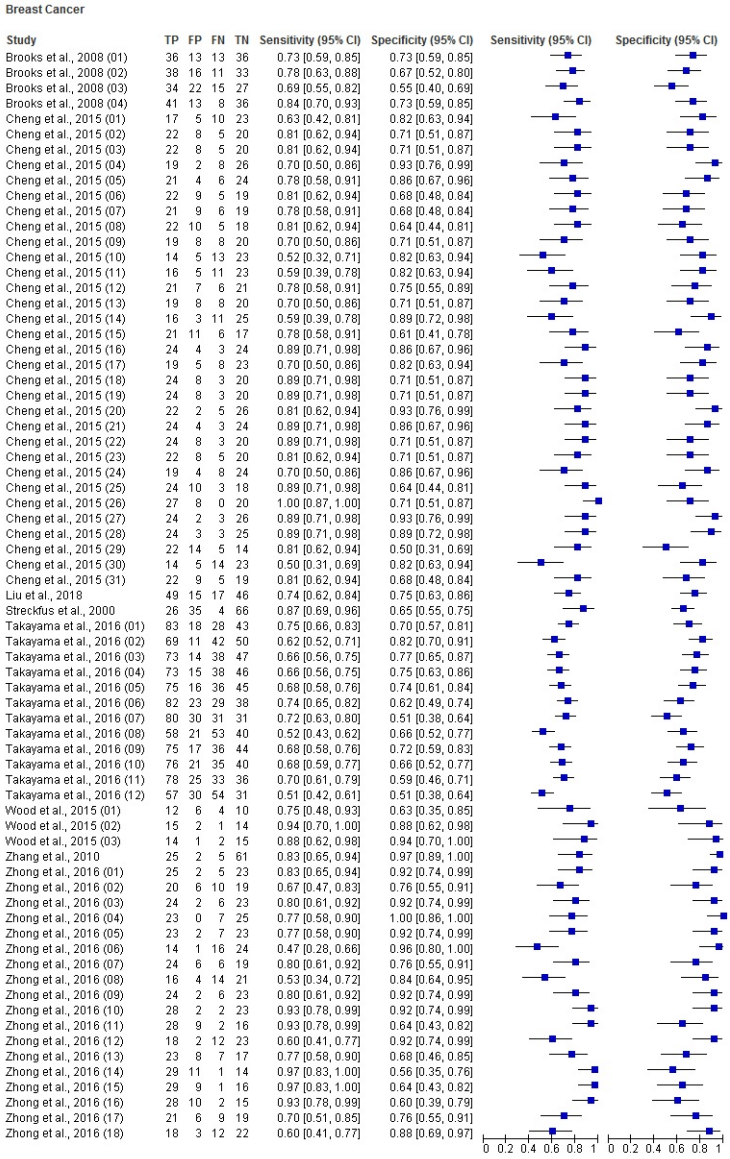

Figure 1. Paired forest plot with the diagnostic test accuracy (sensitivity, specificity and 95% confidence interval) of each unit study for the salivary biomarkers in the diagnosis of breast cancer.

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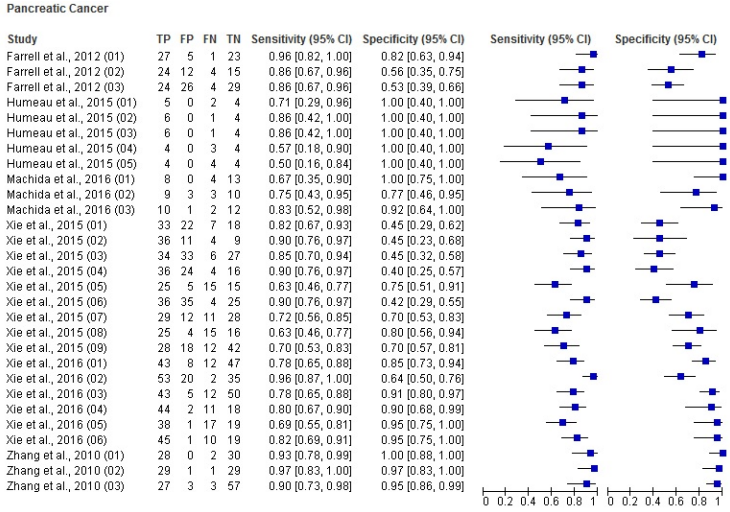

Figure 2. Paired forest plot with the diagnostic test accuracy (sensitivity, specificity and 95% confidence interval) of each unit study for the salivary biomarkers in the diagnosis of pancreatic cancer.

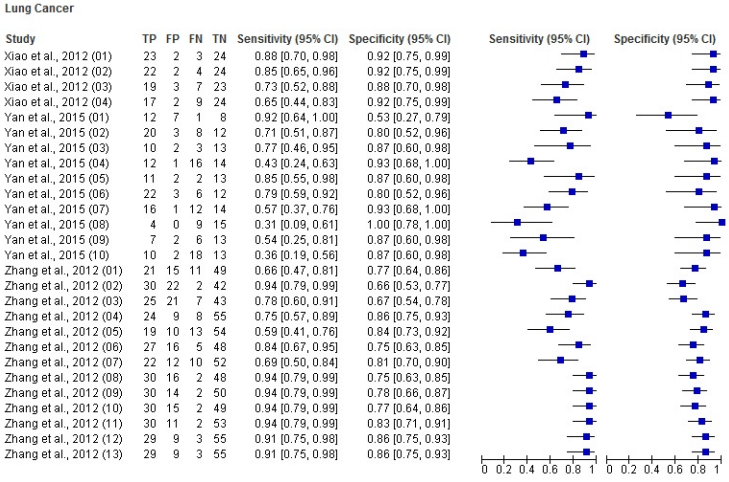

Figure 3. Paired forest plot with the diagnostic test accuracy (sensitivity, specificity and 95% confidence interval) of each unit study for the salivary biomarkers in the diagnosis of lung cancer.

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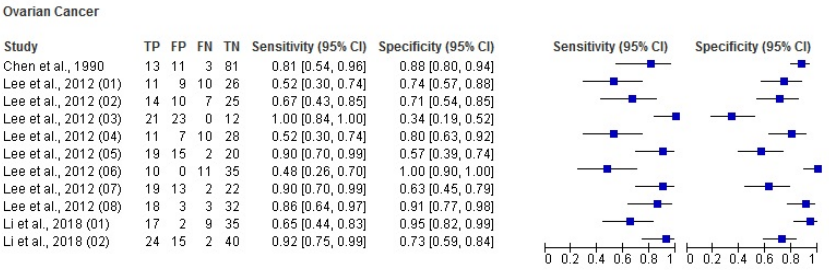

Figure 4. Paired forest plot with the diagnostic test accuracy (sensitivity, specificity and 95% confidence interval) of each unit study for the salivary biomarkers in the diagnosis of ovarian cancer.

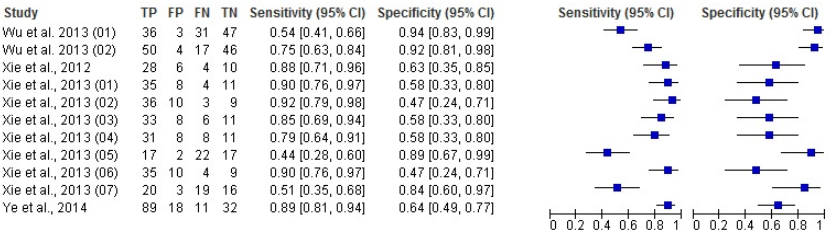

Figure 5. Paired forest plot with the diagnostic test accuracy (sensitivity, specificity and 95% confidence interval) of each unit study for the salivary biomarkers in the diagnosis of oesophageal cancer.

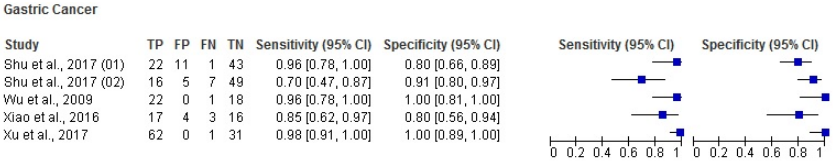

Figure 6. Paired forest plot with the diagnostic test accuracy (sensitivity, specificity and 95% confidence interval) of each unit study for the salivary biomarkers in the diagnosis of gastric cancer.

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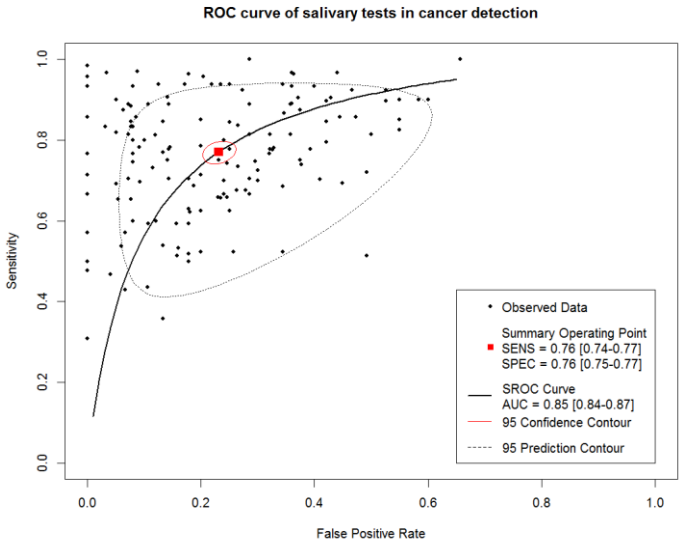

Figure 7. SROC curve with pooled estimates of sensitivity, specificity and AUC on the diagnostic value of salivary biomarkers in cancer.

3.4. Meta-regression and subgroup analysis

The studies included in the meta-analysis showed an overall heterogeneity (I^2 = 61.81%). In order to assess the sources of heterogeneity between the studies, we used the meta-regression with the following covariates as predictor variables: saliva fraction, type of saliva, anatomic tumor location, sample size, type of biomarker and type of control. The results indicated that anatomic tumor location was a potential source of heterogeneity in this study (p = .002; Supplementary Table S1). Consequently, we conducted a subgroup analysis according to the cancer anatomic location. The salivary biomarkers showed a high sensitivity and specificity for detecting each anatomic tumor location (sensitivities ranged from 0.73–0.91 and specificities ranged from 0.71–0.89; Figure 8), indicating "good" diagnostic accuracy for the detection of malignant non oral tumors. Summary data stratified into several subgroups are shown in Table 2 and Supplementary Figures (S3–S7).

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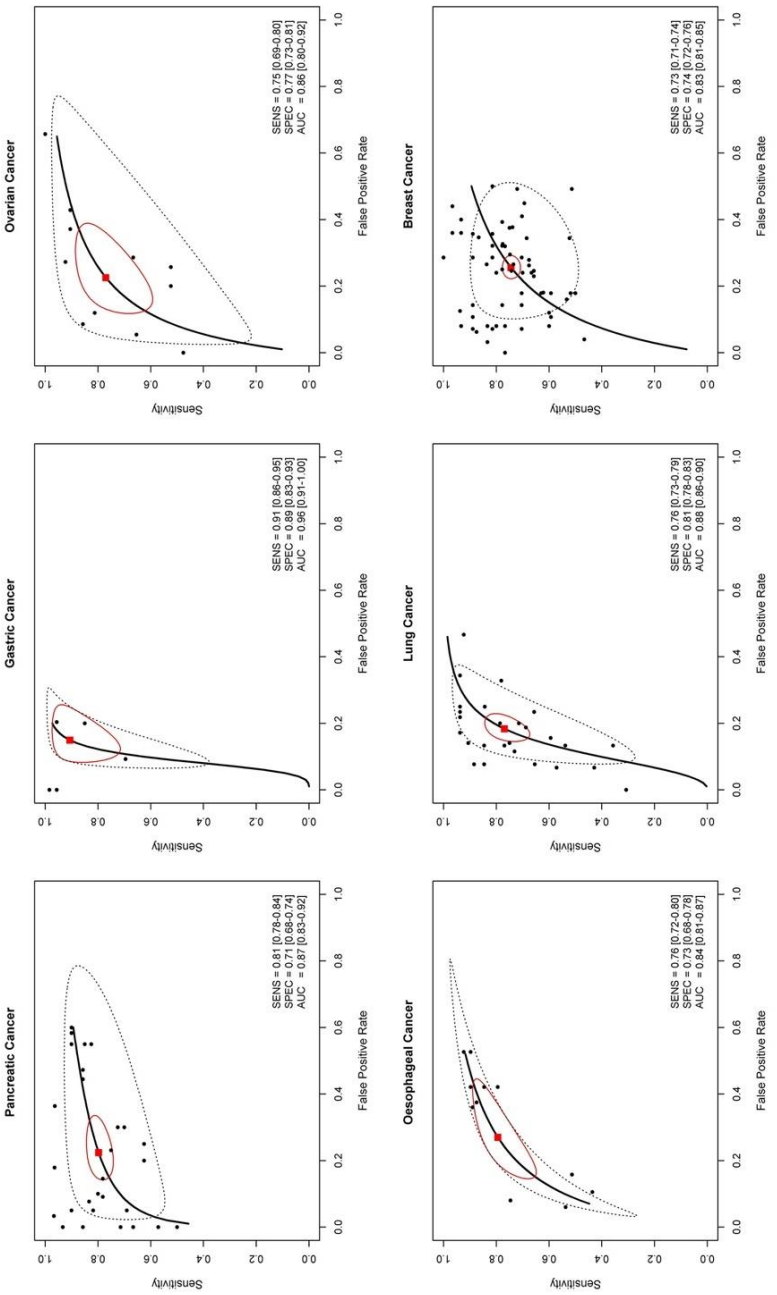

Figure 8. SROC curves for subgroup analysis based on the different anatomic tumor locations.

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Table 2. Subgroup analysis of saliva testing for cancer diagnosis based on different covariates.

	Number of study units	DOR (95% CI)	I^2 (%)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)
All	155	13.42 (12.28-15.96)	61.81	0.85 (0.84-0.87)	0.76 (0.74-0.77)	0.76 (0.75-0.77)	3.22 (2.92-3.55)	0.31 (0.28-0.34)
Saliva fraction								
Whole	12	15.90 (9.91-25.50)	9.24	0.86 (0.84-0.88)	0.76 (0.66-0.83)	0.85 (0.74-0.91)	3.46 (2.30-5.21)	0.27 (0.20-0.38)
Supernatant	129	13.11 (10.85-15.85)	64.76	0.84 (0.83-0.85)	0.77 (0.74-0.79)	0.77 (0.75-0.79)	3.20 (2.89-3.55)	0.31 (0.28-0.34)
Pellet	3	13.97 (3.25-60.02)	64.73	0.85 (0.78-0.91)	0.90 (0.76-0.96)	0.64 (0.42-0.81)	2.40 (1.39-4.15)	0.20 (0.08-0.47)
Type of saliva								
Stimulated	30	10.83 (8.04-15.60)	44.62	0.85 (0.84-0.86)	0.80 (0.75-0.84)	0.71 (0.63-0.78)	2.59 (2.12-3.16)	0.30 (0.25-0.36)
Unstimulated	114	14.33 (11.68-17.57)	65.15	0.83 (0.81-0.84)	0.77 (0.74-0.79)	0.77 (0.75-0.78)	3.39 (3.05-3.78)	0.31 (0.28-0.35)
Anatomic tumor location								
Pancreatic	29	15.86 (10.03-25.08)	59.15	0.87 (0.83-0.92)	0.81 (0.78-0.84)	0.71 (0.68-0.74)	3.32 (2.48-4.44)	0.28 (0.33-0.34)
Oesophageal	11	11.50 (8.03-16.599	0	0.84 (0.81-0.87)	0.76 (0.72-0.80)	0.73 (0.68-0.78)	2.71 (2.26-3.26)	0.34 (0.29-0.40)
Gastric	5	88.22 (18.71-416.07)	63.57	0.96 (0.91-1)	0.91 (0.86-0.95)	0.89 (0.83-0.93)	7.31 (3.16-16.88)	0.10 (0.03-0.31)
Lung	27	18.20 (14.07-25.53)	30.41	0.88 (0.86-0.90)	0.76 (0.73-0.79)	0.81 (0.78-0.83)	4.20 (3.68-4.79)	0.27 (0.33-0.31)
Ovarian	11	14.21 (7.24-27.89)	50.93	0.86 (0.80-0.92)	0.75 (0.69-0.80)	0.77 (0.73-0.81)	3.24 (2.13-4.93)	0.34 (0.23-0.50)
Breast	71	10.40 (8.24-13.13)	64.21	0.83 (0.81-0.85)	0.73 (0.71-0.74)	0.74 (0.72-0.76)	2.88 (2.56-3.24)	0.35 (0.31-0.39)
Sample size								
<50	24	15.60 (10.31-23.93)	0	0.85 (0.83-0.87)	0.71 (0.63-0.79)	0.83 (0.77-0.88)	3.76 (2.83-5.01)	0.34 (0.26-0.45)
>50	131	13.14 (10.91-15.82)	65.69	0.85 (0.78-0.91)	0.78 (0.75-0.80)	0.76 (0.73-0.79)	3.15 (2.85-3.48)	0.31 (0.28-0.34)
Type of biomarker								
Proteomic	49	13.44 (10.43-17.31)	41.13	0.85 (0.84-0.87)	0.78 (0.74-0.81)	0.77 (0.73-0.80)	3.34 (2.90-3.85)	0.30 (0.26-0.34)
Epigenomic	35	11.56 (8.56-15.62)	35.15	0.84 (0.83-0.86)	0.78(0.73-0.82)	0.75 (0.68-0.82)	2.83 (2.27-3.52)	0.31 (0.26-0.37)
Metabolomic	30	8.45 (5.91-12.08)	71.99	0.81 (0.79-0.83)	0.70 (0.66-0.74)	0.74 (0.69-0.78)	2.65 (2.20-3.20)	0.40 (0.35-0.47)
Transcriptomic	24	21.85 (13.31-35.88)	66.25	0.89 (0.87-0.91)	0.82 (0.74-0.88)	0.76 (0.71-0.81)	3.76 (2.91-4.87)	0.22 (0.15-0.31)
Microbiome	13	14.61 (8.32-25.64)	27.21	0.86 (0.84-0.89)	0.77 (0.63-0.87)	0.78 (0.65-0.87)	3.84 (2.46-5.99)	0.36 (0.25-0.52)
Genomic	3	87.89 (11.05-699.02)	68.94	0.96 (0.93-0.99)	0.69 (0.56-0.80)	0.81 (0.70-0.88)	10.96 (1.20-100.36)	0.11 (0.02-0.69)
Type of control								
HC	125	12.98 (10.79-15.62)	61.69	0.87 (0.86-0.88)	0.76 (0.75-0.78)	0.76 (0.74-0.79)	3.17 (2.71-3.51)	0.32 (0.29-0.35)
BC	11	24.88 (11.18-55.40)	57.46	0.92 (0.89-0.94)	0.85 (0.76-0.92)	0.76 (0.59-0.87)	4.72 (2.80-7.97)	0.26 (0.17-0.39)
NCCs	9	11.97 (6.31-22.73)	63.78	0.88 (0.85-0.92)	0.62 (0.51-0.72)	0.74 (0.80-0.92)	2.80 (1.96-3.98)	0.29 (0.21-0.39)

Abbreviations: CI, confidence interval; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio; AUC, area under the ROC curve, HC, healthy controls; BC, benign controls; NCCs, non-cancer controls.

3.5. Publication bias

The analysis of funnel plot asymmetry showed a significant asymmetry ($p < .001$), evidencing the presence of publication bias (Supplementary Figure S8).

4. DISCUSSION

In recent years, numerous studies [50] have highlighted the potential of saliva as a novel liquid biopsy in the field of personalized medicine. In this sense, saliva-based diagnostics offers a unique opportunity for precision medicine in cancer. To the best of our knowledge, this study is the first systematic review and meta-analysis analyzing the potential of salivary biomarkers for the diagnosis of malignant non-oral tumors. A total of 155 study units from 29 eligible articles with 5700 cancer patients and 5453 non-cancer controls were included in this meta-analysis. Overall, our analysis showed that salivary biomarkers had "good" diagnostic accuracy with an AUC of 0.85, sensitivity of 0.76 and specificity of 0.76. To further evaluate the diagnostic accuracy of salivary biomarkers described in the 29 studies, we also examined the dOR, PLR and NLR. The pooled dOR value was 13.42, which indicates a moderate overall accuracy for salivary tests. In addition, the pooled PLR value was of 3.22, indicating that patients with cancer have a 3.22-fold higher chance of having a positive test result compared to cancer free-controls. By contrary, the pooled NLR value was 0.31, indicating that the probability of a patient having cancer is 31% if the salivary test shows a negative result. These results indicate the potential of salivary tests for detection of malignant non-oral tumors, but further controlled studies are necessary to provide evidence of their real clinical utility. Furthermore, we also conducted a meta-regression to determine the effect of saliva fraction, type of saliva, anatomic tumor location, type of biomarker and type of control on heterogeneity. While anatomic tumor location may be a potential source of heterogeneity, our subgroup analysis clearly indicated that salivary tests have a high diagnostic efficiency for each type of cancer. According to the criteria described by Jones and Athanasiou [17], saliva tests showed "very good" accuracy for discriminating gastric cancer

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from cancer-free controls, with an AUC of 0.96, sensitivity of 0.91 and specificity of 0.89. Moreover, analysis of saliva tests in pancreatic, oesophageal, ovarian, lung and breast cancer revealed "good" diagnostic accuracy with AUC values of 0.87, 0.84, 0.86, 0.88 and 0.83, respectively.

Although our study represents the first work which analyze the utility of salivary biomarkers for non-oral cancers diagnosis that taking into account the omic fields and tumor types, two previous meta-analyses addressed the potential of salivary biomarkers as a clinical tool for head and neck carcinoma [51] as well as oesophageal, oral and pancreatic cancer detection [52]. Guerra et al. [51] evaluated a total of 15 articles that analyzed different types of biomarkers such as mRNA, proteins, metabolites and DNA. Biomarker sensitivity and specificity varied from 14 to 100% and 38 to 100%, respectively. However, Ding et al. [52] only evaluated the overall diagnostic values of salivary miRNAs for cancer detection, being the sensitivity and specificity reported of 77%. This data evidenced the interest of salivary miRNAs as a diagnostic tool for cancer detection.

On the other hand, salivary-based tests may be a promising tool for screening and diagnosis of cancer since several biomarkers detected in saliva are able to discriminate cancer patients from healthy individuals with a high degree of sensitivity and specificity. A number of molecular screening tests [2] have been established to detect cancer in early stages. However, current clinical blood-based screening/diagnostic tests are not accurate and specific enough [53]. For example, the prostate specific antigen (PSA), a clinically used biomarker for prostate cancer screening and diagnosis, has been associated with different deficiencies including false-positive results and overdiagnosis [54]. Molecular markers with a higher degree of sensitivity and specificity are urgently needed to improve the cancer screening and diagnosis. Saliva-based tests has shown potential as a tool for clinical diagnostics, accounting with even higher diagnostic sensitivity and specificity compared plasma-based test for cancer diagnosis [30].

To this regard, our study provides relevant information about the accuracy and the applicability of salivary biomarkers to improve cancer

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detection by using a dynamic and non-invasive sample. However, we found some important limitations during our analysis. Publication bias is one of the main limitations. This could be explained by the fact that positive diagnostic accuracy results are easier to publish than studies revealing negative results [55]. Moreover, small-size studies have a higher risk of uncontrolled bias. Therefore, future larger and randomized studies are needed to refute or support the actual scientific evidence. The methodological design of the studies included in the present meta-analysis represents another limitation. Several studies reported sensitive and specificity values without a validation phase. Furthermore, saliva-based diagnosis in cancer is still in its infancy for numerous tumors, limiting the number of studies included for specific types of cancer. Most of the selected studies did not perform a correlation analysis between the salivary biomarkers and TNM stage. From those studies evaluating the correlation with TNM stage, in two the salivary miRNA expression levels were not significantly correlated with cancer stage [29,39]. By contrary, another two studies reported a positive correlation between different salivary biomarkers and the cancer stage [33,44]. In spite of these data, the number of studies that correlated both variables were too low, it was not possible to perform a subgroup analysis based on TNM stage. Although this meta-analysis provides evidence about the potential clinical application of different salivary biomarkers, one of the most important circulating biomarker, the cell free-DNA (cfDNA) [56], was not included in the study because of the unique three studies focused on salivary cfDNA in non-oral cancer did not meet our inclusion criteria [57–59]. Despite of the potential clinical application of salivary cfDNA for detection EGFR mutation, not significant differences were observed among the salivary cfDNA concentration among lung cancer patients and control groups, which suggests that salivary cfDNA might not be an ideal marker for the diagnosis of tumors distant to the oral cavity [57]. In this sense, more research is required to effectively evaluate the potential of salivary cfDNA as diagnostic biomarker in cancer. In addition, most of the studies included in our meta-analysis are based on Asian populations, so subsequent studies should be performed in other ethnic populations. To face these challenges, research efforts should be

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addressed to validate salivary biomarkers for tumors diagnosis, characterization and monitoring. Importantly, our study provides important information on the current virtues and limitations of using salivary biomarkers that can help researchers improve future study design.

5. CONCLUSION

In conclusion, our findings provide evidence on the potential application of saliva as a novel liquid biopsy for cancer screening and early diagnosis. Despite some limitations, our data demonstrate that salivary biomarkers have enough sensitivity and specificity to be applied for non-oral cancer detection. Fortunately, ongoing advances in saliva-omics technology point towards a promising future of salivary biomarkers in cancer management. However, future guidelines on saliva research should require a randomized independent validation phase and multicenter design.

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CHAPTER 2

A Novel Saliva-Based miRNA Signature for Colorectal Cancer Diagnosis

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OBJECTIVE 2

A NOVEL SALIVA-BASED MIRNA SIGNATURE FOR COLORECTAL CANCER DIAGNOSIS*

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ABSTRACT

Salivary microRNAs (miRNAs) are of high interest as diagnostic biomarkers for non-oral cancer. However, little is known about their value for colorectal cancer (CRC) detection. Our study aims to characterize salivary miRNAs in order to identify non-invasive markers for CRC diagnosis. The screening of 754 miRNAs was performed in saliva samples from 14 CRC and 10 healthy controls. The differential expressed miRNAs were validated by RT-qPCR in 51 CRC, 19 adenomas and 37 healthy controls. Receiver operating characteristic (ROC) curves and logistic regression models were performed to analyze the clinical value of these miRNAs. Twenty-two salivary miRNAs were significantly deregulated in CRC patients vs. healthy individuals ($p < 0.05$) in the discovery phase. From those, five upregulated miRNAs (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p*, and *miR-491-5p*) were confirmed to be significantly higher in the CRC vs. healthy group ($p < 0.05$). This five-miRNA signature showed diagnostic value (72% sensitivity, 66.67% specificity, AUC = 0.754) to detect CRC, which was even higher in combination with carcinoembryonic antigen (CEA) levels. Overall, after the first global characterization of salivary miRNAs in CRC, a five-miRNA panel was identified as a promising tool to diagnose this malignancy, representing a novel approach to detect cancer-associated epigenetic alterations using a non-invasive strategy.

Keywords

Liquid biopsy; saliva; colorectal cancer; microRNA; circulating biomarkers; epigenomics.

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1. INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second in women worldwide. Regarding mortality data, it represents the fourth and the third cause of cancer death in men and women, respectively [1]. In 2030, the global burden CRC is expected to increase by 60% to over 2.2 million new cases and 1.1 million deaths [2]. Approximately 50% of CRC patients present metastasis at diagnosis or develop it during the course of the disease [3]. Currently, CRC diagnostic procedures are invasive or show low sensitivity and specificity, and thus research efforts are directed towards the search for less invasive biomarkers with higher accuracy to detect patients.

Recently, a growing number of researchers have reported the potential of microRNAs (miRNAs) as diagnostic biomarkers in CRC [4,5]. MiRNAs are small, noncoding RNA molecules of 18–22 nucleotides that act as regulators of gene expression, interacting mainly with messenger RNA. MiRNAs expression can be deregulated in the pathogenesis and development of human cancer, and they can act as oncogenes or tumor suppressors [6]. Several miRNAs are released by cells [7] in physiological and pathological conditions into the extracellular space and can then be incorporated, free or encapsulated, into the bloodstream and other body fluids, such as saliva, urine, and cerebrospinal fluid [8], as "circulating miRNAs"—they have high stability due to their small size and their association with extracellular vesicles and RNA-binding proteins, such as argonaute-2 or high-density lipoproteins [9]. Due to their unique characteristics, miRNAs detected in liquid biopsies are promising biomarkers for cancer diagnosis, with a clear potential application for patients management in daily clinical practice. To date, several authors have described circulating miRNAs in blood with high diagnostic value and prognostic impact in CRC [10].

Ongoing scientific evidence in the salivary research field reinforces the interest of this biofluid as a source of potential biomarkers, identified in different salivary '-omics' including the genome, the microbiome, the epigenome, the transcriptome, the proteome and the

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metabolome [11]. In this sense, saliva presents many advantages as a diagnostic tool over blood or tissue. The sample collection is even less invasive than blood and easy to store and transport [12]. In addition, saliva can be collected in shorter intervals than other diagnostic samples in order to provide updated tracking and patient follow-up without involving any discomfort for the patient. Aberrant expression profiles of salivary miRNAs have been detected in different types of cancer, showing their power as a discriminatory clinical method [13]. Unlike other tumors, only salivary *miR-21* was analyzed in CRC by Sazanov et al. [14], finding *miR-21* expression significantly higher in the saliva of CRC patients compared to healthy controls.

Based on these findings, we have analyzed miRNAs profiles in a large cohort of saliva samples from CRC patients, adenomas and healthy individuals. With this approach, we were able to identify and validate salivary miRNAs with discriminatory value in detecting CRC.

2. EXPERIMENTAL SECTION

2.1. Study subjects and sample collection

This study was approved by the Galician Ethics Committee of Clinical Research (Ref. No. 2013/371) and written informed consent was obtained from all participants involved in this study at the time of recruitment. The methods were performed in accordance with relevant guidelines and regulations, following the recommendations of the PRoBE design [15]. This study complied the principles outlined in the Declaration of Helsinki [16].

One hundred and seven saliva samples from 51 CRC patients, 19 adenomas and 37 healthy individuals were collected from 2014 to 2018 at Oncology and Digestive Services from Complexo Hospitalario Universitario of Santiago de Compostela (SERGAS) in Galicia, Spain. All CRC and adenoma cases were confirmed by histopathological analysis of the corresponding tissue biopsy. Tumor stage in CRC patients was determined in accordance with the Tumor-Node-Metastasis (TNM)-based staging system promulgated by the American Joint Commission on Cancer (AJCC). None of the patients under study

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received chemotherapy or radiotherapy before sample collection. Eight patients underwent resective surgery before sample collection. Patients with a history of familial adenomatous polyposis, hereditary non-polyposis CRC, inflammatory bowel disease or diagnosis of other cancer in the last 5 years were excluded from this study. This study was designed as two phases: a discovery phase including 24 saliva samples (10 healthy controls and 14 CRC, including 7 non-metastatic and 7 metastatic patients) and a validation phase comprising 107 saliva samples (37 healthy controls, 19 adenomas and 51 CRC), including those used for the discovery phase (Figure S1). Clinicopathologic characteristics and lifestyle habits are summarized in Table S1. The detailed saliva collection and processing can be found in Supplementary Methods.

2.2. Saliva RNA extraction

Total RNA containing miRNAs was extracted from 500 µL of saliva using miRNeasy extraction Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with some modifications (Supplementary Methods). The concentration and quality of total RNA were analyzed using the Agilent 2100 Bioanalyzer and Quantus™ Fluorometer (Promega).

2.3. Salivary miRNA discovery profiling with TaqMan Low-Density Arrays

MiRNA expression profiling was performed using TaqMan® Low-Density Human MicroRNA Arrays (TLDA, Applied Biosystems, Foster City, California, USA). Each TLDA card detects 384 probes including 377 human miRNAs, 3 endogenous control assays and 1 negative control assay. Together, the 2 array cards (A and B Cards v3.0) can detect 754 mature miRNAs present in miRBase v14. This miRNA microarray assay was performed using 3 µL (1–350 ng) of total RNA from each sample, according to the manufacture's protocol (Supplementary Methods).

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2.4. MiRNA expression analysis using RT-qPCR

Expression levels of candidate miRNAs were validated by miRNA TaqMan RT-qPCR assays (Applied Biosystems) according to the manufacturer´s protocol (Supplementary Methods). Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method [17] and normalized with *miR-193b-3p*, which showed high stability among our samples. The selection of this internal control for quantification of salivary miRNA was described in Supplementary Methods (Table S2 and Figure S2).

2.5. Functional enrichment analysis

Pathway enrichment analysis was performed using the DNA Intelligent Analysis (DIANA)-miRPath v3.0 software [18] to determine the miRNA regulatory roles and to identify the significant Kyoto Encyclopedia of Genes and Genomes (KEGG) molecular pathways. The algorithm miRTarBase v7.0 was used to identify the validated targets *miR-186-5p*, *miR-29a-3p*, *miR-29c-3p*, *miR-766-3p* and *miR-491-5p*. We selected the "pathways union" option of the miRPath software. The threshold ($p < 0.05$) and the false discovery rate (FDR) ($p < 0.05$) were calculated by Fisher´s exact test.

2.6. Statistical analysis

All statistical analyses with IBM SPSS Statistics 20 and graphs were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, California, USA). Hierarchical clustering was performed using the R environment (http://www.r-project.org/) and a heatmap was generated using a function of heatmap.2 in gplots package. Two-tailed Mann–Whitney U test or the Kruskal–Wallis test was used to evaluate the differential expression of salivary miRNAs. Multivariate logistic regression analyses were performed to establish the best miRNA panel to discriminate CRC from healthy individuals and those stage IV CRC with bad evolution of the disease (defined as progression or death within the 10 months after sample collection). The following formulas

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were generated after the logistic regression to provide a score for classifying patients vs. healthy controls and good/bad prognosis stage IV CRC patients: score for diagnosis = −9 − (1.4 * expression$_{miR\text{-}29a\text{-}3p}$) + (0.6 * expression$_{miR1\text{-}86\text{-}5p}$) + (2.1 * expression$_{miR\text{-}29c\text{-}3p}$) − (0.38 * expression$_{miR\text{-}491\text{-}5p}$) + (0.01 * expression$_{miR\text{-}766\text{-}3p}$); risk-score for prognosis = 1.09 + (5.4 * expression$_{miR\text{-}29a\text{-}3p}$) − (1.4 * expression$_{miR\text{-}186\text{-}5p}$) − (5.7 * expression$_{miR\text{-}29c\text{-}3p}$) + (1.03 * expression$_{miR\text{-}491\text{-}5p}$) + (0.39 * expressionmiR-766-3p). Receiver operating characteristic (ROC) curves were constructed, and area under the ROC curve (AUC) with 95% of confidence intervals (CIs) was obtained to evaluate the diagnostic accuracy of individual salivary miRNAs and the miRNA panel. Internal validation with the bootstrap method was used to adjust the overfitting. A total of 2000 random samples with replacement were generated. The AUC and the 95% CIs for the sensitivity and specificity were estimated using the pROC package in R software. Cut-off selection was made based on the value that provided the highest sensitivity and specificity to discriminate healthy controls vs. CRC patients and CRC patients with good vs. bad outcomes. For survival analyses Univariate/Multivariate Cox Regression was applied together with the Kaplan–Meier curves and the Log-Rank test. Spearman correlations were performed to determine the relationship among the different miRNAs and between each miRNA and the carcinoembryonic antigen (CEA) blood levels. *p*-value < 0.05 was set as the level of statistical significance.

3. RESULTS

3.1. Salivary miRNA expression profiling identified a specific expression pattern in CRC

During the discovery phase, 754 miRNAs were profiled in saliva samples from 14 CRC patients and 10 healthy controls. From all miRNAs included in the panel, 31 miRNAs (4.11%) were detected in all samples, 194 miRNAs (25.73%) were detected in >50% and 294 miRNAs (38.99%) were not present in any salivary sample. To detect those miRNAs characterizing CRC patients, we selected miRNAs

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present in at least 70% of patients, being significantly differentially expressed in patients (metastatic and/or non-metastatic) vs. healthy controls with a minimum fold change of 1.5 (up or down). Following these criteria, 22 miRNAs were found significantly deregulated in the saliva of patients compared to healthy controls. Expression levels of 20 miRNAs were found upregulated while only two were downregulated. The Venn diagram (Figure S3) shows differentially expressed miRNAs in cancer and controls taking into account the tumor stage. Thus, 18 miRNAs were found differentially expressed in non-metastatic patients, two in metastatic patients and six in both non-metastatic and metastatic patients.

3.2. A panel of salivary miRNAs was validated for CRC

Ten miRNAs (*miR-186-5p, miR-29a-3p, miR-766-3p, miR-16-5p, miR-195-5p, miR-140-3p, miR-142-5p, miR-29c-3p, miR-150-5p,* and *miR-491-5p*) were chosen from a total of the 22 differential miRNAs based on their fold change and their previous description as miRNAs expressed in CRC tumors (Figure 1). To verify whether the selected miRNAs were upregulated in saliva, we first examined their levels in a set of 30 CRC and 15 healthy individuals. Significant differences were found in seven miRNAs (*miR-186-5p, miR-29a-3p, miR-766-3p, miR-16-5p, miR-29c-3p, miR-150-5p* and *miR-491-5p*). These miRNAs were further validated in the full large-scale set of saliva samples from a total of 107 CRC, 19 adenomas and 37 healthy individuals. As shown in Figure 2, after the complete analysis, we found that salivary levels of *miR-186-5p* ($p = 0.0136$), *miR-29a-3p* ($p = 0.0376$), *miR-29c-3p* ($p = 0.0112$), *miR-766-5p* ($p = 0.0381$), and *miR-491-5p* ($p = 0.0366$) were statistically significantly different between the CRC and healthy groups. Salivary expression levels of *miR-16-5p* and *miR-150-5p* were higher in CRC than in healthy individuals, but no significant differences were obtained. In addition, the expression levels of validated miRNAs were also increased in patients with adenomas compared to healthy controls, however no significant differences were found.

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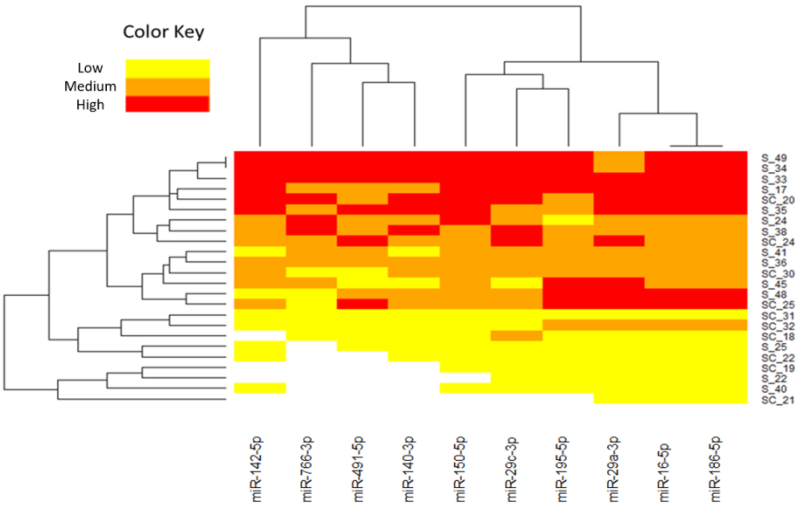

Figure 1. Hierarchical clustering heatmap of 10 miRNAs selected for the validation phase with a > 1.5-fold change difference (p < 0.05) between CRC patients and healthy controls. Each row represents one sample and each column represents the expression profile of a single miRNA. The relative miRNA expression changes are expressed by three colors from red to yellow, as shown at the top, where red represents the high expression, orange medium expression, and yellow low expression. The miRNA clustering tree is shown on the top and the sample clustering dendrogram is presented on the left. S1–S12 correspond to salivary samples of CRC patients and SC1–SC10 correspond to salivary samples of healthy controls. *Note:* miRNAs, microRNAs; CRC, colorectal cancer.

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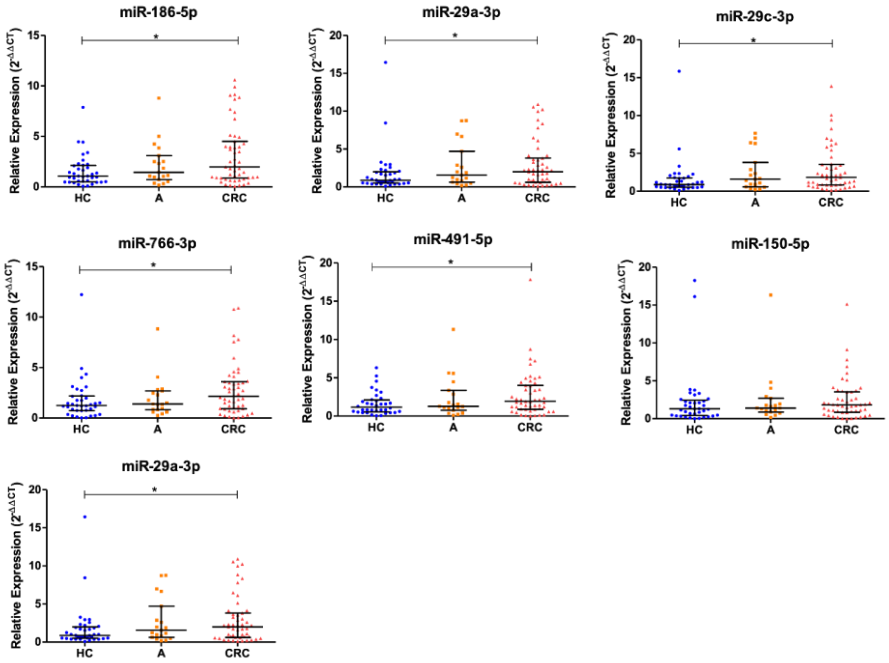

Figure 2. Salivary miRNA expression levels in healthy controls, adenomas and CRC patients in the validation phase of the study. The relative expression levels of selected salivary miRNAs were normalized to endogenous control (*miR-193b-3p*). The two-tailed Mann-Whitney U test was performed to examine the difference between groups of seven miRNAs (* $p < 0.05$). *Note:* HC, healthy controls; A, adenomas; CRC, colorectal cancer; miRNAs, microRNAs.

ROC curve analyses were also performed to determine the diagnostic value of the five validated miRNAs (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p* and *miR-491-5p*) for CRC detection. Importantly, all miRNAs showed good power to discriminate CRC patients from healthy controls with AUC up to 0.659. Importantly, the combined analysis of the five miRNAs showed a higher AUC (0.754), with a sensitivity of 72% and a specificity of 66.67%, differentiating CRC patients from healthy individuals (cut-off value = 0.5132) (Figure 3 and Table S3). Internal validation of the five-miRNA panel showed a bootstrap optimism-corrected AUC value of 0.744 (95% CI = 0.649 to 0.844) for discriminating CRC patients from healthy controls.

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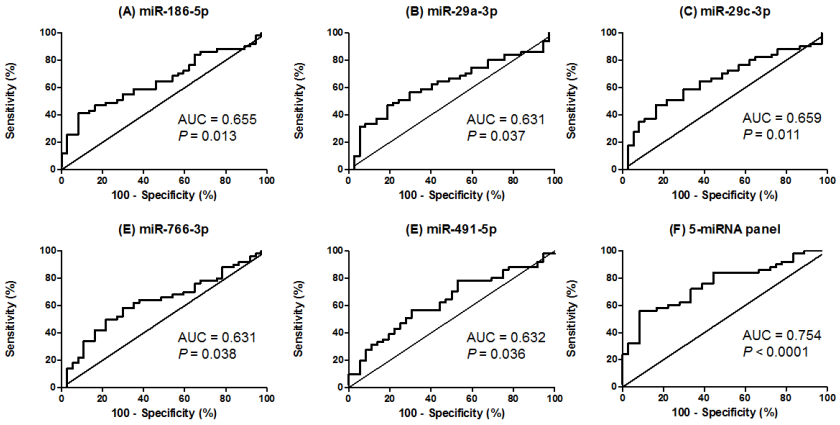

Figure 3. ROC curve analysis using salivary miRNA levels to discriminate CRC patients from healthy individuals. (A) Salivary *miR-186-5p* levels yielded an AUC value of 0.655 (95% CI = 0.5416 to 0.7684); (B) Salivary *miR-29a-3p* levels yielded an AUC value of 0.631 (95% CI = 0.5139 to 0.7474); (C) Salivary *miR-29c-3p* levels yielded an AUC value of 0.659 (95% CI = 0.5452 to 0.7733); (D) Salivary *miR-766-3p* levels yielded an AUC value of 0.631 (95% CI = 0.5132 to 0.7484); (E) Salivary *miR-491-5p* levels yielded an AUC value of 0.632 (95% CI = 0.5150 to 0.7497); (F) ROC curve analysis for the combination of the five miRNAs yielded an AUC value of 0.754 (95% CI = 0.6524 to 0.8554). *Note:* ROC, receiver operating characteristic; CRC, colorectal cancer; AUC, area under the ROC curve; CI, confidence interval; miRNAs, microRNAs.

3.3. Salivary miRNA levels were not associated with demographic and clinicopathological features

The relationship between salivary miRNA expression levels and clinicopathological features of CRC patients were also examined. No significant association was found between the five salivary miRNAs and age, lymph node metastasis, distant metastasis, tumor grading, serum CEA levels, TNM staging, tumor location, body mass index, alcohol intake and smoking status, while the levels of *miR-186-5p*, *miR-29a-3p*, and *miR-29c-3p* levels were significantly increased in males ($p < 0.05$). Further, expression levels of salivary miRNAs were slightly increased in non-metastatic patients although no significant differences were observed compared to the late stages (Table S4). Besides, the

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expression levels of the five validated miRNAs were significantly correlated between them ($p < 0.0001$).

3.4. Combination of salivary miRNA panel and CEA improved CRC detection

We examined the performance of our five-miRNA panel with baseline CEA in 47 CRC patients, a biomarker used for the clinical routine in CRC monitoring. As shown in Figure 4, the five-miRNA panel enabled identifying 33 CRC patients (70.21% sensitivity; cut-off = 0.5132), while CEA levels only enabled identifying 21 CRC patients (44.68% sensitivity; cut-off = 5 ng/mL). Importantly, when we combined the five-miRNA panel and CEA levels, a total of 42 CRC patients were positively detected, showing a diagnostic sensitivity of 89.36%.

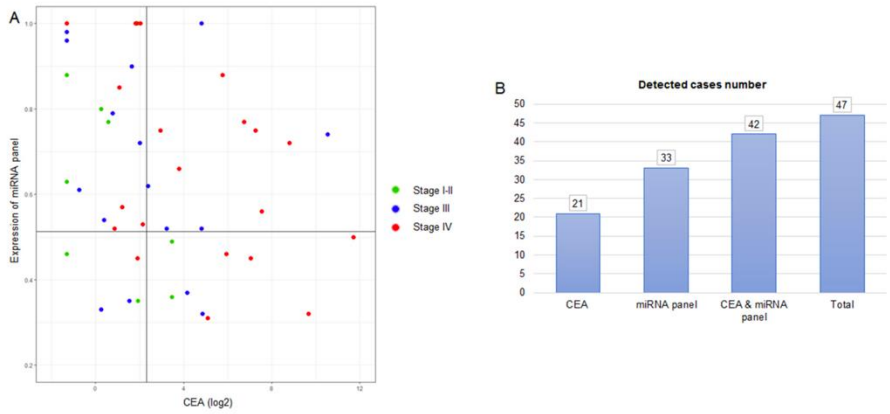

Figure 4. Levels of the five-miRNA panel and serum CEA. (A) A combination of the five-miRNA panel and CEA was used to discriminate CRC. The cut-off value for CEA was 5 ng/mL; and the cut-off value for the five-miRNA panel was 0.5132, defined as the model value that provides the highest sensitivity and specificity to discriminate CRC patients from healthy controls. (B) Detected cases number using the five-miRNA signature, CEA and their combination. *Note:* CEA, carcinoembryonic antigen; CRC, colorectal cancer.

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3.5. Salivary miRNA panel showed value as a prognosis marker in advanced CRC patients

In order to determine whether the salivary miRNA signature was associated with patients outcome, survival analyses were performed only in patients with stage IV tumors, because the follow-up period was not enough to determine good/bad prognosis groups in the early stages. A specific risk score was generated with the five miRNAs to discriminate patients with favorable vs. unfavorable evolution, the unfavorable group being those patients progressing before 10 months after sample collection. The univariate Cox regression analysis with the clinicopathological factors and the miRNA model showed a significant higher risk of progression or *exitus* in patients with higher CEA levels and higher five-miRNA risk score (Figure 5 and Table S5). Importantly, after the multivariate approach, the salivary miRNA model showed independent value in predicting progression-free survival, while serum CEA was a stronger independent predictor for overall survival (Table S6).

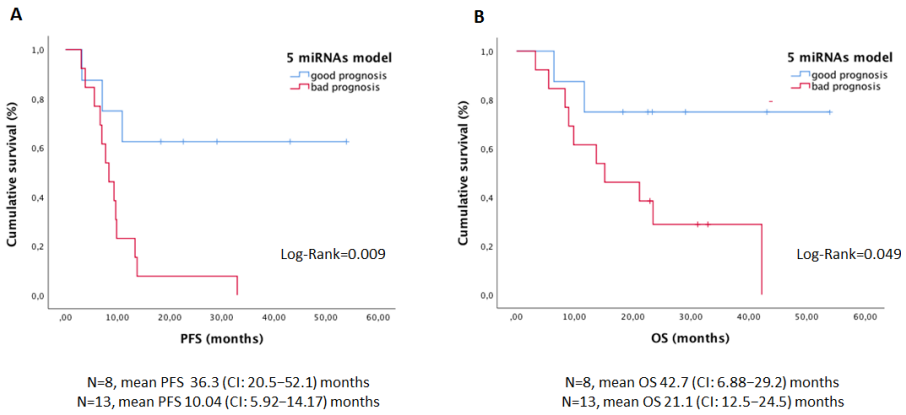

Figure 5. Kaplan-Meier curves to predict PFS (A) and OS (B) according to the five-miRNA model in stage IV CRC patients. *Note:* PFS, progression-free survival; OS, overall survival; CI, confidence interval.

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3.6. Functional annotation analysis of salivary miRNAs

Significant KEGG pathways in our analysis are presented in Table S7 and Figure 6. This analysis showed three miRNAs (*miR-29a-3p*, *miR-186-5p* and *miR-29c-3p*) directly involved in CRC. Importantly, the five miRNAs (*miR-186-5p*, *miR-29a-3p*, *miR-29c-3p*, *miR-766-3p*, and *miR-491-5p*) were linked to the other relevant cell functions and pathways such as p53 or phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), extracellular matrix (ECM) receptor interaction, lipids and amino acids metabolism, cell growth, cell death and cell adhesion.

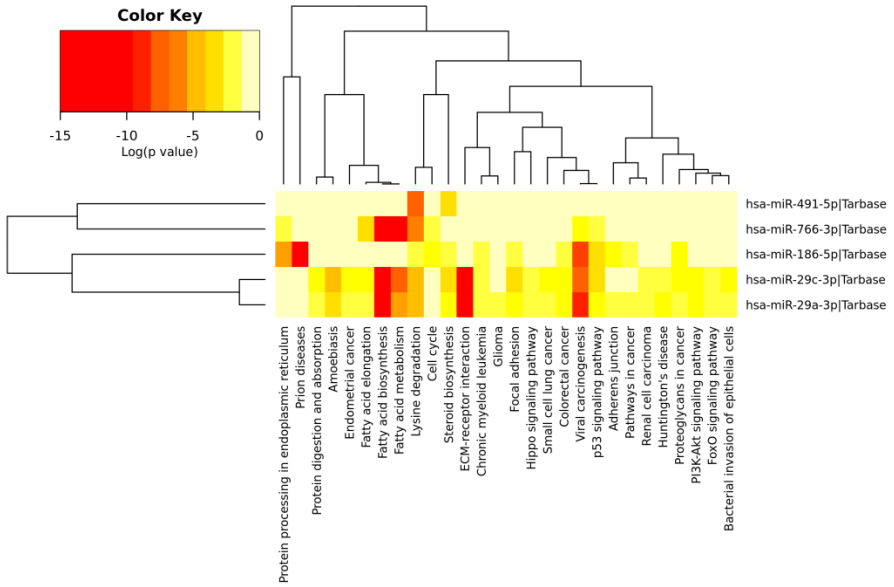

Figure 6. Heatmap of differentially expressed miRNAs vs. significantly enriched functional pathways. In the heatmap, the hottest colors depict higher statistical significance as indicated by the color key at the above. The attached dendrograms on both axes represent hierarchical clustering results for miRNAs and pathways, respectively. Figure was obtained from the output of Diana miRpath V.2.

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4. DISCUSSION

Several studies have identified a wide variety of salivary miRNAs as promising non-invasive biomarkers for cancer detection, such as pancreatic, esophageal, prostatic and head and neck cancer [13]. In fact, various saliva-based miRNA panels have been developed for cancer diagnosis. For instance, Xie et al. identified a model composed by *miR-3679-5p* and *miR-940* with 72.5% sensitivity and 70% specificity for differentiating pancreatic cancer patients from controls [19]. Further, in pancreatic cancer, the combination of *miR-1246* and *miR-4644* yielded 83.3% sensitivity and 92.3% specificity for this tumor diagnosis [20]. These findings demonstrate the potential of saliva as a source of biomarkers for non-oral cancers detection.

Reinforcing the value of saliva characterization to discover new cancer biomarkers, our study described for the first time a saliva-based miRNA signature, composed of *miR-186-5p*, *miR-29a-3p*, *miR-29c-3p*, *miR-766-5p* and *miR-491-5p*, that significantly discriminates CRC patients from healthy individuals, reaching 72% sensitivity and 66.67% specificity. Although diagnostic power should be better explored in a future validation study, including a blinded cohort of patients with a larger number of CRC patients at both early and late stages, these results evidenced the interest of the panel. In this sense, bootstrapping analysis highlights the robustness of our five-miRNA panel, showing its potential value for CRC diagnosis. Although few studies have characterized the miRNA expression profile in saliva from different cancer locations, no other study performed wide profiling of salivary miRNAs in CRC patients to date. Actually, the unique study that evaluated saliva as a source of potential biomarkers for diagnostic purposes in CRC was developed by Sazanov et al. [14] These authors found significantly increased salivary expression levels of *miR-21* in CRC compared to healthy controls. This miRNA was detected in all cases of CRC included in our discovery phase, showing higher mean expression levels in patients than controls but no significant differences, probably because of the lower number of samples analyzed in our discovery phase.

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Another remarkable finding of our study is that the salivary miRNA panel can be combined with CEA levels, the most routinely used colorectal tumor marker [21], for improving the detection rate of CRC in our study. Of note, CEA levels are mainly used for patients monitoring, but some studies have also reported the value of this marker for CRC cancer detection in combination with other biomarkers [22]. In our cohort of patients, CEA levels were increased only in 44.68%, while our panel was found increased in 70.21%. Importantly, the increment of our salivary miRNA panel or/and CEA levels were found in 89.36% of patients, suggesting that the strategy of combining our salivary-miRNA panel with an established marker for clinical practice could improve CRC detection. In fact, a previous study performed by Vychytilova-Faltejskova et al. described that combining a serum-based four-miRNA panel and CEA levels yielded an increase in CRC detection of 47% compared to using the CEA marker alone [23].

Currently, there are several modalities for CRC screening. However, none has been established as a well-accepted screening tool. Colonoscopy or flexible sigmoidoscopy are invasive and expensive tests, associated with low compliance rates, whereas stool-based tests such as guaiac fecal occult blood and fecal immunochemical tests have low sensitivity in detecting premalignant colorectal adenomas or CRC [24]. Our saliva-based miRNA signature represents a really attractive non-invasive method for CRC diagnosis, with similar rates of detection to other plasma-based tests such as CancerSeek [25]. Importantly, in addition to the value of the salivary miRNA signature identified to detect CRC patients, the signature also showed value in determining those patients with stage IV tumors with poorer prognosis, providing useful clinical information to manage CRC patients. Besides, a potential utility of our salivary miRNA signature, not explored in the present study, could be disease monitoring. For this objective, the panel should be analyzed prospectively in longitudinal saliva samples during disease evolution.

On the other hand, regarding the biological function of these miRNAs, functional analysis revealed that they were implicated in a total of 28 pathways, including 22 cancer-related pathways such as ECM-receptor interaction, p53 and PI3K-Akt signaling pathways, and

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cell cycle. It is important to highlight that the salivary miRNA panel found in our study is different from all diagnostic models previously reported in blood [26] and tissue [27], although all of them were described as altered miRNAs in CRC. Interestingly, four (*miR-186-5p, miR-491-5p, miR-766-3p,* and *miR-29a-3p*) of the five miRNAs from our saliva-based panel have never been described in saliva from patients with cancer and other diseases. Altogether, this suggested that the discovered panel is very specific for detecting CRC. Only *miR-29c* was found significantly expressed in saliva from patients with unresectable pancreatic cancer, showing 57% sensitivity and 100% specificity for this tumor detection [28]. In fact, *miR-29a* and *miR-29c* are members of the *miR-29* family, which is known to be associated with tumorigenesis and cancer progression [29]. *MiR-29a* might act as either an oncogene or tumor suppressor in a wide variety of malignancies [30]. Besides, numerous studies [4,5] have identified the significant upregulation of *miR-29a* in serum and plasma from CRC patients compared to controls, as we have demonstrated in the current study. On the other hand, *miR-29c* was described as a tumor suppressor in several human cancers although its role in CRC pathogenesis is unclear. Zhang et al. showed *in vitro* that *miR-29c-3p* overexpression decreased significantly the secreted protein acidic and cysteine rich (*SPARC*) expression, as a potential target in CRC. Further, the silencing of *SPARC* significantly inhibited proliferation and migration of the CRC cells, while the *miR-29c-3p* inhibitor promoted it, which indicated that *miR-29c-3p* acts as a tumor suppressor [31]. Further, *miR-29c* is thought to be involved in the progression of advanced CRC because its downregulation was observed in primary CRC tissues, while, in metastasis (lung and liver), this miRNA was upregulated [32,33]. Nevertheless, in the present study, no differences were observed for salivary *miR-29c-3p* between early and late stages. Further, other authors reported circulating *miR-29c* as a diagnostic and recurrence predictor biomarker for CRC [34,35].

The remaining salivary miRNAs from our saliva-based five-miRNA panel (*miR-186-5p, miR-491-5p* and *miR-766-3p*) were also previously associated with CRC. Islam et al. found *miR-186-5p* overexpressed in CRC tissues compared to non-cancer samples and

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moreover, this expression was correlated with distant metastasis, lymphovascular permeation, and poor prognosis. In addition, the overexpression of *miR-186-5p* promotes cell proliferation, migration and colony formation by inhibiting the tumor suppressor family with sequence similarity 134, member B (*FAM134B*) [36]. Recently, Qu et al. found significant overexpression of *miR-186-5p* in serum samples from CRC patients with lymph node metastasis, which could suggest its involvement in the metastatic process [37]. On the other hand, data regarding *miR-491-5p* are contradictory. Nakano et al. showed that *miR-491* inhibited cell proliferation of CRC cells *in vitro* and *in vivo*, inducing apoptosis via the downregulation of B-cell lymphoma extra-large (*Bcl-XL*) [38]. However, recent findings revealed upregulated levels of *miR-491* in serum samples from patients with CRC and adenomas compared to control subjects [39]. This result is in line with our results in saliva from CRC patients. Another miRNA involved in the cancer cell biological process is *miR-766*, which can act as a tumor suppressor or tumor promoter in several cancers [40]. *MiR-766* is involved in CRC—its overexpression promotes cell proliferation by repressing SRY-box transcription factor 6 (*SOX6*), which indicates that *miR-766* acts as an oncogene in the development of CRC [41]. Besides, high expression levels of *miR-766-3p* were found in the plasma of CRC patients compared to healthy individuals [42] but, unlike in our study, no significant differences were observed.

5. CONCLUSIONS

Overall, this work represents the first massive characterization of salivary miRNAs in patients with benign and malignant colorectal disease. Our approach identified a set of salivary miRNAs with potential value for discriminating CRC patients from healthy controls and predicting disease outcome in advanced stages non-invasively. These data demonstrated the value of saliva as an important liquid biopsy for CRC detection and prognosis, setting up the basis for a further validation study to really decipher the clinical value of this approach. For this purpose, a large cohort of patients and controls should be evaluated to determine the diagnostic and prognostic

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accuracy of the panel in a prospective multicenter study including longitudinal samples to also test the panel as a monitoring tool.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/12/2029/s1, Figure S1: Study design for CRC salivary miRNAs discovery and validation, Figure S2: Cq values of the selected reference gene, Figure S3: Venn diagram analysis showing overlapping and non-overlapping of differentially expressed miRNAs, Table S1: Clinicopathological characteristics of study participants, Table S2: Candidate reference genes based on expression stability calculated by NormFinder and Coefficient of Variation score, Table S3: Discriminatory power of salivary miRNAs for the detection of CRC, Table S4: Association of salivary miRNA expression levels with clinical variables, Table S5: Univariate Cox regression analysis for clinicopathological parameters and salivary miRNAs in stage IV CRC patients included in the study, Table S6: Multivariate Cox regression analysis for clinicopathological parameters and salivary miRNAs in stage IV CRC patients included in the study, and Table S7: Significantly enriched signaling pathways in our five-miRNA salivary panel ($p < 0.05$).

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CHAPTER 3

Biomarkers in Head and Neck Cancer Liquid Biopsies

3.1. MiRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic Review and Meta-analysis.

3.2. Integrity and Quantity of Salivary Cell-Free DNA as a Potential Molecular Biomarker for Oral Cavity Cancer Diagnosis.

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CHAPTER 3.1

MiRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic Review and Meta-analysis

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OBJECTIVE 3.1

MIRNAS IN LIQUID BIOPSY FOR ORAL SQUAMOUS CELL CARCINOMA DIAGNOSIS: SYSTEMATIC REVIEW AND META-ANALYSIS*

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is often diagnosed at advanced stages and is associated with poor survival rates. Increasing evidence suggests that microRNAs (miRNAs) present in liquid biopsies could be potential biomarkers for non-invasive OSCC diagnosis. Here, we performed a comprehensive meta-analysis to evaluate the overall diagnostic accuracy of blood and salivary miRNAs in detecting OSCC. A literature search using PubMed EMBASE, Web of Science, LILACS, Scopus, and the Cochrane Library was undertaken up to February 2019. Study quality was assessed with the Quality Assessment for Studies of Diagnostic Accuracy-2, and sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, diagnostic odds ratio, and their corresponding 95% confidence intervals (CIs) were calculated using a bivariate random-effect meta-analysis model. Meta-regression and subgroup analyses were performed to assess the heterogeneity. Twenty-five study units from 16 articles with 2562 subjects were included in this meta-analysis. The pooled sensitivity and specificity of blood and salivary miRNAs in the diagnosis of OSCC were 0.78 (95% CI, 0.76–0.80) and 0.82 (95% CI, 0.79–0.84), respectively, and the pooled positive and negative likelihood ratios were 4.31 (95% CI, 3.38–5.51) and 0.25 (95% CI, 0.20–0.32), respectively. The overall area under the curve was 0.91 (95% CI, 0.88–0.93), with a diagnostic odds ratio of 21.46 (95% CI, 13.37–34.45). These findings provide evidence regarding the potential clinical application of blood and salivary miRNAs as a novel, non-invasive, and accurate diagnostic tool for OSCC.

Keywords

MicroRNA; oral cancer; biomarkers; liquid biopsy; systematic review; meta-analysis.

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1. INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck cancer, accounting for > 90% of all oral malignancies [1]. According to GLOBOCAN, OSCC is one of the leading causes of morbidity and mortality worldwide, with an estimated 354,864 cases and 177,384 OSCC-related deaths occurring in 2018 [2]. Oral carcinogenesis has been associated with several genetic and/or lifestyle associated risk factors, including smoking, alcohol consumption, poor oral hygiene, chronic mechanical trauma, and viral infections, such as those involving human papillomavirus or Epstein–Barr virus [1,3]. Despite progress in OSCC diagnosis and treatment, most patients are diagnosed in advanced stages, which are associated with poor prognosis and high rates of local recurrence and distant metastasis [4]. Overall, the 5-year survival rate is ~80% when diagnosed at early stages and decreases to 20% at later stages [5]. Therefore, there is an urgent need to develop novel approaches that improve OSCC diagnosis.

MicroRNAs (miRNAs) are a large family of single-stranded non-coding RNAs comprising 19–25 nucleotides and that regulate gene expression at the post-transcriptional level. They usually bind to complementary sequences in the 3′ untranslated region of mRNAs, resulting in their degradation and inhibited translation [6]. miRNAs can target hundreds or thousands of different mRNAs, resulting in potential regulation of individual mRNAs by multiple different miRNAs [7]. Importantly, growing evidence implicates miRNAs in cancer hallmarks by acting as both tumor suppressors and promoters [7,8]. Additionally, dysregulated miRNAs have been detected in different body fluids [9], including plasma, serum, saliva, or urine, resulting in their classification as circulating miRNAs. Circulating miRNAs are highly stable under harsh conditions, such as high temperature, extreme pH, and in the presence of RNase activity, suggesting their potential as non-invasive biomarkers for cancer diagnosis [10]. Furthermore, several studies report altered expression of blood and salivary miRNAs in different tumor types [11,12], including OSCC [13,14]; however, there is not a clear consensus regarding the clinical value of miRNAs in liquid biopsy for OSCC diagnosis.

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Therefore, the purpose of this systematic review and meta-analysis was to summarize the clinical results of published studies regarding the use of miRNAs as liquid biopsy biomarkers for OSCC diagnosis and assess their overall diagnostic accuracy in discriminating OSCC.

2. METHODS

2.1. Protocol and registration

This study was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines [15], and the protocol was registered with the International Prospective Register of Systematic Reviews (reference No. CRD42019122960).

2.2. Search strategy and study selection

The search strategy was designed to identify studies describing the diagnostic value of miRNAs present in liquid biopsies for identifying patients with OSCC. In this sense, a detailed literature search was performed in PubMed, EMBASE, Web of Science, LILACS, Scopus, and the Cochrane Library for eligible articles published up to 31 February 2019, without language restrictions. The following combinations of keywords and medical subject headings were used: (microRNA OR microRNAs OR miRNAs) AND (OSCC OR oral squamous cell carcinoma OR oral cancer OR oral tumor) AND (plasma OR blood OR whole blood OR serum OR saliva) AND (diagnosis OR sensitivity OR specificity OR ROC curve) AND (marker OR biomarker). All studies were screened based on the title and abstract, and eligible manuscripts were retrieved for full-text review. Additionally, we performed manual searches of reference lists in each original and review article in order to avoid missing potential studies. The literature search was performed in- dependently by two investigators (ORG and MMSC), and disagreements during the selection process were resolved by consensus. The studies selected through the search strategy and other references were man- aged with RefWorks software (https://www.refworks.com/content/

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path_learn/faqs.asp), and duplicate items were removed using the associated tools.

2.3. Selection criteria

The inclusion criteria were as follows: (1) diagnostic studies with significantly dysregulated miRNAs from body fluids between OSCC and healthy controls; (2) study subjects included cancer patients and healthy controls; and (3) studies included enough data to generate a 2 × 2 contingency table containing true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) values. The exclusion criteria were as follows: (1) reviews, letters, personal opinions, book chapters, case reports, conference abstracts, and meetings; (2) duplicate publications; and (3) studies only reporting associations between saliva and oral cancer based on *in vitro* or *in vivo* animal experiments.

2.4. Data extraction

Two investigators (ORG and MMSC) independently assessed each eligible manuscript, extracted data using a pre-established form, and collated the data into a Microsoft Excel spreadsheet (Microsoft Corp. Redmond, WA, USA). Any disagreement among reviewers was resolved by consensus. The following information was extracted from each study: author, publication year, country, sample size, gender, mean age, TNM stage, type of fluid sample (blood, plasma, serum, or saliva), detection method, specific significantly dysregulated miRNAs, miRNA-expression pattern(s), and outcomes of statistical analysis, including the reference gene used for data normalization and diagnostic accuracy outcomes. If the required data were incomplete, attempts were made to contact the authors to obtain the missing information.

We defined a “study unit” as analysis of a relationship between miRNAs and OSCC. Because different miRNAs could be reported in the same manuscript, a single publication could then potentially report more than one study unit.

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2.5. Quality assessment of individual studies

Quality assessment of the selected studies was separately conducted by two independent investigators (ORG and MMSC) using the Quality Assessment of Diagnostic Accuracy Studies-2 checklist (QUADAS-2) [16], as recommended by the Healthcare Research and Quality Agency. Discrepancies between the two investigators were resolved by a third reviewer (LMR). The QUADAS-2 checklist is a revised tool developed to assess the quality of studies of diagnostic tests and that analyzes four key domains: (1) patient selection, (2) index tests, (3) reference tests, and (4) flow and times. Each domain was evaluated in terms of its risk of bias, and the first three domains were also evaluated in terms of applicability concerns, with risk of bias and concerns of applicability for each domain considered "low", "high", or "unclear". If the response to the risk of bias and applicability questions were "low" risk or "low" concern, respectively, one point for each item was applied. The articles were grouped based on their score into high (6–7 points), moderate (4–5 points), and low (0–3 points) quality categories.

2.6. Statistical analysis

Statistical analysis was conducted using MetaDiSc software (v.1.4) [17], free R software (v.3.4.4; https://www.r-project.org), and STATA (v.14.0; https://www.stata.com). For the diagnostic meta-analysis, the number of subjects with a TP, FP, FN, and TN for each study unit was extracted to calculate the pooled sensitivity [TP/(TP + FN)], specificity [TN/(TN + FP)], positive likelihood ratio (PLR) [(sensitivity/ (1−sensitivity)], negative likelihood ratio (NLR), [(1−specificity)/ specificity)], diagnostic odds ratio (dOR) [PLR/NLR], and their corresponding 95% confidence intervals (CIs) using a bivariate random-effect meta-analysis model. The summary receiver operator characteristic (SROC) curve was plotted, and the area under the SROC curve (AUC) was calculated to evaluate the pooled diagnostic performance of blood and salivary miRNAs in OSCC detection. Heterogeneity in the meta-analysis was explored to understand the factors that influence accuracy indicators and evaluate the statistical model applied to the meta-analytic database [17]. The

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heterogeneity of included studies caused by a threshold effect was assessed by Spearman's correlation analysis and ROC plane plots. The heterogeneity of non-threshold effects was assessed using the Cochran's Q statistic test-based Chi-squared test and I^2 statistics. Heterogeneity was considered significant when $I^2 > 50\%$ and/or presence of a $p < 0.05$ for the Cochran's Q test. If significant heterogeneity was detected, the DerSimonian and Laird random-effects model was applied; otherwise, the Mantel–Haenszel fixed-effects model was used. Meta-regression and subgroup analyses were applied to analyze the potential sources of heterogeneity unrelated to the threshold effect among included studies. Additionally, Fagan's nomo-gram was used to assess the predictive value of the post-miRNAs test for OSCC diagnosis using likelihood ratios to calculate a post-test prob- ability based on Bayes theorem. Publication bias was assessed using Deeks' funnel-plot asymmetry test [18], with statistical significance defined at a $p < 0.05$.

3. RESULTS

3.1. Study selection

The search strategy identified 174 records across the six electronic databases and two additional reports from the reference lists. After removing the duplicates, 137 records were screened, of which 29 records were excluded after title and abstract review. Therefore, full-text articles were retrieved for the remaining 68 articles. After a full-text review, 23 articles were excluded for the following reasons: not related to the diagnosis (2); reviews, letters, personal opinions, book chapters, case reports, conference abstracts, and meetings (7); not reporting sensitivity and specificity (13); and insufficient information for meta-analysis (1). Finally, 16 articles met the inclusion criteria and were included in the final analysis [13,14,19–32]. A flowchart detailing the selection process is shown in Figure 1.

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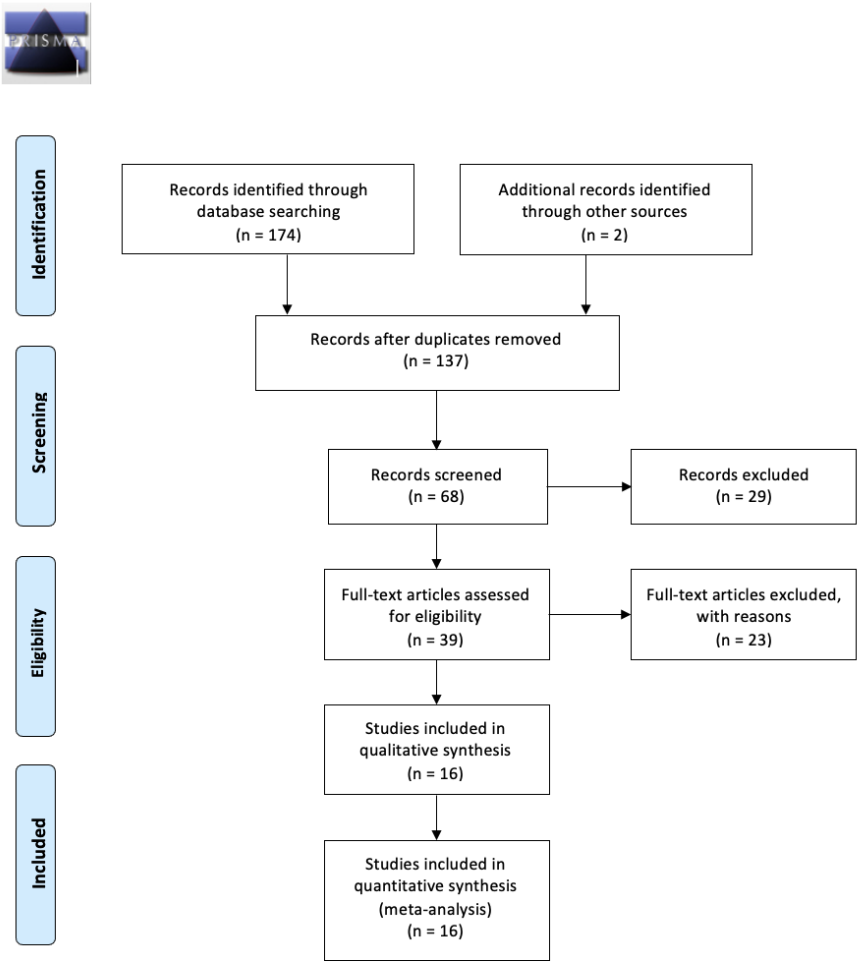

Figure 1. PRISMA flow diagram. *Abbreviations:* PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-analysis.

3.2. Characteristics of the included studies

A total of 25 study units from 16 articles (14 in English and 2 in Chinese) evaluating 22 different miRNAs were included in this meta-analysis. The sample size of the studies ranged from 18 to 204 and

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comprised a total of 2562 subjects according to unit studies (1547 patients with OSCC and 1015 healthy individuals). Sixteen published studies (from 2010 to 2018) were performed in six countries, including six in Taiwan, six in China, one in the United States, one in Canada, one in Japan, and one in Turkey. Expression levels of single miRNAs (n = 25) and/or a panel of miRNAs (n = 2) were detected by real-time quantitative polymerase chain reaction. In terms of sample sources, miRNAs were analyzed in plasma (n = 13), serum (n = 7), saliva (n = 4), and whole blood (n = 1). According to single miRNA-expression levels, 16 miRNAs were upregulated and seven were downregulated. The main characteristics of the included studies are shown in Table 1.

3.3. Quality assessment of the included studies

Assessment of the methodological quality of the included studies was performed using the QUADAS-2 checklist (Figure S1). A total of 10 studies showed a low risk of bias in three of four domains, four studies showed a low risk of bias in two of four domains, and two studies showed a low risk of bias in all domains. The patient selection and index-test domains represented most of the methodological concerns, showing unclear risk in nine studies and high risk in eight studies, respectively. Several studies did not report a detailed description of the inclusion/exclusion criteria or whether they used a blind method. Although the threshold was used, it was not pre-specified, which could indicate an overestimation of diagnostic value. All studies were of moderate-to-high quality, with QUADAS-2 scores between 5 and 7.

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Table 1. Summary of descriptive characteristics of included studies.

Author	Country	Healthy controls			OSCC patients				Type of sample	Reference gene	miRNA profiling	TP	FP	TN	FN	Cut-off	Expression Level
		N	Sex (male/ female)	Mean age (SD, range)	*N*	Sex (male/ female)	Mean age (SD, range)	TNM stage (*N*)									
Lin et al. (2010)	Taiwan	10	…	…	33	…	…	I–III (10) IV (33)	Plasma	RNU6B and let-7a	miR-24	23	1	10	9	1.09	▲
Lu et al. (2012)	Taiwan	36	31/5	50.5 (± 12.6)	54	51/3	52.0 (± 12.8)	I/II (20) III/ IV (34)	Plasma	RNU6B	miR-10b	51	7	3	29	…	▲
Liu et al. (2012)	Taiwan	24	23/1	51.1 (± 1.7)	45	43/2	53.7 (± 1.4)	I/II (21) III/ IV (24)	Saliva	miR-16	miR-31	36	8	9	16	…	▲
MacLellan et al. (2012)	Canada	26	13/13	62 (50–75)	30	21/9	CIS: 64 (50–84) OSCC: 62 (51–93)	…	Serum	global mean	miR-338-3p	24	5	6	21	…	▼
											miR-29a	23	6	7	20	…	▼
											miR-223	29	10	1	16	…	▼
											miR-16	18	2	12	24	…	▲
											let-7b	24	5	6	21	…	▲
Hung et al. (2013)	Taiwan	12	…	…	51	…	…	I-III (19) IV (40)	Plasma	miR-16	miR-146a	40	1	10	11		▲
Ren et al. (2014)	China	32	…	…	58	39/19	61 (25–92)	…	Blood	RNU6B	miR-21	36	3	22	29	9.646	▲
Momen-Heravi et al. (2014)	USA	9	5/3	60.16 (± 9.57, 32–77)	9	8/1	60.66 (± 11.83, 41–78)	…	Saliva	miR-191	miR-27b	8	0	1	9	14.73	▲
											miR-136	8	0	1	9	10.44	▼
Gu et al. (2015)	China	46	38/8	63.80 (± 10.42)	85	56/29	…	…	Plasma	cel-miR-39	miR-125b	76	3	9	43	…	▲
Gu et al. (2015)	China	44	35/9	63.68 (± 10.41)	83	54/29	…	…	Plasma	cel-miR-39	miR-4677	66	4	17	40	0.625	▼
Xu et al. (2015)	China	103	75/28	52.4 (± 9.0)	101	77/ 24	53.2 (± 10.3)	I/II (51) III/ IV (50)	Serum	RNU6B	miR-483-5p	86	26	15	77	…	▲
Lu et al. (2015)	Taiwan	53	37/16	47.2 (± 11.8, 22–74)	90	82/8	54.0 (± 11.7, 25–77)	I/II (30) III/ IV (60)	Plasma	…	miR-196a	60	2	30	50	…	▲
											miR-196b	88	10	2	43	…	▲
											miR-196a and miR-196b	79	4	11	49	…	▲
Duz et al. (2016)	Turkey	25	21/4	46.88 (± 3.63)	25	19/6	54.08 (± 2.38)	…	Saliva	RNU6b	miR-139-5p	18	4	6	21	…	▼
Tachibana et al. (2016)	Japan	31	…	…	31	20/11	75.74 (± 8.96, 58–91)	I/II (12) III/ IV (19)	Plasma	let-7a	miR-223	21	12	10	19	40.67	▲
Sun et al. (2018)	China	80	36/44	53.9 (± 4.2)	80	35/45	54.8 (± 6.4)	T1-2 (43) T3-4 (37)	Plasma	cel-miR-39	miR-200b-3p	72	9	8	71	…	▲
Chen et al. (2018)	China	55	…	…	121	73/48	…	I/II (75) III/ IV (46)	Serum	cel-miR-39	miR-99a	97	9	24	46	…	▼
Chang et al. (2018)	Taiwan	70	68/2	…	114	111/3	…	I/II (61) III/ IV (53)	Plasma	miR-130b-3p and miR-221-3p	miR-150-5p	69	16	45	54	5.010	▲
											miR-423-5p	67	19	47	51	2.185	▲
											miR-150-5p and miR-423-5p	81	19	33	51	0.510	▲

Abbreviations: (▲), upregulated; (▼), downregulated; TP, true positive; FP, false positive; TN, true negative; FN, false negative; CIS, oral carcinoma in situ.

3.4. Synthesis of results

The pooled estimates for sensitivity and specificity of blood and salivary miRNAs to discriminate OSCC from healthy controls were 0.78 (95% CI: 0.76–0.80) and 0.82 (95% CI: 0.79–0.84), respectively (Figure 2), corresponding to a PLR of 4.31 (95% CI: 3.38–5.51) and an NLR of 0.25 (95% CI: 0.20–0.32) (Figure 3). The overall dOR was 21.46 (95% CI: 13.37–34.45), and the AUC was 0.91 (95% CI: 0.88–0.93) (Figure 4). As shown in Figure 5, when the pre-test probability of OSCC was 55%, a positive measurement improved the post-test probability of having cancer to 84%, whereas the post-test probability reduced to 23.4% when a negative measurement occurred. These results indicate the good discriminative ability of miRNAs as liquid biopsy biomarkers for OSCC detection.

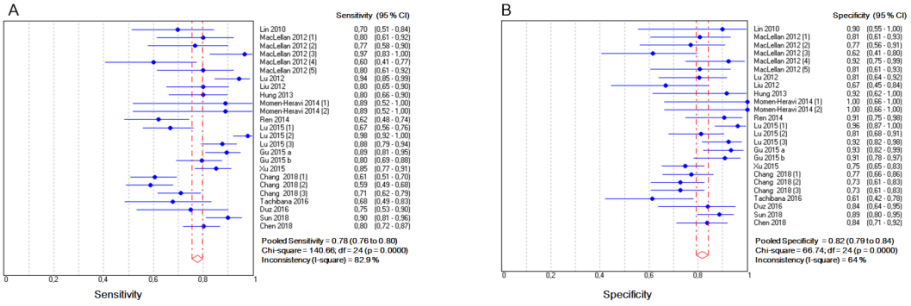

Figure 2. Forest plot of (A) sensitivities and (B) specificities from test-accuracy studies of blood and salivary miRNAs for predicting OSCC diagnosis.
Abbreviations: **miRNA, microRNAs; OSCC, oral squamous cell carcinoma.**

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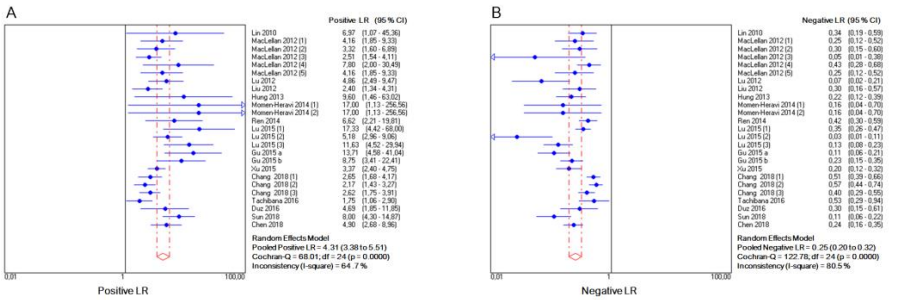

Figure 3. Forest plot of likelihood ratios for (A) positive and (B) negative test results from blood and salivary miRNAs studies for predicting OSCC diagnosis. *Abbreviations:* miRNA, microRNAs; OSCC, oral squamous cell carcinoma.

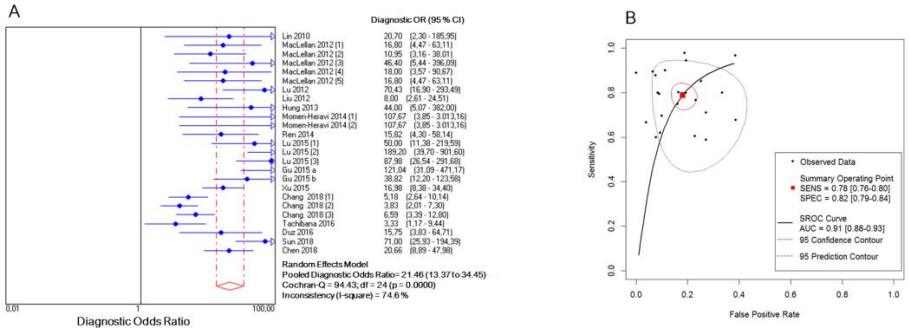

Figure 4. Overall performance of blood and salivary miRNAs in diagnosing oral cancer. (A) Forest plot of the pooled dOR of miRNAs for diagnosing oral cancer. (B) SROC curve with pooled estimates of sensitivity, specificity, and the AUC for all included studies of miRNAs for detecting OSCC. *Abbreviations:* AUC, area under the SROC curve; miRNA, microRNAs; dOR, diagnostic odds ratio; OSCC, oral squamous cell carcinoma; SROC, summary receiver operator characteristic.

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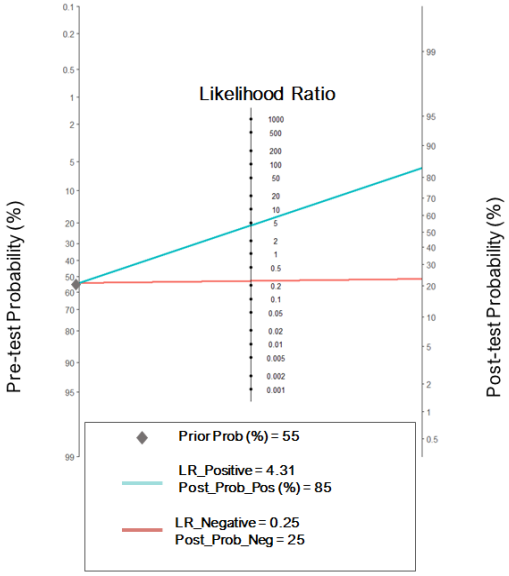

Figure 5. Fagan's monogram evaluating the clinical utility of blood and salivary miRNAs for differentiating OSCC patients. *Abbreviations:* miRNA, microRNAs; OSCC, oral squamous cell carcinoma.

3.5. Heterogeneity and subgroup analysis

The results of diagnostic meta-analysis indicated the existence of significant heterogeneity in the overall sensitivity (I^2 = 82.9%; p < 0.001), specificity (I^2 = 64%; p < 0.001), PLR (I^2 = 64.7%; p < 0.001), NLR (I^2 = 80.5%; p < 0.001), and dOR (I^2 = 74.6%; p < 0.001). Further analysis of heterogeneity showed no "shoulder arm" pattern in the ROC plane, suggesting the absence of a threshold effect (Figure S2). Moreover, Spearman's correlation coefficient was −0.059 (p = 0.779), also indicating no obvious heterogeneity detected as a result of the threshold effect. To determine possible sources of heterogeneity, we performed meta-regression analysis with the following covariates as predictor variables: ethnicity (Asian vs. others), sample size (n ≥ 100 vs. n < 100), miRNA-expression pattern (upregulated vs. downregulated), and miRNA profiling (single- vs. multiple- miRNA assay). Significant heterogeneity was not observed between the groups (Table S1). Although the meta-regression results were negative, we

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conducted subgroup analysis to further explore the potential diagnostic value of miRNAs in liquid biopsy (Table 2).

3.6. Evaluation of publication bias

Performance of Deeks' funnel-plot asymmetry test to explore the potential publication bias from each miRNA liquid biopsy study revealed a slope coefficient *p*-value of 0.59 in the overall studies, indicating a symmetric pattern for the data and, therefore, absence of publication bias (Figure S3).

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Table 2. Subgroup analysis of blood and salivary miRNAs for OSCC detection based on different covariates.

Subgroups	No of study units	Sensitivity (95% CI)	I^2 (%)	Specificity (95% CI)	I^2 (%)	PLR (95% CI)	I^2 (%)	NLR (95% CI)	I^2 (%)	dOR (95% CI)	I^2 (95%)	AUC (95% CI)
Ethnicity												
Asian	17	0.78 (0.76–0.80)	87.22	0.82 (0.79–0.84)	68.50	4.47 (3.28–6.09)	73.63	0.25 (0.19–0.34)	85.93	21.83 (11.97–38.82)	23.23	0.91 (0.88–0.93)
Others	8	0.79 (0.73–0.85)	53.83	0.82 (0.75–0.87)	56.11	3.64 (2.66–4.98)	4.63	0.29 (0.21–0.39)	18.51	18.37 (10.38–23.50)	0	0.90 (0.86–0.94)
Samples size												
≥100	11	0.78 (0.75–0.80)	81.60	0.83 (0.80–0.85)	52.96	4.92 (3.39–7.16)	60.22	0.23 (0.16–0.34)	80.70	25.88 (12.09–55.41)	75.27	0.92 (0.87–0.96)
< 100	14	0.76 (0.74–0.81)	65.07	0.80 (0.75–0.84)	55.06	3.66 (2.69–4.98)	39.73	0.29 (0.23–0.38)	47.64	16.31 (9.89–29.91)	31.29	0.90 (0.86–0.93)
MiRNA expression												
Upregulated	18	0.77 (0.74–0.79)	86.88	0.82 (0.79–0.84)	68.37	4.41 (3.24–6)	70.83	0.26 (0.19–0.35)	84.76	21.48 (11.72–39.38)	80.60	0.91 (0.87–0.94)
Downregulated	7	0.81 (0.76–0.85)	27.27	0.82 (0.76–0.87)	53.78	4.15 (2.86–6.02)	35.48	0.24 (0.19–0.30)	0	21.32 (13.07–34.77)	0	0.91 (0.87–0.95)
MiRNA profiling												
Single-miRNA assay	23	0.78 (0.75–0.80)	83.32	0.82 (0.73–0.84)	30.34	4.28 (3.32–5.51)	62.14	0.26 (0.20–0.33)	80.12	21.46 (13.10–35.15)	72.56	0.91 (0.88–0.94)
Multiple-miRNA assay	2	0.78 (0.72–0.84)	88.52	0.81 (0.73–0.88)	87.97	5.23 (1.05–26.04)	89.81	0.24 (0.78–0.72)	91.66	22.91 (1.79–292.71)	92.78	0.71 (0.71–0.71)

Abbreviations: miRNAs, microRNAs; AUC, area under the SROC; dOR, diagnostic odds ratio; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval.

4. DISCUSSION

Despite easy accessibility to the oral cavity for exploring tumor lesions, OSCC is still diagnosed at late stages, which are associated with poor prognosis. To achieve better survival in OSCC patients, a non-invasive molecular test using saliva or blood could be applied for screening purposes in high-risk patients, in tumoral lesions with apparent benign appearance, in multifocal lesions, and in suspicious malignant lesions. There are currently no validated circulating biomarkers used in clinical practice in OSCC diagnosis, generally based on conventional clinical (visual and tactile) oral examination and tissue biopsy. Although tumor biopsy still represents the "gold standard" for clinical molecular analysis, it is an invasive procedure that is temporally and spatially limited and unable to reflect the complete molecular landscape of the tumor [33]. To overcome these limitations, circulating biomarkers might represent a new opportunity to understand the molecular profile and dynamic behavior of the tumor [34]. Accumulating evidence demonstrates that small non-coding RNAs (mainly miRNAs) play an important role in cancer pathogenesis [35], and recently, tumor-derived cell-free miRNAs obtained using liquid biopsies have emerged as potential biomarkers for oral cancer [36].

To the best of our knowledge, this represents the first meta-analysis evaluating the diagnostic accuracy of miRNAs from body fluids, including blood and saliva, for discriminating OSCC patients. A previous systematic review by Troiano et al. [37] reported the potential of serum and plasma miRNAs as oral cancer biomarkers. The present study included a total of 16 studies (25 study units) comprising 1547 OSCC patients and 1015 healthy individuals, with study quality determined by the QUADAS-2 checklist for all included studies revealing moderate-to-high quality. According to the overall analysis, blood and salivary miRNAs demonstrated a high diagnostic accuracy to differentiate OSCC patients from healthy individuals, with sensitivity, specificity, and AUC values of 0.78, 0.82, and 0.91, respectively. Additionally, the pooled dOR was 21.46, which indicated high diagnostic effectiveness of miRNAs for detecting OSCC, and the pooled PLR value was 4.31, suggesting that the probability of cancer in a person with a positive

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miRNA test was ~4.31-fold higher than that in a healthy individual. By contrast, the pooled NLR indicated that the probability of having cancer in a person with a negative test was 25%. Furthermore, Fagan's nomogram results showed that when the pre-test probability was 55%, the correct diagnosis of OSCC increased to 84% after a positive miRNA test, whereas when the test was negative, OSCC was present in 23.4% of patients. Therefore, these results confirmed miRNAs as potentially useful diagnostic liquid biopsy biomarkers in OSCC patients.

Because heterogeneity is often present in meta-analysis of test-accuracy data, we explored possible causes that can potentially contribute to the existence of inconsistencies in accuracy estimates across studies. Given that we observed significant heterogeneity among the studies, we applied a bivariate random-effects model and assessed the threshold effect based on its being a primary cause of heterogeneity in test-ac- curacy studies. The results indicated no significant negative correlation between sensitivities and specificities in the included studies, and the ROC plane revealed a non-typical shoulder-arm pattern, which indicated the absence of a threshold effect. Because heterogeneity can arise from different factors in addition to the threshold effect, we performed meta-regression analysis to test the effect of ethnicity, sample size, miRNA-expression pattern, and miRNA profiling and found no significant differences. Therefore, it is possible that the heterogeneity present in the studies can be explained by other potential factors related to clinicopathological characteristics of subjects or the different approaches used for miRNA analysis, sample types, data normalization, and the use of different cut-off values. Additionally, we observed a higher (but non-significant) accuracy between studies with large ($n \geq 100$) and small ($n < 100$) sample sizes and study populations ranging from nine to 121 OSCC patients and nine to 103 healthy individuals. Furthermore, a total of 22 different miRNAs were classified according to their expression patterns, with no significant differences found between upregulated and downregulated miRNAs. Interestingly, analysis of diagnostic accuracy between single- and multiple-miRNA assays revealed no differences. It is important to note that only two studies included in our meta-analysis [14,26] used miRNA panels. This indicates that future translational and clinical

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investigations should be focused on the combined use of multiple miRNAs to investigate their effect on diagnostic accuracy.

This meta-analysis revealed the potential of miRNAs as promising liquid biopsy biomarkers in OSCC; however, this study had some limitations. First, only a few studies investigated blood and/or salivary miRNAs in OSCC patients, with some of these involving a small sample size. Additionally, several studies focused on liquid biopsies described only miRNA-expression profiles in OSCC patients but did not analyze their diagnostic power. Moreover, there was a lack of clarity in the level of detail applied and/or the data-reporting techniques used by some of the studies, which hindered our analysis; therefore, specific miRNAs potentially useful for diagnosing OSCC were not included in our meta-analysis. Second, considerable heterogeneity was detected among the included studies. Although meta-regression and subgroup analyses were performed, we were unable to elucidate the potential sources contributing to this heterogeneity. Additionally, some variables, including TNM stage, fluid type, age distribution, gender proportion, and normalization strategy, were not specified by various studies, thereby precluding further subgroup analysis. Third, the studies included in the meta-analysis applied diverse cut-off values and different normalization methods, including global mean expression, exogenous synthetic miRNAs (*i.e., cel-miR-39*) and endogenous reference genes as miRNAs (*i.e., miR-191*) or small-nucleolar RNAs (*i.e., RNU6B*), as controls for miRNA quantification, which could substantially affect the results of our analysis and reports of potential clinical efficacy. This suggests that a consensus in the scientific community should be acquired to determine the optimal reference molecules for use as “gold standard” controls. Finally, several studies included in the meta-analysis were mainly developed in Asian population and only a few studies involved patients from a different ethnic population.

5. CONCLUSION

Overall, the results of this meta-analysis suggest that blood and salivary miRNAs represent potentially promising biomarkers for oral

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cancer diagnosis. Although these findings offer insight into possible clinical application of miRNAs present in liquid biopsies as a novel, non-invasive, and accurate diagnostic tools for OSCC detection, further research focusing on large-scale prospective, multicenter, and blinded studies involving both novel single- and combined-miRNA assays is necessary to validate the diagnostic efficacy of blood and salivary miRNAs in OSCC.

Declaration of Competing Interest: The authors declared that there is no conflict of interest.

Author Contributions: Study concepts, L.M.-R., and M.M.S-C.; study design, L.M.-R. and M.M.S.-C.; data acquisition, O.R.-G. M.M.S-C.; quality control of data and algorithms, O.R.-G., M.M.S-C., L.M.-R., C.R.-M. and A.S.-B.; data analysis and interpretation, O.R.-G., M.M.S-C., C.R.-M. and A.S.-B.; statistical analysis, C.R.-M. and A.S.-B.; manuscript preparation, O.R.-G., L.M.-R. and M.M.S-C.; manuscript editing, O.R.-G., L.M.-R. and M.M.S-C.; and manuscript review, L.M.-R., R.L.-L., M.M.S-C. and A.S.-B. All authors have read and agreed to the published version of the manuscript.

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Chapter 3.2

Integrity and Quantity of Salivary Cell-Free DNA as a Potential Molecular Biomarker for Oral Cavity Cancer Diagnosis: A Preliminary Study

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OBJECTIVE 3.2

INTEGRITY AND QUANTITY OF SALIVARY CELL-FREE DNA AS A POTENTIAL MOLECULAR BIOMARKER FOR ORAL CAVITY CANCER DIAGNOSIS: A PRELIMINARY STUDY*

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ABSTRACT

Introduction: Differences in cell-free DNA (cfDNA) fragments have been described as a valuable tool to distinguish cancer patients from healthy individuals. Our study aims to investigate the concentration and integrity of cfDNA fragments in saliva (scfDNA) from oral cavity squamous cell carcinoma (OCSCC) patients and healthy individuals to explore their value as diagnostic biomarkers.

Materials and methods: Saliva samples were collected from a total of 34 subjects (19 OCSCC patients and 15 healthy controls). The total concentration of scfDNA was determined using a fluorometry method and quantitative real-time PCR (qPCR). To evaluate the scfDNA quantity and integrity, qPCR targeting Arthobacter luteus (*ALU*) sequences at three amplicons of different lengths (60 bp, 115 bp, and 247 bp, respectively) was carried out. ScfDNA integrity indexes (ALU115/ALU60 and ALU247/ALU60) were calculated as the ratio between the absolute concentration of the longer amplicons 115 bp and 247 bp and the total scfDNA amount (amplicon 60 bp).

Results: The total scfDNA concentration (ALU60) was higher in OCSCC than in healthy donors, but this trend was not statistically significant. The medians of scfDNA integrity indexes, ALU115/ALU60 and ALU247/ALU60, were significantly higher in OCSCC, showing area under the curve (AUC) values to discriminate patients and controls of 0.8211 and 0.7018, respectively.

Conclusion: Our preliminary results suggest that scfDNA integrity indexes (ALU115/ALU60 and ALU247/ALU60) have potential to identify the presence of OCSCC.

Keywords

Oral cavity squamous cell carcinoma (OCSCC); salivary cell-free DNA (scfDNA); DNA integrity; liquid biopsy; saliva; ALU repeat.

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1. INTRODUCTION

Oral cavity squamous cell carcinoma (OCSCC) is the most common head and neck malignancy, responsible for 355,000 new cases and 178,000 deaths worldwide in 2018 [1]. There is an urgent need for developing non-invasive approaches for improving early detection and therapeutic success rates [2].

Liquid biopsies have emerged as a potential non-invasive source of tumor-related biomarkers [3]. In particular, the analysis of cell-free DNA (cfDNA) has aroused great interest as a non-invasive biomarker [4]. The cfDNA comprises both nuclear and/or mitochondrial double-stranded DNA released from the cells into the physiological body fluids, such as blood, saliva, urine, cerebrospinal fluid, or pleural fluid, among others [5]. The structural composition of cfDNA varies depending on its origin, which includes apoptosis, necrosis, or active release. Apoptotic cells secreted small and uniform DNA fragments, with a length around 167 bp as a result of caspase-activated DNase (CAD) cleavage at inter-nucleosomal chromatin regions. Conversely, necrotic cells release high molecular weight DNA due to random and incomplete genomic DNA fragmentation by DNases [6]. The determination of cfDNA size distribution in plasma from cancer patients showed the presence of short and long DNA fragments derived from both apoptotic and necrotic cells, respectively [7], while plasma cfDNA from healthy individuals is mainly derived from apoptotic cells [8]. Several studies have reported the presence of elevated levels of long cfDNA fragments in serum, plasma, or urine from different types of tumors. Therefore, the DNA integrity index calculated as the ratio of the quantity of long to short DNA fragments has been described as a promising tumor biomarker [9,10].

Salivary cell-free DNA (scfDNA) has emerged as a potential biomarker in cancer [11,12], however, there is little information about its characteristics. Since saliva is the biological fluid of the oral cavity, scfDNA fragmentation could reflect a different origin under physiological and pathological conditions, representing a non-invasive biomarker for tumor detection. Our proposed hypothesis is that scfDNA fragments from OCSCC patients should be longer than those of healthy

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individuals due to tumor necrosis. Therefore, this pilot study aimed was to investigate whether the total scfDNA quantity and DNA integrity could be used for discriminating OCSCC patients from healthy individuals.

2. MATERIAL AND METHODS

2.1. Study population

A total of 34 participants, including 19 OCSCC patients and 15 controls were included in this cross-sectional study. All primary OCSCC patients were recruited at the Department of Oral and Maxillofacial Surgery from Complexo Hospitalario Universitario of A Coruña (CHUAC, SERGAS) in Galicia, Spain, between September 2020 and April 2021. Baseline clinical and histopathological characteristics of subjects are shown in Table 1. There was no statistical difference between groups regarding gender (p = 0.730) or age (p = 0.5085). All cases were confirmed by histopathological analysis of the corresponding tissue biopsy and classified based on tumor human papillomavirus (HPV) status in negative or positive OCSCC. None of the patients under study received surgery, chemotherapy, or radiotherapy before sample collection. Patients with the presence of a synchronous tumor were excluded from the study. The staging of OCSCC was established in accordance with the 8th edition of the Tumor-Node-Metastasis (TNM)-staging system promulgated by the American Joint Commission on Cancer (AJCC) and the International Union Against Cancer (UICC). Saliva samples were obtained before tissue biopsy, on the same day to avoid disease bias. The control group included healthy individuals without visible oral lesions and any acute or chronic inflammatory conditions. All participants provided written informed consent before enrollment. The present study was approved by the Galician Ethics Committee of Clinical Research (Ref. No. 2018/003) and carried out in accordance with principles outlined in the Declaration of Helsinki (World Medical Association, 2013).

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Table 1. Clinicopathological characteristics of study subjects.

	OCSCC N=19	Healthy individuals N=15
Age, years		
Median (IQR 25–75)	72 (67-76)	68 (64-75)
Gender, n (%)		
Male	11 (57.9)	7 (46.7)
Female	8 (42.1)	8 (53.3)
Smoking status, n (%)		
Current	4 (21.05)	
Never	15 (78.9)	
Daily alcohol consumption		
Yes	2 (10.5)	
No	17 (89.5)	
OPMD, n (%)		
Yes	5 (26.3)	
No	14 (73.7)	
Tumor Location		
Tongue	12 (63.2)	
Floor of mouth	5 (26.3)	
Gingiva	2 (10.5)	
Tumor Stage (TNM)		
Early-stages (I-II)	12 (63.2)	
Late-stages (III-IV)	7 (36.8)	
Histologic differentiation		
Well	2 (10.5)	
Moderate	16 (84.2)	
Poor	1 (5.3)	
Tumor HPV-status, n (%)		
HPV-positive	2 (10.5)	
HPV-negative	17 (89.5)	

Abbreviations: OPMD, oral potentially malignant disorders; HPV, human papillomavirus; IQR, interquartile range.

2.2. Saliva collection and DNA extraction

All the participants were asked to refrain from eating, drinking, smoking, and using oral hygiene products for at least 1 h prior to sample collection. Unstimulated saliva samples were collected using Danasaliva sample collection kit (Danagene, Barcelona, Spain)

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according to the manufacturer's instructions. Then, saliva was centrifuged at 2600 xg for 15 min at room temperature. The salivary supernatant was removed from the pellet and centrifuged again at 13000 xg for 1 min (2 times) to avoid cell contamination. Salivary supernatants were stored at -80ºC until further analysis.

DNA from salivary supernatants was extracted with the QIAamp DNA Circulating Nucleic Acid Kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. DNA samples were stored at −20 °C until use. Total DNA from each sample was quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, Agilent's TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA) was used to assess the fragment distribution of the extracted salivary DNA through Agilent Genomic DNA Screen Tape assay, in order to have a visual approach of DNA integrity.

2.3. Bacterial salivary cell-free DNA quantification assay

To determine the percentage of bacterial scfDNA, we carried out a quantitative real-time PCR (qPCR) based assay using microbial DNA qPCR Assay kit (Qiagen, Venlo, Netherlands), which is designed to detect bacterial 16S rRNA gene. The reaction mixture for each direct qPCR contained 12.5 uL microbial qPCR mastermix, 1uL microbial DNA qPCR Assay, and 11.5 uL of scfDNA in a total reaction volume of 25 uL. In addition, for the qPCR, a positive PCR control, a negative control, and a microbial DNA positive control were included following the manufacturer's protocol. A standard curve was performed with serial dilutions (20 ng/uL to 0.02 ng/uL) of ZymoBIOMICS™ microbial community DNA standard. Thermal cycling was conducted on the QuantStudioTM 3 Real-Time PCR system (Life Technologies, Foster City, CA, USA) using the following parameters: initial denaturation at 95°C for 10 min followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 2 min.

2.4. Real-time quantitative PCR of ALU fragments

The quantity of the cfDNA in saliva was evaluated by qPCR targeting the human Arthobacter luteus (*ALU*) gene. The length of the

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amplicons designed for this study were 60, 115, and 247 bp respectively. The sequences of ALU60 primers were forward: 5´-ACCAGCCTGGCCAAC-3´ and reverse 5´-GCCCGGCTAATTTTTGTA-3´; ALU115 primers were forward: 5´-CCTGAGGTCAGGAGTTCGAG-3´and reverse: 5´-CCCGAGTAGCTGGGATTACA-3´; and ALU247 primers were forward: 5´-GTGGCTCACGCCTGTAATC-3´ and reverse: 5´-CAGGCTGGAGTGCAGTGG-3´. The primer set for ALU60 amplified both short (apoptotic) and long (non-apoptotic) DNA fragments representing the total amount of scfDNA.

The reaction mixture for each direct qPCR contained 10 µl TaqManTM Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 4 µl H20, 1 µl of each primer, and 5 µl of DNA in a total reaction volume of 20 µl. For each unknown reaction, 30 ng of DNA extract was added and for the negative control reaction, 5 µl of DNAse-free water was added. Thermal cycling setting was performed in order to achieve a comparable qPCR efficiency among the three different amplicons. The qPCR protocol included an initial denaturation step at 95°C for 10 min and 50 cycles of PCR as follows: i) 15 s at 95 °C, 60 s at 60 °C and 60 s at 72 °C for the for 60 bps amplicon, ii) 15 s at 95 °C, 60 s at 60 °C and 60 s at 72 °C for the longer amplicons for 115 and 247 bps amplicons using QuantStudio^{TM} 3 Real-Time PCR system. The absolute equivalent amount of DNA fragments in each sample was determined by a standard curve with serial dilutions (20 ng−2 pg) of prepared human genomic DNA (Sigma-Aldrich, St. Louis, MO, USA). All the measurements were performed in duplicate.

2.5. Salivary cell-free DNA integrity index

Each integrity index was calculated as the ratio between the absolute concentration of the longer amplicons (amplicon 115 bp and 247 bp) and the total scfDNA amount (amplicon 60 bp). Then, cfDNA integrity was considered as ALU115/ALU60 and ALU247/ALU60. Additionally, we evaluated the fraction of scfDNA fragments with lengths varying from 60 to 115 bp and from 115 to 247 bp by subtracting the absolute concentration of the longer fragment to that of

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the shorter one. Also, longer scfDNA fragments (≥247 bp) were calculated. These results were normalized on the total scfDNA amount and expressed as percentages.

2.6. Statistical analysis

All statistical analyses with IBM SPSS Statistics 20 and graphs were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, California, USA). Continuous variables were expressed as medians, interquartile range (IQR 25-75), and categorical variables were expressed as counts and percentages. Statistical differences between quantitative data were evaluated by a two-tailed Mann–Whitney U test while the relationship between continuous with categorical variables was investigated using the Chi-square test or, where appropriate, Fisher's exact test. Receiver operating characteristic (ROC) curves were constructed, and area under the ROC curve (AUC) with 95% of confidence intervals (CIs) was obtained to evaluate the diagnostic accuracy of using scfDNA integrity index for OCSCC. The cut-off selection was made based on the value that provided the highest sensitivity and specificity to discriminate OSCC patients vs. healthy controls. p-value < 0.05 was set as the level of statistical significance.

3. RESULTS

3.1. Salivary cell-free DNA quantitation and integrity

The median (IQR) concentration of scfDNA obtained by a fluorometric method (Qubit) in OCSCC was 6.200 ng/mL (2.500−11.233) whereas in healthy individuals was 4.333 ng/mL (1.080−14.467). Although scfDNA concentration was found increased in cancer patients, no significant differences were observed (Figure 1). In addition, we analyzed the potential contamination of bacterial-derived DNA in the samples. Thus, after applying the qPCR assay to detect bacterial DNA in 19 samples from OCSCC patients, the median (IQR) concentration was 71.67 (26.28−145.8) ng/mL. The mean percentage of bacterial DNA, as we expected, was negligible in

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comparison with the human DNA since only represents the 3.5% of the total scfDNA quantified in OCSCC patients.

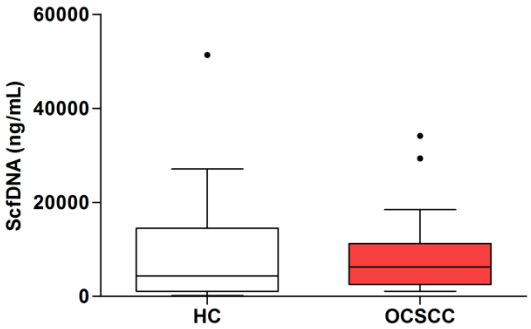

Figure 1. Distribution of scfDNA concentration measured by Qubit fluorometer (ng/mL) in OCSCC patients and HC. *Abbreviations*: scfDNA, salivary cell-free DNA; OCSCC, oral cavity squamous cell carcinoma; HC, healthy controls.

The qPCR assay was used to determine more specifically the concentration of short and long scfDNA fragments (Figure 2). The median (IQR) ALU60 concentration in OCSCC patients and healthy controls was 6.404 ng/mL (2.721−9.748) and 2.715 ng/mL (1.997−19.938) ng/mL, respectively. The median value of ALU115 amplicon was again higher in OCSCC patients (3.524 ng/mL; IQR: 1.439−6.150) than in healthy controls (1470 ng/mL; IQR: 675,3−7.426) ng/mL. Finally, the median of ALU247 in OCSCC patients and healthy controls was 1.907 (838.5−4.898) and 1.205 (477.3−3.270) ng/mL, respectively. Overall, the results show a decrease in scfDNA concentration as the amplicon dimension increase both in OCSCC patients and healthy controls.

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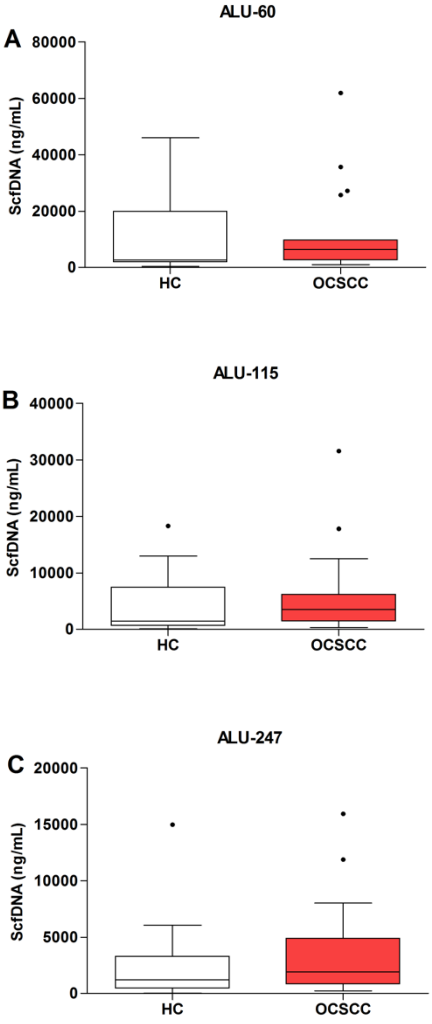

Figure 2. Distribution of absolute scfDNA fragments concentration (ng/mL) measured by qPCR targeting ALU sequences in OCSCC patients and HC: (A) ALU60 scfDNA levels, (B) ALU115 scfDNA levels, and (C) ALU247 scfDNA levels. *Abbreviations:* scfDNA, saliva cell-free DNA; ALU, Arthobacter luteus; OCSCC, oral cavity squamous cell carcinoma; HC, healthy controls.

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3.2. Salivary cell-free DNA integrity indexes by qPCR

The median salivary DNA integrity ALU115/ALU60 was significantly higher in OCSCC patients (0.5120; IQR: 0.4540−0.5886) compared to healthy individuals (0.3725; IQR: 0.3141−0.5342) (p = 0.0016) (Figure 3A). Similarly, the median salivary DNA integrity ALU247/ALU60 was significantly higher in OCSCC patients (0.3503; IQR: 0.2248−0.4612) compared to healthy individuals (0.2531; IQR: 0.1640−0.3247) (p = 0.048) (Figure 3B).

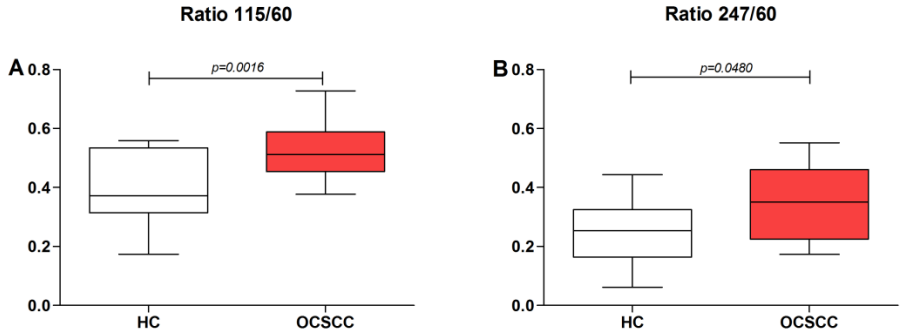

Figure 3. ScfDNA integrity indexes in OCSCC patients and HC: (A) Ratio ALU115/ALU60 and (B) Ratio ALU247/ALU60. *Abbreviations:* scfDNA, salivary cell-free DNA; OCSCC, oral cavity squamous cell carcinoma; HC, healthy controls.

Analyzing the amount of scfDNA fragments by qPCR (Figure 4), we observed that 62% of the total scfDNA in healthy controls comprised fragments with lengths ranging from 60 to 115 bp. However, this percentage was significantly lower in OCSCC patients (p = 0.0016). Regarding the percentage of fragments comprised between 115 and 247 bp, no significant differences were observed between OCSCC patients and healthy individuals, representing around 35% of total scfDNA fragments. On the contrary, the percentage of longer fragments (≥247 bp) with respect to total scfDNA quantity was significantly higher in OCSCC patients (35%) than in healthy controls (25%) (p = 0.048).

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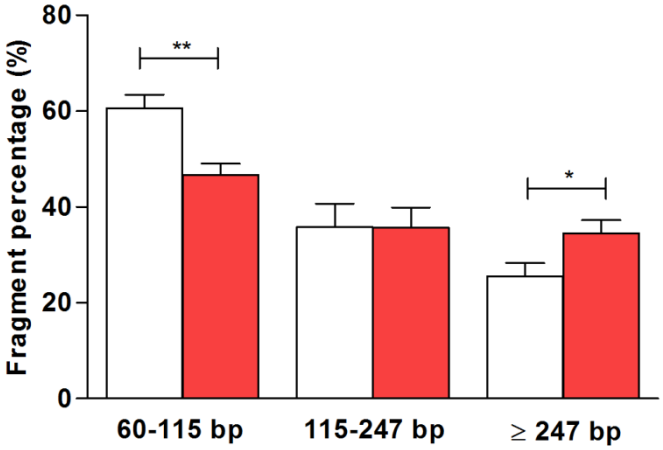

Figure 4. Percentages of scfDNA fragments of different length ranging 60-115 bp, 115-247 bp and ≥247 bp in OCSCC patients (red column) and HC (white column). $p < 0.05$; ** $p < 0.01$. *Abbreviations*: scfDNA, salivary cell-free DNA; OCSCC, oral cavity squamous cell carcinoma; HC, healthy controls; bp, base pairs.

3.3. Diagnostic utility of salivary cell-free DNA integrity indexes

With the goal of determining the diagnostic utility of each salivary DNA integrity index we calculated its sensitivity and specificity using ROC analysis. The ROC curves for discriminating OCSCC patients from healthy individuals showed AUC values of 0.8211 (95% CI, 0.6722 to 0.9699) for scfDNA integrity index ALU115/ALU60 and 0.7018 (95% CI, 0.5253 to 0.8782) for scfDNA integrity index ALU247/ALU60 (Figure 5). At the optimal cut-off level of >0.4479 ng/mL the scfDNA integrity index ALU115/ALU60 showed 83.33% sensitivity and 73.33% specificity. The scfDNA integrity index ALU247/ALU60 yielded also 83.33% sensitivity and 73.33% specificity at the cut-off level of >0.2661 ng/mL.

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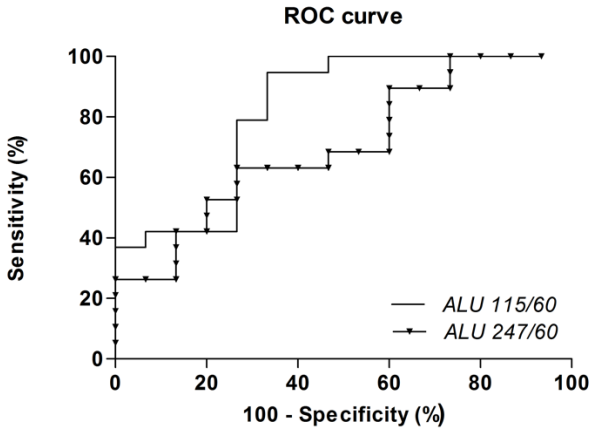

Figure. 5. ROC curves of scfDNA integrity indexes ALU115/ALU60 and ALU247/ALU60 for discriminating OCSCC patients from HC. *Abbreviations:* scfDNA, salivary cell-free DNA; OCSCC, oral cavity squamous cell carcinoma; HC, healthy controls.

4. DISCUSSION

Liquid biopsies based on tumor biomarkers represent an opportunity for improving early cancer detection. Among the various liquid biopsy samples, saliva has attracted great interest for identifying tumor-related biomarkers since its collection is easy, non-invasive, and inexpensive [13]. Like in blood, in addition to the cellular fraction, saliva comprises an extracellular fraction that reflects local and systemic biological information [14,15]. As we previously noted, scfDNA could be released by apoptosis, necrosis, or active secretion from malignant and non-malignant cells [12]. Recently, it has been demonstrated the feasibility to detect tumor-derived circulating DNA in saliva [11]. Nevertheless, the potential clinical role of scfDNA concentration and integrity for OCSCC diagnosis was not already established.

To the best of our knowledge, this is the first study that investigates the potential application of cfDNA concentration and integrity characterization in using saliva samples for discriminating OCSCC patients from healthy controls. The cfDNA concentration has been shown to act as a diagnostic biomarker due to its ability to differentiate

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cancer patients from controls subjects [16,17]. Opposite to other tumor types, few studies analyzed the quantity of cfDNA in oral cancer [18–20]. In this preliminary study, we have observed higher scfDNA in OCSCC patients compared to healthy controls using fluorometry and ALU60-qPCR quantification methods, although this increase was not statistically significant, probably because of the limited cohort analyzed. Similarly, Ding et al. analyzed the concentration of scfDNA in lung cancer by LINE1-qPCR, but no significant differences were observed among the groups [21]. Previously, Shukla et al. failed to find significant differences in plasma cfDNA levels in oral cancer patients, precancerous lesions, or patients with oral cancer after surgery compared with healthy controls [19]. By the contrary, Lin et al. reported a significant increase of cfDNA levels in oral cancer, showing an elevation of plasma cfDNA concentration together with tumor progression [18]. Based on our findings, the total concentration of scfDNA may have limited diagnostic value for oral cancer detection and does not provide information about the mechanism of cfDNA release into saliva.

In addition to the total scfDNA concentration, the scfDNA fragment size may be used to differentiate cancer patients from healthy controls. In the last few years, several studies have focused on the analysis of cfDNA integrity as a promising tumor biomarker [9,22,23]. Since apoptosis is the main source of uniform and small fragments of circulating cfDNA in healthy individuals, the presence of longer cfDNA fragments has been referred to a marker of necrotic tumor cell death. Therefore, the ratio of long to short fragments, known as cfDNA integrity, reflects the relative amount of non-apoptotic cell death [9]. ALU-targeting qPCR represents the most common method for assessing DNA integrity. Specifically, ALU115 (amplicons ≥115bp) and ALU247 (amplicons ≥247bp) are the sequences analyzed to determine DNA originating from apoptotic and necrotic cell death, respectively [9,10,24,25]. Like Ren et al. [26] we have used three sets of ALU primers: two primer sets for the amplicons with 60 bp and 115 bp lengths that amplify both short and long DNA fragments, and a primer set for the amplicon 247 bp that amplifies only long DNA fragments. ALU sequences are transposable DNA elements (short

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interspersed elements) of around 300 bp length that comprise more than 10% of the mass of the human genome [27]. In our study, we have observed that the medians for the two scfDNA integrity indexes (ALU115/ALU60 and ALU247/ALU60) were significantly higher in OCSCC patients compared to healthy controls. In line with our results, Desai et al. [20] showed in plasma from oral cancer patients a significantly higher DNA integrity compared to healthy or oral precancerous groups, using the ratio of longer (253 bp) to shorter (102 bp) beta-actin gene fragments. Furthermore, considering the diagnostic potential of each scfDNA integrity indexes, we observed that the scfDNA integrity index 115/60 had the highest diagnostic accuracy (AUC = 0.8211) for discriminating OCSCC with a sensitivity and specificity of 83.33% and 73.33%, respectively. In line with our findings, Pinzani et al. [22] reported after the quantification of four amplicons length of the Amyloid Precursor Protein *(APP)* gene that the ratio 180/67 had the better diagnostic accuracy for differentiating melanoma patients compared to the ratios 307/67 and 406/67. Recently, the study performed by Azab et al. [28] based on cfDNA from oral rinses found a poor diagnostic accuracy of DNA integrity index ALU247/ALU115 for discriminating oral cancer from oral potentially malignant disorders (leukoplakia and oral lichen planus), showing an AUC of 0.652. Additionally, our results regarding the scfDNA fragment size revealed that longer scfDNA fragments (>247 bp) are significantly more prevalent in OCSCC while in healthy controls there is a prevalence of shorter fragments between 60 and 115bp, suggesting that tumor necrosis would be increasing the relative concentration of longer DNA fragments in OCSCC patients. Therefore, these results indicate that OCSCC patients have scfDNA with higher integrity, probably for the presence of a higher content of necrotic DNA and this integrity represents a potential diagnosis tool that should be further explored in this type of tumor. Interestingly, our study also revealed for the first time the presence of scfDNA of high molecular weight in healthy individuals indicating that non-tumoral scfDNA is innately a mixture of long and short DNA fragments. We hypothesized that the global profile of scfDNA could be conditioned by the content of

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salivary exosomes and the mechanisms of epithelial cell death modulated by the oral microbiota [29,30].

However, our study is not exempt from shortcomings. Our findings should be validated in longitudinal studies with larger sample sizes for better understanding the potential clinical impact of scfDNA, not only for diagnosis but also for prognosis and monitoring the response to therapy in OCSCC. Also, future studies should investigate the correlation between cfDNA integrity in saliva and plasma to determine the effectiveness of each biofluid in early- and late-stage cancer. To optimize the diagnostic accuracy, the combination of scfDNA integrity with other exploratory tumor-specific biomarkers such as proteins, microRNAs, or other biomolecules should be explored.

In conclusion, scfDNA integrity indexes (ALU115/60 and ALU247/60) represent promising tools for discriminating OCSCC patients from healthy controls. Our results, although coming from a pilot study, open new avenues to explore scfDNA as a clinical tool.

Author Contributions: Study concepts, O.R.-G., L.M.-R., and M.M.S-C.; study design, O.R.-G., L.M.-R., R.L.-L., and M.M.S-C.; data acquisition, O.R.-G., R.M.L.-L., and A.A.; data analysis and interpretation, O.R.-G., L.M.-R., and M.M.S.-C.; statistical analysis, O.R.-G. and L.M.-R.; manuscript preparation, O.R.-G. and M.M.S-C.; manuscript editing, O.R.-G.; and manuscript review, L.M.-R., R.L.-L., J.L.L.-C. and G.R.-R. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 3.3

Somatic Mutations in Tumor and Plasma of Locoregional Recurrent and/or Metastatic Head and Neck Cancer Using a Next-Generation Sequencing Panel

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OBJECTIVE 3.3

SOMATIC MUTATIONS IN TUMOR AND PLASMA OF LOCOREGIONAL RECURRENT AND/OR METASTATIC HEAD AND NECK CANCER USING A NEXT-GENERATION SEQUENCING PANEL*

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ABSTRACT

Next-generation sequencing (NGS) of cell-free DNA (cfDNA) has emerged as a potential non-invasive approach for tumor genome profiling. We explored the utility of TruSight Tumor 170 panel (TST170) for detecting somatic mutations in tumor and cfDNA from recurrent and/or metastatic head and neck squamous cell carcinoma (HNSCC) patients. Targeted NGS sequencing of tumor DNA and plasma cfDNA was performed using this panel. In addition, a set of somatic mutations previously described in HNSCC were selected for being validated in tumor, plasma, and saliva by digital droplet PCR. The TST170 panel identified 24 somatic mutations in tissue, of which 14/24 were detected in plasma cfDNA, showing an overall concordance rate of 58.3%. A total of 9 variants (39.13%) identified in plasma were not identified in tumor tissue. This proof-of-principle study shows that targeted NGS analysis of tumor and cfDNA with the TST170 panel is technically feasible in patients with advanced HNSCC.

Keywords

Head and neck squamous cell carcinoma; cell-free DNA; next-generation sequencing, somatic mutations; liquid biopsy; saliva.

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1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignancy characterized by a heterogeneous nature both at clinical and molecular point of view. Despite advances in diagnosis and multimodal treatment strategies, 65% of HNSCC patients develop recurrent or metastatic disease, associated with an unfavorable prognosis [1]. The characterization of the molecular landscape of HNSCC has identified several driver genes involved in this tumor carcinogenesis, such as *TP53*, *CDKN2A*, *PIK3CA*, *NOTCH1*, *FAT1*, or *EGFR*, representing an opportunity for developing molecular targeted therapies. Furthermore, molecular subtyping has shown different genetic alterations in human papilloma virus (HPV) negative head and neck cancer (HNC), which are mainly characterized by the accumulation of mutations in *TP53* and *CDKN2A* tumor suppressor genes [2]. However, nowadays epidermal growth factor receptor (*EGFR*) is the only proven molecular target for HNC therapy. The anti EGFR-specific monoclonal antibody cetuximab has been approved by the United States Food and Drug Administration for HNC management, showing a significant clinical benefit in locally regional advanced disease and recurrent and/or metastatic disease [3]. Unfortunately, still today the standard of care in HNC is only based on the clinical and histopathological characteristics of the tumor, so there is an urgent need for the identification of real-time tumor biomarkers for guiding therapy selection and improving the therapeutic response rates in these patients.

Liquid biopsies based on circulating tumor DNA (ctDNA), a fraction of total cell-free DNA (cfDNA), have emerged as a potential non-invasive approach for tumor genome profiling [4]. Ongoing advances in Next Generation Sequencing (NGS) technology have allowed the development of multiple-gene panels for characterizing tumors in both tissue and cfDNA samples. The feasibility to identify tumor somatic genomic alterations, such as single nucleotide variants (SNVs), insertions and deletions (InDels), or copy number alterations (CNVs), in cfDNA supports its clinical value for targeted therapy-decision making and monitoring treatment response in cancer [5–7]. Due to the molecular landscape of HNSCC harbors potentially

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actionable alterations, cfDNA profiling could represent an alternative to tumor biopsy for detecting and monitoring somatic alterations over the course of the disease, which will favor the application of precision medicine in HNC. Recently, some studies have been focused on the identification of somatic mutations in liquid biopsies using a tumor-tissue informative approach [8,9] or a tumor-agnostic approach [10,11]. The aim of this pilot study was to explore whether a targeted NGS panel (TST170) previously used for tissue characterization could be suitable for detecting somatic mutations in both tumor and cfDNA from recurrent and/or metastatic HNSCC patients. Furthermore, we also carried out an orthogonal validation of somatic mutations in the tumor, plasma cfDNA and salivary DNA.

2. MATERIAL AND METHODS

2.1. HNSCC patients's recruitment

The present study was approved by the Galician Ethics Committee of Clinical Research (Ref. No. 2018/003) and carried out in accordance with the principles of the Declaration of Helsinki (World Medical Association, 2013). Written consent was obtained for each patient for use of their tissues and liquid biopsies (blood and saliva) samples. This retrospective study enrolled patients diagnosed with locoregional recurrent and/or metastatic HPV-negative HNSCC. Patients were diagnosed by histopathological analysis of the corresponding tissue biopsy and HPV-status was determined using Anyplex II HPV28 (Seegene®) detection kit. All HNSCC patients (n=3) were recruited at Department of Oral and Maxillofacial Surgery from Complexo Hospitalario Universitario of A Coruña (CHUAC, SERGAS) and at Department of Radiation Oncology, Centro Oncológico of Galicia, in Galicia, Spain. Blood and saliva samples were collected at diagnosis of locoregional recurrent and/or metastatic disease and primary tumor samples were obtained of tissue biopsy specimens from biobank. Clinical and histopathological characteristics of patients are shown in Table S1.

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2.2. Plasma and saliva samples collection

Peripheral blood samples were collected by drawing blood into Streck Cell-Free DNA blood collection tubes (Streck BCT®, Streck, Omaha, NE, USA). A two-step centrifugation was performed to isolate the plasma. First, blood was centrifuged at 1600 xg for 10 min at room temperature. Then, plasma was collected without disturbing buffy coat layer and centrifuged again at 5500 xg for 10 min at room temperature to remove the remaining cells. Plasma samples were stored at -80 °C until further analysis.

Saliva samples were collected using the Oragene® DNA Sample Collection Kit (DNA Genotek OG-500; Ottawa, Ontario, Canada) according to the manufacturer's instructions. This kit provides a collection tube with 1 mL of suspension buffer containing proprietary reagents that stabilize the whole saliva sample prior to DNA extraction. Then, saliva samples were stored at -80 °C until further analysis.

2.3. DNA extraction

Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissues using the AllPrep® DNA/RNA FFPE kit (Qiagen, Hilden, Germany) according to the manufacture's recommendations. Genomic DNA from tumor samples was eluted in 100 µL buffer AE and stored at -20 °C for further use. DNA from saliva and plasma was isolated using the QIAamp DNA Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Samples were eluted in 100 µL of buffer AVE and stored at -20 °C until further use. Isolated DNA from tumor, plasma, and saliva was quantified by Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Agilent's TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA) was used to assess the fragment distribution of the extracted DNA.

2.4. NGS-based mutational analysis

Targeted NGS sequencing of genomic DNA and plasma cfDNA was performed using the Illumina TruSight Tumor 170 panel (TST170;

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Illumina, Inc., San Diego, California, US). This hybrid-capture panel is designed to target DNA variants of 170 cancer-related genes. The panel includes 55 genes for fusions and splice variants, 148 SNVs and InDels, and 59 amplifications characterization, providing a comprehensive coverage of the variants that are most likely to play a role in tumorigenesis (Table S2). A total of 60 ng of DNA were used as input for sequencing library preparation following the manufacture's reference guides. Then, a pool of oligos specific to the 170 genes was hybridized to DNA libraries for a minimum of 8 h to a maximum of 24 h at 57 °C. To ensure high specificity of the captured regions a second hybridization step was performed for a minimum of 1.5 h to a maximum of 4 h at 57 °C. After amplification of the captured regions, the quantity of enriched libraries was assessed using a fluorometric method (Qubit) and further, a bead-based normalization was performed to ensure a uniform library representation in the pooled libraries. Sequencing was carried out on NextSeq 500 sequencing system (Illumina), according to the TruSight® Tumor 170 protocol (Illumina).

2.5. Bioinformatic analysis

FASTQ sequencing reads were assessed using the quality control tool, FastQC [12]. Reads were mapped to the hg19 reference genome by Burrows-Wheeler Aligner (BWA)-MEM [13]. SAMtools were used to convert the aligned SAM files to BAM files [14], applying Bammarkduplicates2 to mark duplicated reads [15]. mosDepth was used for quantifying coverage from BAM input files (htttps://github.com/brentp/mosdepth). Variant calling was performed using the Genome Analysis Tool Kit (GATK) [16], together with the Mutect2 tool in GATK [17], following best practices (https://gatk.broadinstitute.org/hc/en-us/articles/360035894731-Somatic-short-variant-discovery-SNVs-Indels-). The Ensembl Variant Effect Predictor (VEP) was used to determine the effect of genetic variants [18], using SIFT and Polyphen scores to establish the effect of differently called genetic variants [19,20]. Following VEP annotations, variants were filtered in order to preserve just those classified as probably pathogenic both by SIFT and Polyphen, as well as those with a high impact. As an additional control for somatic variants, each of

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them must present an allele frequency observed in any population from 1KGP, ESP or gnomAD lower than 0.01 or not described, to assess they are not common in the population [21].

2.6. Validation of somatic mutations by ddPCR

Somatic mutations were validated by digital droplet PCR (ddPCR) (Bio-Rad Laboratories Hercules, CA, USA) using specific-mutation assays and the QX200 ddPCR system (Bio-Rad Laboratories Hercules, CA, USA). Data analysis was performed on the QuantaSoffTM v.1.7 software (Bio-Rad Laboratories Hercules, CA, USA) according to the manufacture's recommendations.

3. RESULTS

3.1. Analysis of somatic gene alterations in tumor and plasma cfDNA of HNSCC patients by TST170

The targeted NGS of tumoral DNA by TST170 provided a mean coverage of 1190x (range: 0x-5314x). Allelic frequency of mutant copies ranged from 0.011−0.562 in tumor samples (Table S3). Importantly, the panel identified 24 non-synonymous somatic mutations in tumor tissue of the 3 patients included in the study (Figure 1). Overall, the detected somatic gene alterations correspond to frameshifts (62.5%), missense (20.83%), and stop gain 8.3%) variants. *MCL1* and *SLX4* genes showed the same somatic variant in all patients. Interestingly, in tumor samples we found 10 somatic variants in patient No.3, 8 in patient No.2, and 6 in the patient No.6. The application of the TST170 on cfDNA provided a mean coverage of 2043x (range: 0x-4276x) and allelic frequency of mutant copies ranged from 0.012−0.287. Among the 24 somatic mutations detected in tissue, 14 were detected in plasma cfDNA, showing an overall concordance rate of 58.3%. In addition, 39.13% variants (9) identified in plasma were not identified in tumor tissue showing that liquid biopsy analysis provides additional information.

After analyzing the intra-patient concordance, we detected in cfDNA the 50% (4/8) of the somatic mutations present in tumor tissue

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from patient No.2, the 50% (5/10) variants in patient No.3 and the 83.33% (5/6) in patient No.6.

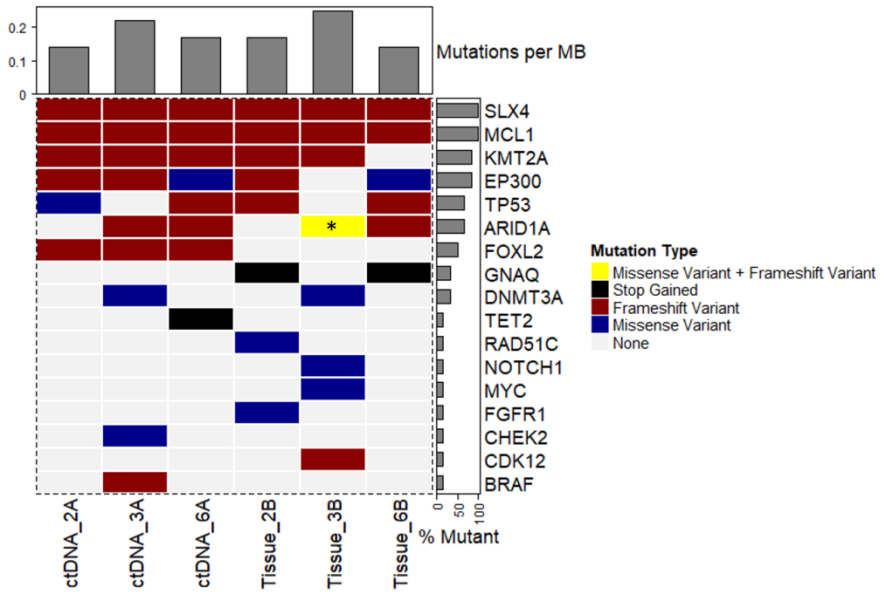

Figure 1. Matrix of somatic mutations detected in tumor and plasma cfDNA of three advanced HNSCC (head and neck squamous cell carcinoma) patients. *Somatic gene mutations including one missense variant and two frameshift variants.

3.2. Analysis of selected somatic mutations in tumor, plasma cfDNA and salivary DNA of HNSCC patients by ddPCR

A total of four somatic genetic alterations previously described in HNC (*EP300* c.4241A>G, *NOTCH1* c.6208C>T, *TP53* c.723delA, and *TP53* c.485T>A) were selected to orthogonally validate our NGS analysis using ddPCR. In addition to tumor tissue and plasma-derived DNA, we included paired salivary DNA from the three HNSCC patients. The *EP300* c.4241A>G mutation was validated in tumor DNA and plasma cfDNA with an allele frequency of mutant copies of 43.2% and 0.72%, respectively (Patient No.6). The *NOTCH1* c.6208C>T variant, detected only in tumor DNA by the NGS approach was found

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in tumor and plasma samples with an allele frequency of 8.3% and 1.7%, respectively (Patient No.3). The *TP53* c.723delA mutation, detected in the tumor tissue by NGS, was only validated in tumor DNA with an allele frequency of mutant copies of 58.7% (Patient No.2). The *TP53* c.485T>A variant detected in plasma by NGS, was validated by ddPCR in plasma and saliva samples with an allele frequency of mutant copies of 0.26% and 0.07%, respectively (Patient No.2) (Figure 2 and Table S4).

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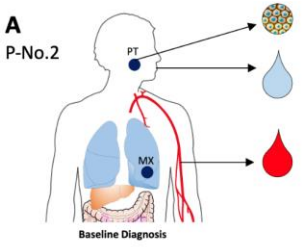
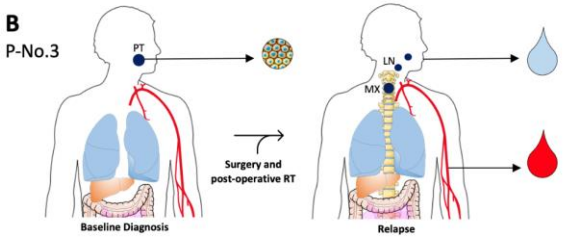
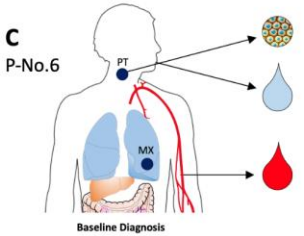


Assay		Tumor	Blood	Saliva
TP53 c.485 T>A	NGS	wt	5.4%	NP
	ddPCR	wt	2.7%	0.07%

Assay		Tumor	Blood	Saliva
TP53 c.723delA	NGS	39.1%	wt	NP
	ddPCR	58.7%	wt	wt



Assay		Tumor	Blood	Saliva
NOTCH c.6208 C>T	NGS	5.5%	wt	NP
	ddPCR	8.3%	1.7%	wt



Assay		Tumor	Blood	Saliva
EP300 c.4241 A>G	NGS	32.7%	1.8%	NP
	ddPCR	43.2%	0.72%	wt

Figure 2. Validation of selected somatic mutations detected by NGS (TST170) panel on tissue, plasma and saliva from HNSCC patients by ddPCR. A) Patient No. 2 with tongue squamous cell carcinoma and lung metastasis (T3N2M1) at baseline diagnosis; B) Patient No. 3 with a gingival squamous cell carcinoma at baseline diagnosis (), and after surgery and post-operative radiotherapy (RT) treatment, locoregional lymph node recurrence and distant metastasis in vertebra (T4N3M0); and C) Patient No. 6 with hypopharynx tumor and lung metastasis at baseline diagnosis (T3N0M1). *Abbreviations:* NGS, next-generation sequencing; ddPCR, digital droplet PCR; WT, wild-type; PT, primary tumor; MX, metastasis; LN, lymph nodes; NP, not performed.

4. DISCUSSION

Our pilot study showed the ability to detect somatic mutations in cfDNA from HNSCC patients using TST170 targeted NGS-panel designed for screening genetic alterations in solid tumors. Recently, our research group demonstrated the potential clinical utility of the TST170 panel for detecting gene variants in cfDNA from metastatic CRC patients. Specifically, *KRAS* variants identified in cfDNA by the TST170 panel showed a high concordance with tumor tissue and cfDNA analyzed by BEAMing, indicating that TST170 can successfully identify mutations with variant allele frequency (VAFs) ≥ 0.5% [22]. In the present study, we evaluated for the first time the applicability of the TST170 panel on tumor tissue and cfDNA from HNSCC patients. The molecular characterization of HNC patients using cfDNA has emerged as an opportunity for identifying predictive biomarkers and developing targeted therapies. In our study we detected somatic variants (SNVs and InDels) in a total of 19 genes in tumor and/or cfDNA, of which *TP53*, *NOTCH1*, and *EP300* are described as common driver genes in HPV-negative HNC.

The feasibility of detecting somatic alterations in liquid biopsies from HNSCC has been previously explored in different studies [11,23,24]. Wang et al. analyzed the presence of HPV and somatic mutations in *TP53, PI3KCA, CDKN2A, FBXW7, HRAS,* and *NRAS* in plasma cfDNA and salivary DNA of HNSCC patients. Although a higher rate of ctDNA detection was observed in plasma for advanced-stage disease and in saliva for early-stage disease, this PCR approach was restricted to a limited set of genes previously identified in tumor biopsy [23]. Later, Perdomo et al. analyzed by ctDNA sequencing 65 tumor mutations in 5 genes (*TP53, NOTCH1, CDKN2A, CASP8*, and *PTEN*) observing ctDNA alterations in 42% of plasma samples, with an overall concordance rate of 28%. Using a more comprehensive genomic approach, we evaluated the performance of the TST170 panel in plasma cfDNA and tumor DNA from HNSCC due to this panel covers 60% of the most frequently mutated tumor suppressor genes and oncogenes characterized in HNC and accessible on the Catalogue of Somatic Mutations in Cancer (COSMIC) database. One of the questions of the

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present work is how representative of the tumor molecular landscape could be the cfDNA samples. In this regard, an overall concordance rate around 60% was found between plasma cfDNA and tumor tissue. This concordance data, although coming from a limited cohort are higher than previously described. In fact, Galot et al. sequenced both cfDNA and the solid tumor using a custom panel of 604 genes in a cohort of 39 HNSCC patients showing a concordance rate of 42% in metastatic patients. However, this rate decreased to 18% in patients with locally advanced disease [11]. Interestingly, lower concordance rates have been reported when different clinically available NGS panels were applied for sequencing tumor tissue and cfDNA [25,26]. To overcome this challenge, we evaluated the applicability of a targeted NGS tumor panel for detecting somatic alterations in cfDNA. The use of targeted NGS panels that allows the detection of gene variants in tumor tissue and cfDNA at the same time may provide a potential strategy for the molecular characterization of the tumor in a single assay without the need of designing a specific cfDNA panel. It is also important to remark that ctDNA sequencing at primary diagnosis of the metastatic disease can reflect the somatic gene alterations in both primary tumor and metastatic lesions contributing to capturing the molecular heterogeneity that may be underestimated by single tissue biopsy [27–29].

Regarding this important point, our study included cfDNA from three metastatic HNSCC patients. Importantly, liquid biopsies were obtained at the same time as tumor biopsy in two patients (No.2 and 6), whereas in the third patient (No.3) liquid samples were taken at disease recurrence but tumor tissue biopsy was obtained at diagnosis. The patient No.6 showed a high concordance rate (83.33%) between cfDNA and tumor tissue biopsy. In this case, the tumor was located in the hypopharynx and the metastasis in the lung. Interestingly, the profiling of cfDNA revealed three somatic mutations in plasma that were not detected in tumoral tissue, which could be present in metastatic lesions. The patient No.3 showed a concordance of 50% between cfDNA and tumor tissue. This lower concordance rate could be the result of the tumor evolution between the different moments of samples collection. In this case, the primary tumor was located at the gingiva and the recurrence was in the cervical region with distant metastasis in the

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vertebra. A total of 4 variants were detected exclusively in plasma probably reflecting the molecular evolution of the disease. Interestingly, the patient No.2, with a tongue tumor and lung metastasis showed a concordance of 50%. This partial concordance rate could be explained by the intratumor heterogeneity and specific metastatic profile.

Since saliva is the biofluid of the oral cavity, proximal and distant tumors may shed DNA into saliva representing an opportunity for detecting tumor-associated mutations [23,30]. Due to tumor DNA has been detected in saliva at frequencies <1% [8,23] we analyzed a set of gene variants, identified in tissue samples with the TST170, in total salivary DNA from our patient cohort by ddPCR. We were able to detect the variant *TP53* c.485T>A with a mutant allele fraction of 0.079% in saliva from one patient with tongue cancer which conforms the possibility to detect somatic variants with allele frequency <0.1% in saliva using the ddPCR. As previously reported Wang et al., the sensitivity for detecting tumor-DNA in saliva was anatomic site-dependent, showing the higher rate of detection and fraction of mutant alleles in oral cavity tumors compared to oropharynx, larynx, and hypopharynx tumors [23]. Although in the other two cases (No. 3 and 6), tumor DNA was not detected in saliva, the selected somatic mutations were validated in plasma samples. These findings suggest that plasma could be a more suitable fluid for detecting tumor DNA than saliva in patients with advanced HNC.

One of the strengths of our work is the application of the same sequencing technology to tumor and cfDNA samples. TST170 allows the detection of somatic variations identified with a VAF <0.1% using the ddPCR. Also, thanks to the high sensitivity of ddPCR the analysis of somatic tumor-derived variants is feasible in liquid biopsies, as reinforced by our study, representing a valuable tool for the detection and monitoring of predictive biomarkers to improve the management of HNC patients. However, our study has several limitations. The main limitation of our study is related to its sample size and the heterogeneous nature of this cohort. All patients included were males with advanced-stage of HNSCC. Also, the tumoral origin of the genetic variants detected exclusively in cfDNA should be interpreted with caution due to the lack

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of data on peripheral blood leukocytes to assess the contribution of mutations from clonal hematopoiesis. Although we corrected this issue by using a bioinformatic approach, our recommendation is to analyze matched germline DNA from each patient for identifying the contribution of clonal hematopoiesis to false positive detection. Furthermore, we only performed mutation analysis in plasma and saliva at a single time point which did not allow us to monitor mutations during treatment and we couldn´t sequence the metastatic lesions to confirm the origin of the variants detected in cfDNA.

5. CONCLUSION

This proof-of-principle study shows that targeted NGS analysis of tumor and cfDNA with the TST170 panel is technically feasible for patients with advanced HNSCC. This approach allows to detect somatic mutations using a single assay that could promote the clinical implementation of personalized medicine in the clinic. However, further research is necessary to validate our findings.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Clinicopathological characteristics, Table S2: List of genes analyzed in DNA by the TST170 panel, Table S3: Somatic mutations detected by NGS, Table S4: Absolute and relative quantification of mutant and wild-type DNA in tumor, plasma and saliva samples by ddPCR.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used Conceptualization, L.M.-R and M.M.S.-C.; methodology, L.M.-R, A.D.-L., and M.M.S.-C.; validation, O.R.-G. and L.M.-R.; formal analysis, M.M.-F. and J.B.-I.; investigation, O.R.-G, A.R.-C, and A.B.-C.; resources, J.L.L.-C. and G.T.-M.; data curation, X.X.; writing—original draft preparation, O.R.-G. and M.M.S.-C.; writing—review and editing, O.R.-G., M.M.S.-C., L.M.-R., A.D.-L.; visualization, O.R.-G. and R.D.-P.; supervision, O.R.-G., M.M.S.-C. and L.M.-R.; project administration, M.M.S.-C.;

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CHAPTER 3.4

Salivary DNA Methylation in Head and Neck Cancer

3.4.1. ***Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-Analysis***

3.4.2. *Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-Analysis*

3.4.3. *Genome-Wide DNA Methylation Profiling of Oral Tongue Squamous Cell Carcinoma*

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CHAPTER 3.4.1

Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-analysis

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OBJECTIVE 3.4.1

SALIVARY DNA METHYLATION AS AN EPIGENETIC BIOMARKER FOR HEAD AND NECK CANCER. PART I: A DIAGNOSTIC ACCURACY META-ANALYSIS *

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ABSTRACT

DNA hypermethylation is an important epigenetic mechanism for gene expression inactivation in head and neck cancer (HNC). Saliva has emerged as a novel liquid biopsy representing a potential source of biomarkers. We performed a comprehensive meta-analysis to evaluate the overall diagnostic accuracy of salivary DNA methylation for detecting HNC. PubMed EMBASE, Web of Science, LILACS, and the Cochrane Library were searched. Study quality was assessed by the Quality Assessment for Studies of Diagnostic Accuracy-2, and sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (dOR), and their corresponding 95% confidence intervals (CIs) were calculated using a bivariate random-effect meta-analysis model. Meta-regression and subgroup analyses were performed to assess heterogeneity. Eighty-four study units from 18 articles with 8368 subjects were included. The pooled sensitivity and specificity of salivary DNA methylation were 0.39 and 0.87, respectively, while PLR and NLR were 3.68 and 0.63, respectively. The overall area under the curve (AUC) was 0.81 and the dOR was 8.34. The combination of methylated genes showed higher diagnostic accuracy (AUC, 0.92 and dOR, 36.97) than individual gene analysis (AUC, 0.77 and dOR, 6.02). These findings provide evidence regarding the potential clinical application of salivary DNA methylation for HNC diagnosis.

Keywords

DNA methylation; epigenetics; head and neck cancer; saliva; biomarkers; liquid biopsy; meta-analysis.

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1. INTRODUCTION

Head and neck cancer (HNC) comprises a heterogenous group of epithelial malignancies arising from mucosal linings of the oral cavity, oropharynx, larynx, and hypopharynx. According to data from the World Health Organization's GLOBOCAN network, HNC is highly prevalent worldwide, accounting for an estimated 890,000 new cases and 450,000 deaths in 2018 [1]. Despite improvements in diagnosis and therapeutic strategies, the 5-year survival rate for HNC has remained around 50% for the last decade. Unfortunately, HNC patients are frequently diagnosed in advanced stages involving a high risk of locoregional recurrence and distant metastasis [2,3]. Therefore, it is necessary to identify new biomarkers for diagnosis and prognosis to allow early detection and improved overall survival. HNC arises through multistep carcinogenic pathways as a result of cumulative genetic and epigenetic aberrations resulting from risk factors including alcohol and tobacco consumption, human papillomavirus infection, chronic inflammation, and genetic predisposition, and leading to reduced tumor suppressor gene function as well as oncogene activation [2,4]. In recent years, accumulating scientific evidence has highlighted the important role in tumorigenesis of epigenetic mechanisms, which represent a cancer hallmark [5,6]. Epigenetic alterations such as DNA methylation, histone covalent modifications, chromatin remodeling, and non-coding RNAs have been implicated in the landscape of phenotypical changes occurring in a wide variety of malignancies, including HNC [7]. DNA hypermethylation has been shown to be an important epigenetic mechanism for gene expression inactivation. The hypermethylation of cytosine–phosphodiester bond–guanine (CpG) islands within promoter regions plays an important role in carcinogenesis through the transcriptional silencing of different tumor suppressor genes or dysfunction in DNA repair genes [7,8]. Several studies have focused on the identification of aberrant promoter methylation patterns in HNC tissue and liquid biopsies [9,10]. Moreover, a number of investigations have detected promoter hypermethylation in various genes using saliva from HNC patients [10,11,12]. Therefore, salivary DNA hypermethylation represents a promising biomarker for non-invasively diagnosing HNC.

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DNA methylation is a heritable and stable epigenetic mechanism implicated in the regulation of gene expression that plays an important role during normal development, regulating X chromosome inactivation, genomic imprinting, and preventing the transcription of DNA repetitive sequences, inserted viral sequences, and transposons [8,13]. DNA methylation is characterized by the covalent addition of a methyl group to the 5'-position of the pyrimidine ring of cytosines by DNA methyltransferases, giving rise to 5-methylcytosine. This enzymatic process occurs predominantly within CpG dinucleotides which are concentrated at CpG-rich DNA stretches named CpG islands (CGIs), which overlap the promoter region of 60–70% of protein-coding genes [14]. In the human genome, approximately 80% of CpG dinucleotides are heavily methylated whereas CGIs in gene promoters are mostly unmethylated, allowing active gene transcription [13]. Dysregulation of DNA methylation has been found to be involved in several diseases, representing an early epigenetic event in carcinogenesis. These alterations of normal DNA methylation patterns in cancer have been characterized as global hypomethylation and gene-specific hypermethylation. The global hypomethylation of repetitive sequences and transposable elements within the genome induces genomic instability and mutagenesis. In this line, the loss of DNA methylation may also activate latent viral sequences, promoting carcinogenesis. By contrast, in addition to global hypomethylation, aberrant promoter hypermethylation can drive the inactivation of key tumor suppressor genes, which are unmethylated in non-malignant tissues [15]. In this sense, although silencing by DNA hypermethylation of some genes is common in many types of tumors, the methylation profile of gene promoters is different for each human cancer, allowing the identification of cancer-specific hypermethylation patterns [16]. Although tissue biopsy of the primary tumor or metastatic lesions remains the gold standard method for diagnosis, DNA methylation biomarkers can be assessed in different liquid biopsies, representing a non-invasive alternative for early cancer detection [10,17,18].

Recently, saliva has emerged as an attractive liquid biopsy for genomic and epigenomic analysis. Saliva-based liquid biopsy is a fast, reliable, cost-effective, and non-invasive approach to analyze epigenetic

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alterations involved in the onset and course of the disease. Some researchers have found comparable methylation profiles between saliva and tissue, representing a non-invasive alternative for epigenomic profiling [19,20]. Additionally, although similar methylation DNA patterns have been reported between saliva and blood [21,22], methylation differences between both biofluids can be identified due to tumor shedding and tissue-specific methylation [23,24]. Focusing on promoter hypermethylation, a number of investigations have detected various methylated genes in saliva, representing a promising biomarker for non-invasive HNC detection [10,11,12].

The purpose of this systematic review and meta-analysis was to summarize the results of published clinical studies to assess the overall diagnostic accuracy of salivary DNA hypermethylation for discriminating HNC.

2. MATERIAL AND METHODS

2.1. Protocol and registration

This study was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines [25], and the protocol was registered with the International Prospective Register of Systematic Reviews (reference No. CRD42020199114).

2.2. Search strategy and study selection

The systematic literature search of eligible articles published up to 27 August 2020 was carried out without language restrictions using PubMed, EMBASE, Web of Science, LILACS, and the Cochrane Library. The search strategy was based on the following combinations of keywords and medical subject headings: (methylation OR hypermethylation OR epigenomics) AND (saliva OR oral rinse OR mouthwash) AND (head and neck cancer OR head and neck neoplasm OR head and neck carcinoma OR head and neck squamous cell carcinoma OR HNSCC OR oral cancer OR pharyngeal cancer OR laryngeal cancer). Studies were screened based on title and abstract, and eligible manuscripts were retrieved for full-text review. In addition,

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reference lists from each original and review article were searched manually in order to find further relevant studies. The literature search was performed independently by two investigators (ORG and MMSC), and disagreements during the selection process were resolved by consensus. The studies selected by means of the search strategy and other references were managed using RefWorks software (https://www.refworks.com/content/path_learn/faqs.asp, accessed 28 October 2020), and duplicate items were removed using the associated tools.

2.3. Selection criteria

The inclusion criteria were as follows: (1) studies that evaluated the diagnostic accuracy of gene promoter hypermethylation in saliva samples from HNC patients; (2) inclusion of a control group consisting of healthy controls; (3) sufficient data for generating a two-by-two (2 × 2) contingency table containing true positive (TP), false positive (FP), true negative (TN), and false negative (FN) values. The exclusion criteria were as follows: (1) reviews, letters, personal opinions, book chapters, case reports, conference abstracts, and meetings; (2) duplicate publications; (3) *in vitro* and *in vivo* animal experiments.

2.4. Data extraction

All eligible studies were assessed independently by two investigators (ORG and MMSC) and data were extracted using a pre-established form designed on a Microsoft Excel spreadsheet (Microsoft Corp. Redmond, WA, USA). Any disagreement among reviewers was resolved by consensus. The following information was extracted from each study: name of first author, year of publication, country, anatomic tumor location, number of cases and controls, positive methylated cases, positive methylated controls, method for DNA methylation detection, type of saliva sample (saliva or oral rinse), methylated gene names, and statistical analysis outcomes, including diagnostic accuracy and cut-off values. If the required data were incomplete, attempts were made to contact the authors to obtain the missing information. We defined "study unit" as the analysis of a relationship between gene

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promoter hypermethylation and HNC. Therefore, a single publication could potentially include more than one study unit as a result of reporting promoter hypermethylation for multiple genes.

2.5. Quality assessment of individual studies

Following Healthcare Research and Quality Agency recommendations, two independent researchers (ADL and LMR) applied the Quality Assessment of Diagnostic Accuracy Studies-2 checklist (QUADAS-2) [26]. Any discrepancies were resolved by a third reviewer (MMSC). The QUADAS-2 checklist assesses study quality by analyzing four key domains: (1) patient selection, (2) index tests, (3) reference tests, and (4) flow and times. Risk of bias and applicability concerns for each domain were assessed as "low", "high", or "unclear". One point was assigned to each item assessed as "low". Thus, articles were grouped into the following quality categories based on their cumulative score: "high" quality (6–7 points), "moderate" quality (4–5 points), and "low" quality (0–3 points).

2.6. Statistical analysis

MetaDiSc software (v.1.4) [27], free R software (v.3.4.4; https://www.r-project.org, accessed 30 November 2020), and STATA (v.14.0; https://www.stata.com, accessed 30 November 2020) were used to carry out statistical analysis. The numbers of TP, FP, FN, and TN in each study unit in the diagnostic meta-analysis were extracted to calculate pooled sensitivity [TP/(TP + FN)], specificity [TN/(TN + FP)], positive likelihood ratio (PLR) [(sensitivity/(1 − sensitivity)], negative likelihood ratio (NLR), [(1 − specificity)/specificity)], diagnostic odds ratio (dOR), and their corresponding 95% confidence intervals (CIs) using a bivariate random or fixed effect meta-analysis model. The pooled diagnostic performance of salivary DNA promoter hypermethylation for HNC detection was determined by plotting the summary receiver operator characteristic (SROC) curve and calculating the area under the SROC curve (AUC). Heterogeneity analysis was used to identify factors influencing accuracy indicators and the statistical model applied [27]. Spearman's correlation analysis and

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ROC plane plots were used to assess heterogeneity due to the threshold effect. Cochran's Q statistic test-based chi-squared test and I^2 statistics were used to assess non-threshold heterogeneity. When $I^2 > 50\%$ and/or $p < 0.05$ for the Cochran's Q test, heterogeneity was considered to be significant. The DerSimonian and Laird random effects model was applied when heterogeneity was significant; otherwise, we applied the Mantel–Haenszel fixed effects model. Potential sources of non-threshold heterogeneity were explored by meta-regression and subgroup analyses. In addition, the predictive value of post-salivary DNA promoter hypermethylation for HNC diagnosis was evaluated by Fagan's nomogram. Post-test probability was calculated using Bayes theorem under the assumption of prior probabilities of 25%, 50%, and 75%, respectively [28]. Deeks' funnel plot asymmetry test [29] was used to ascertain publication bias (statistical significance: $p < 0.05$).

3. RESULTS

3.1. Study selection

A PRISMA flowchart for the literature identification and selection process is shown in Figure 1. A total of 576 studies were identified based on the search strategy across the five electronic databases, which was reduced to 470 after removing duplicates. After title and abstract review, 27 articles were submitted for full-text reading, of which nine were excluded for the following reasons: non-independent cancer group (two articles); reviews, letters, personal opinions, book chapters, case reports, conference abstracts, and meetings (three articles); absence of a healthy control group (one article); saliva enriched with brush oral cytology (two articles); and insufficient information for meta-analysis (one article). In the end, 18 articles met the inclusion criteria for final analysis [10,11,12,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44].

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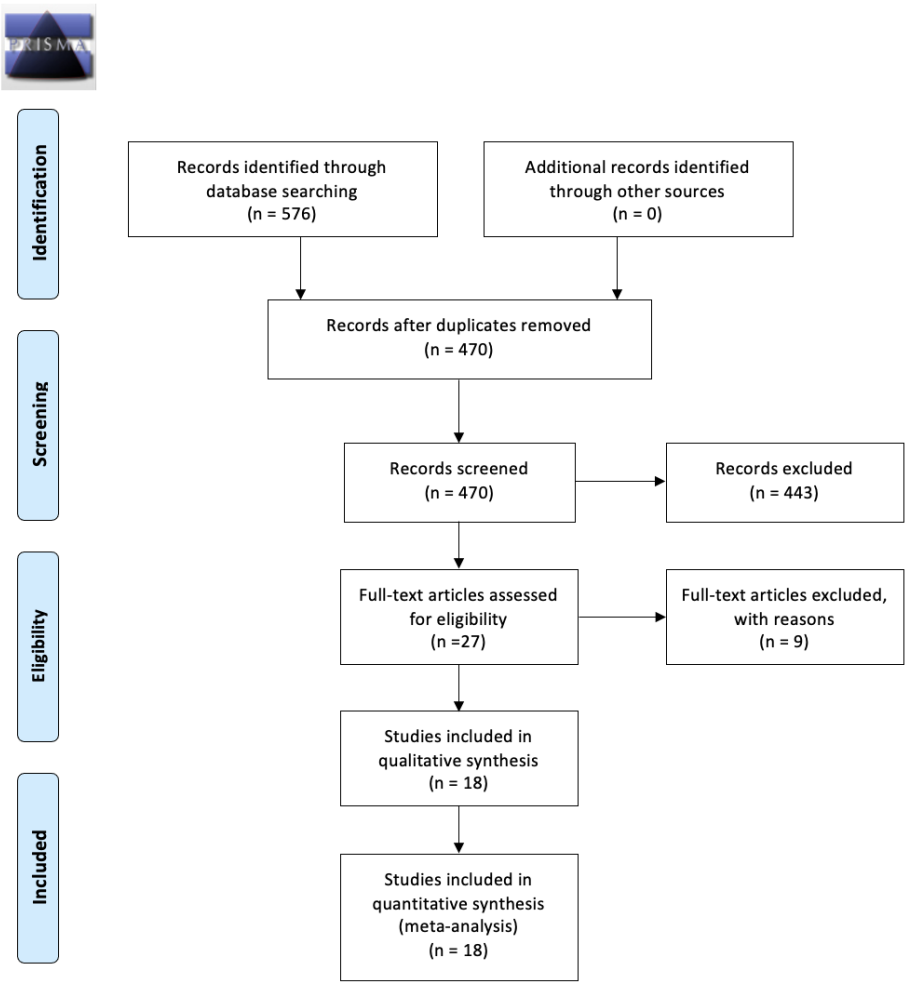

Figure 1. PRISMA flow diagram.

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3.2. Characteristics of included studies

The characteristics of the included studies are shown in Table 1. The 18 articles comprised a total of 84 study units, including 4758 HNC patients and 3605 healthy individuals (the sample size ranged from 13 [31] to 210 [41]). All articles were published between 2001 and 2020, and studies were conducted in the following geographical regions: the United States (5), Australia (3), Thailand (2), Japan (2), France (1), Brazil (1), India (1), Taiwan (1), Colombia (1), and Italy (1). Saliva samples included oral rinses (12) and whole saliva (6). Salivary DNA methylation was detected by different methods, including methylation-specific polymerase chain reaction (MSP) (11) and quantitative MSP (7). A total of 34 different genes were identified in the studies. Five studies evaluated the methylation status of a single gene [31,34,36,39,41] and 13 studies evaluated two or more genes [10,11,12,30,32,33,35,37,38,40,42,43,44]. Ten studies focused on gene promoter methylation panels combining two to four genes, whereas eight studies evaluated only single genes.

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Table 1. Summary of descriptive characteristics of included studies.

First Author	Anatomic Tumor Location	Type of Sample	Method	Biomarker	Cancer Group N (M+)	Control Group N (M+)
Rosas 2001	HNC	Oral rinse (NaCl)	MSP	p16	30 (11+)	30 (1+)
				DAPK	30 (6+)	30 (0+)
				MGMT	30 (4+)	30 (1+)
Righini 2007	HNC	Oral rinse (NaCl)	MSP	TIMP3	60 (17+)	30 (0+)
				ECAD	60 (12+)	
				p16	60 (16+)	
				MGMT	60 (13+)	
				DAPK	60 (9+)	
				RASSF1A	60 (10+)	
				p15	60 (7+)	
				p14	60 (2+)	
				APC	60 (4+)	
				FHIT	60 (2+)	
				hMLH1	60 (0+)	
Franzmann 2007	HNC	Oral rinse (NaCl)	MSP	CD44	11 (9+)	10 (0+)
Guerrero-Preston 2011	HNC	Oral rinse (NaCl)	qMSP	HOXA9	32 (20+)	19 (9+)
				NID2	32 (23+)	19 (12+)
				HOXA9+NID2	32 (25+)	19 (6+)
	OC			HOXA9	16 (11+)	19 (9+)
				NID2	16 (14+)	19 (12+)
				HOXA9+NID2	16 (14+)	19 (6+)
	OPC			HOXA9	16 (9+)	19 (9+)
				NID2	16 (9+)	19 (12+)
				HOXA9+NID2	16 (11+)	19 (6+)
Nagata 2011	OC	Oral rinse (NaCl)	MSP	ECAD	34 (32+)	24 (5+)
				TMEFF2	34 (29+)	24 (3+)
				RARβ	34 (28+)	24 (2+)
				MGMT	34 (26+)	24 (5+)
				FHIT	34 (27+)	24 (8+)
				WIF1	34 (24+)	24 (5+)
				DAPK	34 (19+)	24 (6+)
				p16	34 (13+)	24 (2+)
				HIN	34 (10+)	24 (2+)
				TIMP3	34 (8+)	24 (1+)
				p15	34 (22+)	24 (9+)
				APC	34 (18+)	24 (9+)
				SPARC	34 (14+)	24 (8+)
				ECAD+TMEFF2+RARβ+MGMT	34 (34+)	24 (3+)
				ECAD+TMEFF2+MGMT	34 (33+)	24 (2+)
				ECAD+TMEFF2+RARβ	34 (32+)	24 (1+)
				ECAD+RARβ+MGMT	34 (31+)	24 (2+)
Ovchinnikov 2012	OC	Saliva	Nested MSP	p16+RASSF1A+DAPK1	143 (117+)	46 (6+)
Rettori 2012	HNC	Oral rinse (NaCl)	qMSP	DCC	143 (75+)	50 (5+)
				CCNA1	146 (17+)	60 (2+)
				DAPK	146 (12+)	39 (1+)
				MGMT	146 (11+)	57 (2+)
				TIMP3	146 (7+)	60 (2+)
				MINT31	68 (3+)	20 (0+)
				AIM1	71 (2+)	41 (0+)
				SFRP1	71 (2+)	20 (0+)
				APC	62 (2+)	20 (0+)
				CDKN2A	69 (1+)	20 (0+)
				HIN1	134 (16+)	57 (11+)
				CCNA1+DAPK+DCC+MGMT+TIMP3	NA	NA
				CCNA1+DAPK+MGMT+TIMP3	NA	NA
				CCNA1+DAPK+MGMT	NA	NA
				CCNA1+MGMT+TIMP3	NA	NA
				CCNA1+DAPK+TIMP3	NA	NA

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Table 1. Cont.

First Author	Anatomic Tumor Location	Type of Sample	Method	Biomarker	Cancer Group N (M+)	Control Group N (M+)
				DAPK+MGMT+TIMP3	NA	NA
				CCNA1+MGMT	NA	NA
				CCNA1+DAPK	NA	NA
				CCNA1+TIMP3	NA	NA
Ksumoto 2012	OC	Oral rinse	MSP	p16	10 (4+)	3 (0+)
Ovchinnikov 2014	HNC	Saliva	MSP	PCQAP5′	62 (42+)	49 (17+)
				PCQAP3′	60 (41+)	45 (19+)
Gaykalova 2015	HNC	Oral rinse	qMSP	ZNF14	59 (5+)	35 (0+)
				ZNF160	59 (10+)	35 (0+)
				ZNF420	59 (8+)	35 (0+)
				ZNF14+ZNF160+ZNF420	59 (13+)	35 (0+)
Lim 2016	HNC	Saliva	MSP	RASSF1α	88 (36+)	122 (10+)
				p16	88 (41+)	122 (38+)
				TIMP3	88 (33+)	122 (22+)
				PCQAP5′	88 (72+)	122 (66+)
				PCQAP3′	88 (30+)	122 (18+)
				RASSF1α+p16+TIMP3+PCQAP5′+ PCQAP3′	88 (62+)	122 (24+)
Ferlazzo 2017	OC	Saliva (Oragene DNA kit)	MSP	P16	58 (10+)	90 (5+)
				MGMT	58 (16+)	90 (7+)
				P16 + MGMT	58 (12+)	90 (0+)
Cheng 2017	OC	Oral rinse (0.12% clorhexidine)	qMSP	ZNF582	94 (62+)	65 (10+)
				PAX1	94 (64+)	65 (7+)
				ZNF582+PAX1	94 (75+)	65 (14+)
Puttipanyalears 2018	HNC OC OPC	Oral rinse (NaCl)	qMSP	TRH	66 (57+) 42 (37+) 24 (20+)	54 (4+)
Liyanage 2020	HNC	Saliva	MSP	p16	88 (62+)	NA
				RASSF1 α	88 (59+)	NA
				TIMP3	88 (68+)	NA
				PCQAP/MED15	88 (66+)	NA
				p16+RASSF1α+TIMP3+PCQAP	84 (80+)	60 (5+)
	OC			p16	54 (39+)	NA
				RASSF1α	54 (37+)	NA
				TIMP3	54 (43+)	NA
				PCQAP/MED15	54 (43+)	NA
				p16+RASSF1α+TIMP3+PCQAP	54 (46+)	60 (5+)
	OPC			p16	34 (23+)	NA
				RASSF1α	34 (22+)	NA
				TIMP3	34 (25+)	NA
				PCQAP/MED15	34 (23+)	NA
				p16+RASSF1α+TIMP3+PCQAP	34 (34+)	60 (5+)
Srisuttee 2020	OC	Oral rinse (NaCl)	qMSP	NID2	43 (34+)	90 (0+)
Shen 2020	OPC	Oral rinse (NaCl)	qMSP	EDNRB	21 (15+)	40 (2+)
				PAX5	21 (15+)	40 (4+)
				p16	21 (3+)	40 (0+)
González-Pérez 2020	OC	Saliva	MSP	p16	43 (19+)	40 (4+)
				RASSF1A	43 (10+)	40 (2+)
				p16+RASSF1A	43 (23+)	40 (5+)

Abbreviations: HNC, head and neck cancer; OC, oral cancer; OPC, oropharyngeal cancer; MSP, methylation-specific polymerase chain reaction; qMSP, quantitative MSP; NA, not available.

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3.3. Quality assessment of the included studies

All included articles were evaluated for risk of bias using the QUADAS-2 checklist (Figure S1). The major risk of bias in this study was patient selection domain, as 13 out of 18 publications were unclear or lacked detail on whether the patient sample was consecutive or random. Additionally, 14 out of 18 studies did not provide a detailed description of the inclusion/exclusion criteria. Moreover, there was high risk of bias in the index test, since some studies lacked a setting threshold. All domains were considered to have a low risk of bias in terms of applicability concern. All studies were of moderate-to-high quality, with an average QUADAS-2 score of 5.6.

3.4. Diagnostic accuracy of salivary DNA promoter hypermethylation

The diagnostic accuracy (sensitivity, specificity, and 95% confidence interval) of each study unit (n = 74) included in this meta-analysis is shown in Figure 2 and Figure 3. The pooled sensitivity and specificity of salivary DNA hypermethylation genes in the diagnosis of HNC were 0.39 (95% CI: 0.38–0.41) and 0.87 (95% CI: 0.86–0.88), respectively. The PLR and NLR were 3.68 (95% CI: 2.97–4.57) and 0.63 (95% CI: 0.57–0.69) (Figure 4 and Figure 5), respectively; the summary dOR was 8.34 (95% CI: 6.10–11.39) (Figure S2); and the area under the SROC was 0.81 (95% CI 0.77–0.84) (Figure 6). As shown in Fagan's nomogram (Figure S3), given a pre-test probability of 27.8%, a positive measurement leads to a post-test cancer probability of 59%, whereas a negative measurement leads to a post-test probability of 20%.

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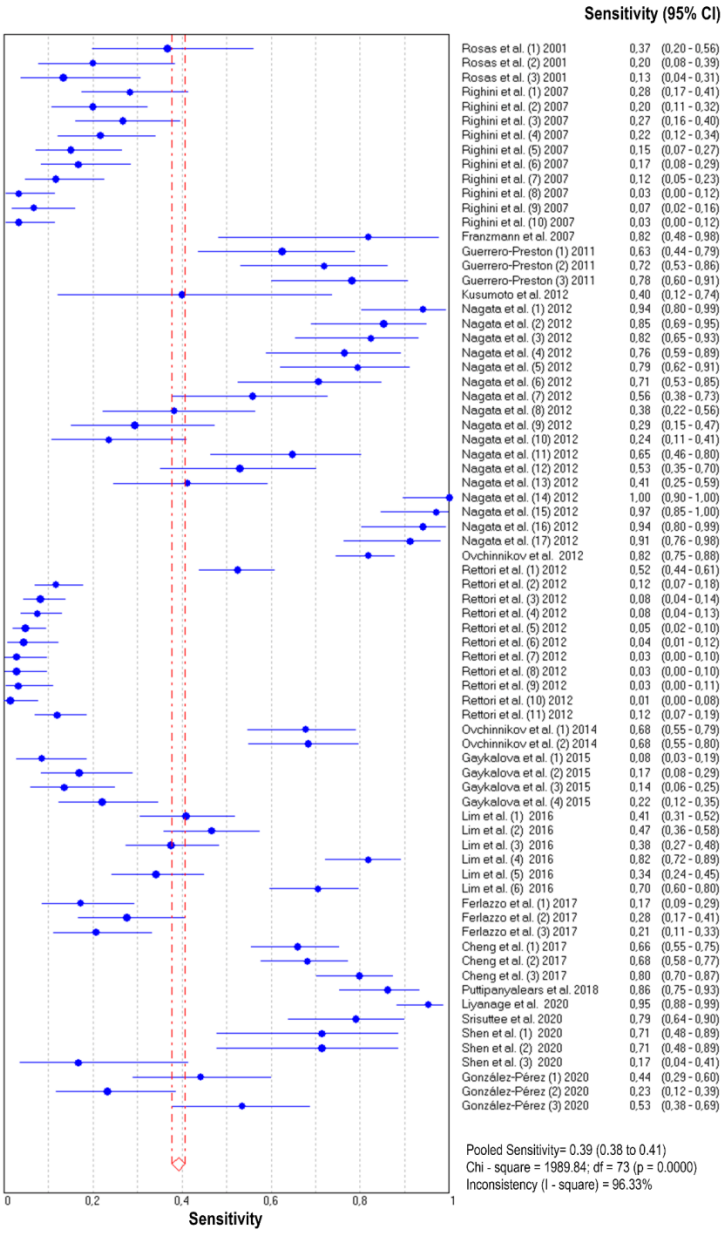

Figure 2. Forest plot of sensitivities from test accuracy studies of salivary DNA methylation for predicting HNC diagnosis.

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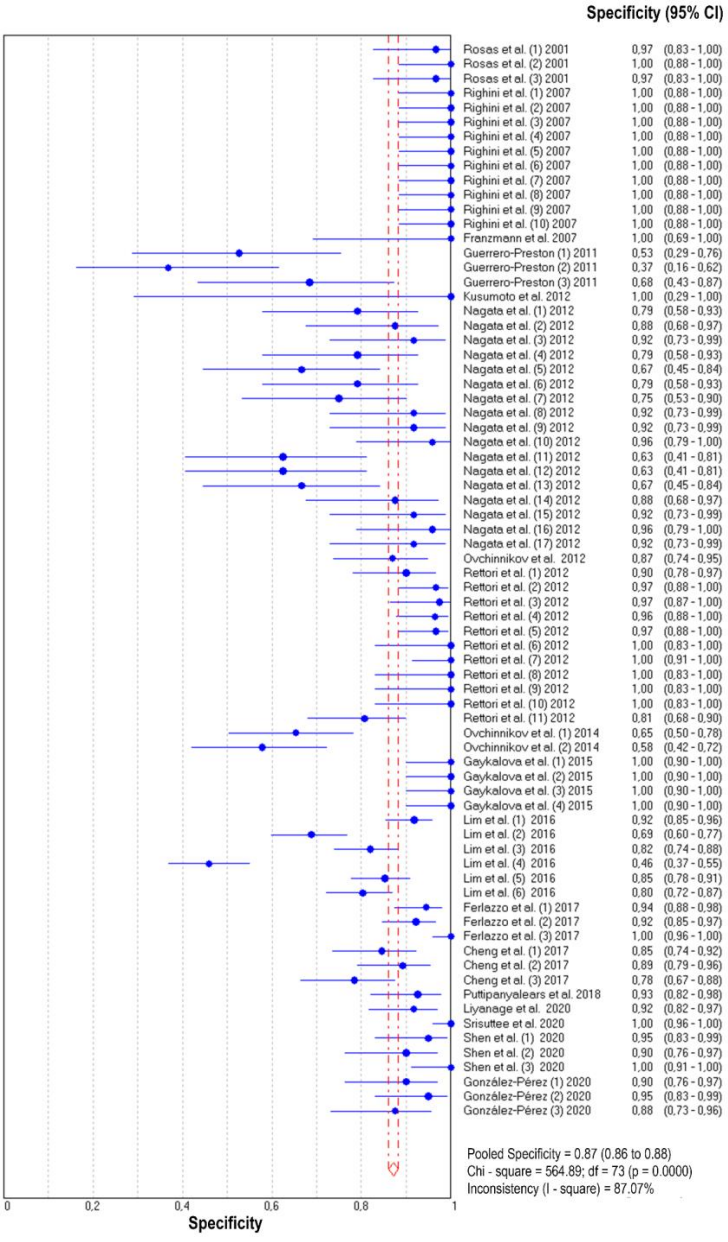

Figure 3. Forest plot of specificities from test accuracy studies of salivary DNA methylation for predicting HNC diagnosis.

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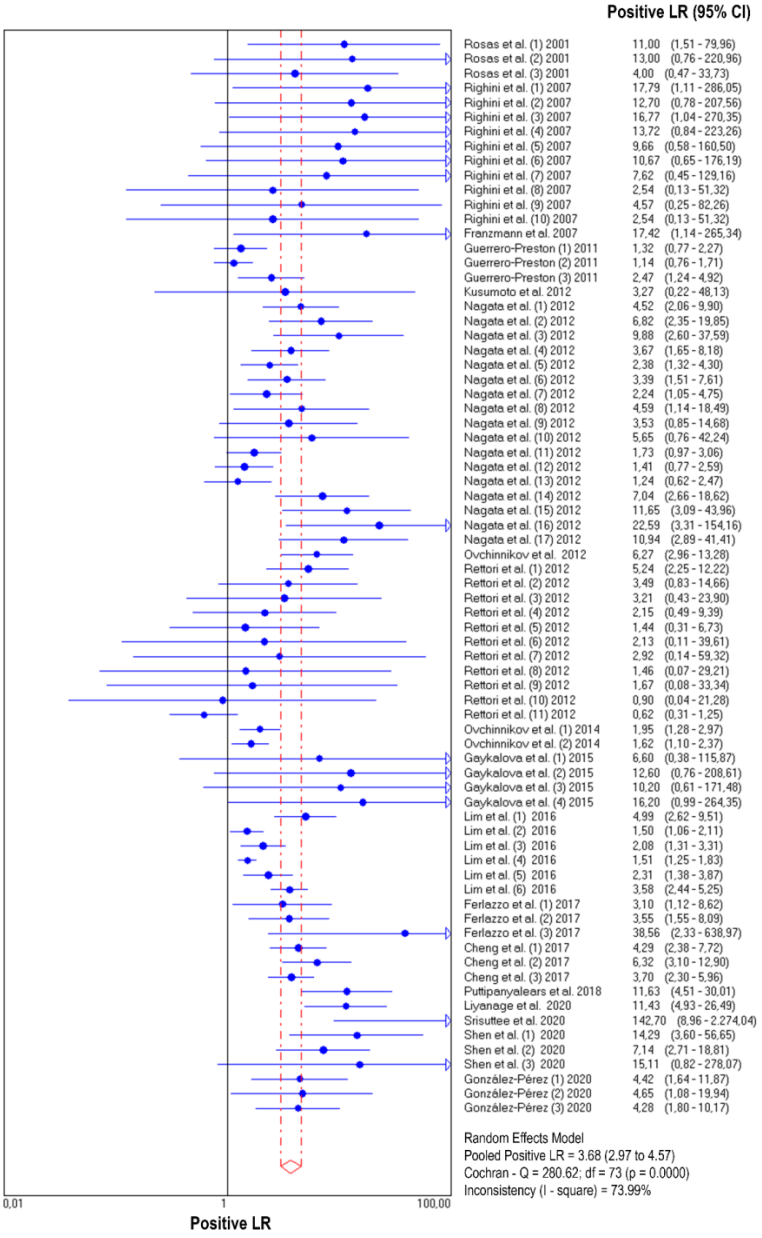

Figure 4. Forest plot of likelihood ratios for positive test results from salivary DNA methylation studies for predicting HNC diagnosis.

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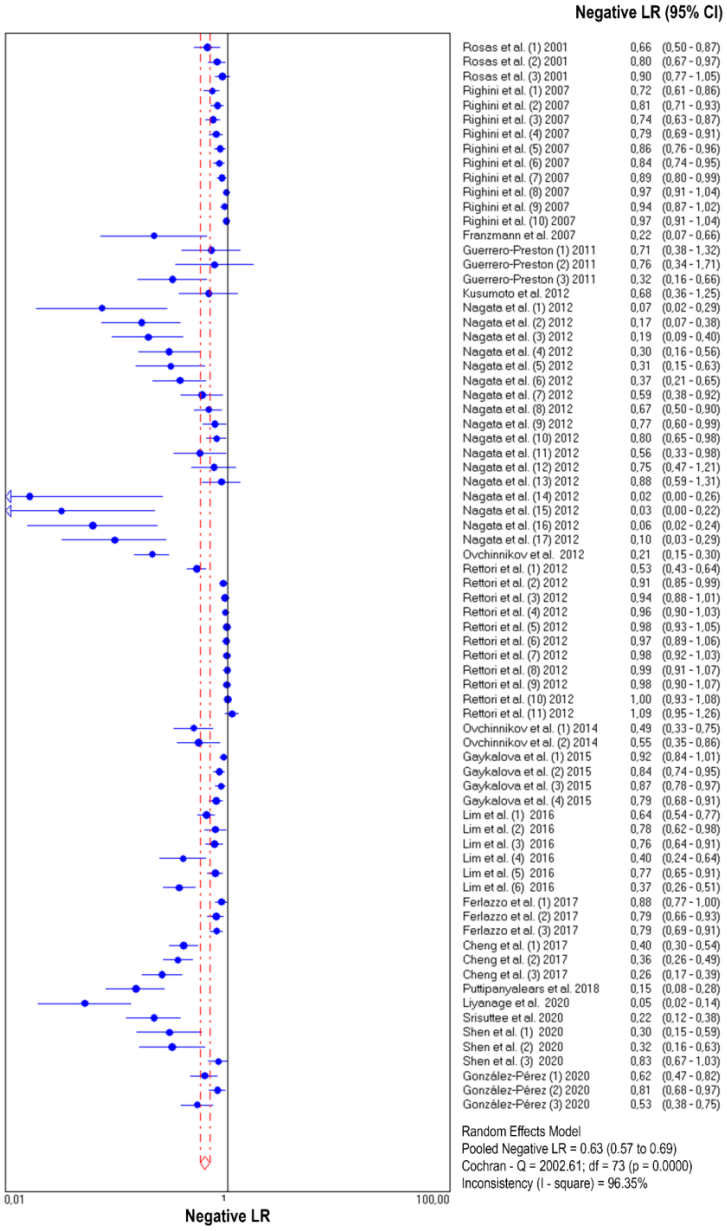

Figure 5. Forest plot of likelihood ratios for negative test results from salivary DNA methylation studies for predicting HNC diagnosis.

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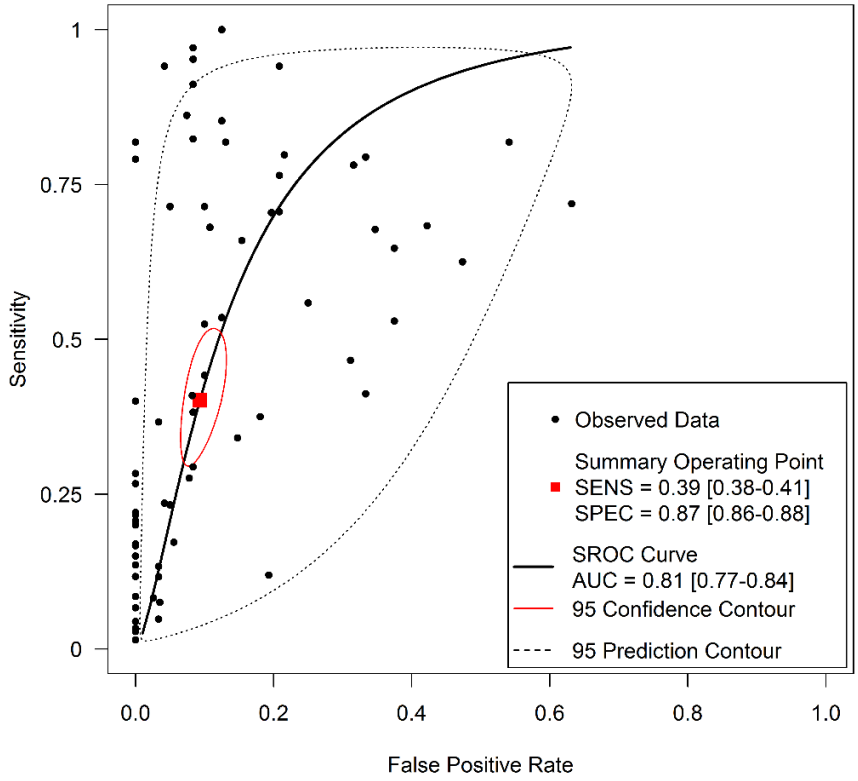

Figure 6. SROC curve with pooled estimates of sensitivity, specificity, and the AUC for all included studies of the salivary DNA methylation studies for detecting HNC.

3.5. Heterogeneity and subgroup analysis

As shown in Figure 2, Figure 3, Figure 4 and Figure 5 and Figure S2, significant heterogeneity was observed regarding the pooled sensitivity (I^2 = 96.33%; p < 0.001), specificity (I^2 = 87.07%; p < 0.001), PLR (I^2 = 73.99%; p < 0.001), NLR (I^2 = 96.35%; p < 0.001), and dOR (I^2 = 71.83%; p < 0.001). The representation of accuracy estimates from each study in the SROC space revealed a typical pattern of a "shoulder arm", suggesting the presence of a threshold effect (Figure S4). Moreover, Spearman's correlation coefficient between the

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logit of the true positive rate and the logit of the false positive rate was 0.633 ($p = 0.000$), which showed further indication of a threshold effect. In addition to the variations due to the threshold effect, meta-regression was performed to determine the possible sources of heterogeneity using the following covariates as predictor variables: sample type, sample size, anatomic tumor location, DNA methylation methods, and methylation gene profiling. The results indicated that anatomic tumor location ($p = 0.002$) and gene profiling ($p < 0.001$) were potential sources of heterogeneity in this study (Table S1). Consequently, subgroup analysis based on anatomic tumor location (HNC vs. oral cancer vs. oropharyngeal cancer) and gene profiling (single vs. combination of genes) was performed. As shown in Table S2, the results indicated similar accuracy for salivary methylated genes in oral cancer (sensitivity, 0.63; specificity, 0.87; PLR, 4.02; NLR, 0.40; dOR, 13.07; AUC, 0.88) and oropharyngeal cancer (sensitivity, 0.70; specificity, 0.86; PLR, 3.67; NLR, 0.41; dOR, 13.26; AUC, 0.87). However, differences in diagnostic accuracy were observed in the HNC group (sensitivity, 0.31; specificity, 0.86; PLR, 3.03; NLR, 0.75; dOR, 5.78; AUC, 0.81). When basing the meta-analysis on gene profile, the combination of methylated genes showed higher diagnostic accuracy (sensitivity, 0.73; specificity, 0.88; PLR, 5.76; NLR, 0.22; dOR, 36.97; AUC, 0.92) compared to individual genes (sensitivity, 0.32; specificity, 0.87; PLR, 3.17; NLR, 0.71; dOR, 6.02; AUC, 0.77). Although the meta-regression results were negative for other covariates, we conducted subgroup analyses based on these factors to further explore the diagnostic potential of salivary DNA methylated genes (Table S2).

3.6. Publication bias

The potential publication bias in each salivary DNA methylation study was explored by Deeks' funnel plot asymmetry test, which yielded a slope coefficient *p*-value of 0.711 overall. This indication of a symmetric data pattern suggests the absence of publication bias (Figure S5).

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4. DISCUSSION

Over the last few years, saliva has aroused great interest in the scientific community due to its potential as a non-invasive liquid biopsy in cancer. Several studies have evidenced the diagnostic capability of salivary biomarkers for diagnosing both HNC [45] and tumors distant from the oral cavity [46]. In this sense, a wide variety of biomolecules have been assessed as tumor biomarkers using saliva-omics approaches, including genomic, epigenomic, transcriptomic, metabolomic, proteomic, and microbiomic technologies [47]. In the field of epigenomics, DNA promoter hypermethylation represents one of the most intensively studied epigenetic alterations in human cancer. Promoter hypermethylation of critical pathway genes has been recognized as an important epigenetic mechanism of carcinogenesis [13]. Its potential role as an early diagnostic biomarker stems from the fact that gene promoter hypermethylation is an early event in cancer development [13]. DNA hypermethylation as a common event in cancer plays an important role in HNC development and progression [7]. The detection of DNA methylation in body fluids has emerged as an opportunity to assess the methylation status non-invasively and cost-effectively. In this line, several studies have investigated the promoter methylation of different tumor-suppressor genes in saliva from HNC patients [10,11,12,38]. Therefore, salivary DNA methylation biomarkers could be potentially used in the screening and early detection of HNC.

To the best of our knowledge, this study represents the first meta-analysis evaluating the diagnostic accuracy of promoter hypermethylation genes in saliva for differentiating HNC patients from healthy individuals. The present analysis included a total of 18 articles (84 study units) involving 4758 HNC patients and 3605 healthy individuals. According to QUADAS-2 quality evaluation, most of the included studies were of moderate quality. As for the overall accuracy of salivary hypermethylated genes for discriminating HNC from healthy individuals, the pooled diagnostic parameters of sensitivity, specificity, and AUC values were 0.39, 0.87, and 0.81, respectively. The summary dOR of 8.34 reflects the diagnostic capacity of salivary

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hypermethylated genes for HNC. The pooled PLR value of 3.68 indicates that a person testing positive had approximately 3.68 times higher probability of having cancer than a healthy individual. On the other hand, the pooled NLR indicated that a person testing negative had a 63% probability of having cancer. Additionally, given a pre-test probability of 27.8% in Fagan's nomogram, correct HNC diagnosis increased to 59% after a positive test, and reduced to 20% after a negative test. Overall, these results show a low sensitivity for HNC detection, indicating a high false negative rate. Therefore, the salivary gene methylation evaluated in this meta-analysis presented limitations as a screening biomarker for HNC. However, the diagnostic specificity of gene methylation for HNC was very high, suggesting that detection in saliva may aid assessment in HNC diagnosis. New molecular biology techniques such as next-generation sequencing platforms and digital PCR represent an opportunity for improving the sensitivity of methylation assays and for discovering new methylation patterns.

Due to the fact that heterogeneity is inherent to any diagnostic accuracy meta-analysis, an evaluation of the reasons contributing to inconsistencies across studies should be carried out. In the present research, overall heterogeneity among studies was high, so a bivariate random effects model was applied. This significant heterogeneity was reflected numerically in Cochran's Q test and the I^2 statistic. Further exploration of the heterogeneity revealed the presence of a threshold effect. The threshold effect is a major source of heterogeneity in meta-analyses of diagnostic tests. It arises from differences in sensitivities and specificities or likelihood ratios due to different cut-offs among studies for defining positive (or negative) test results [27]. In the present meta-analysis, the threshold effect was suggested by the visual inspection of accuracy estimates in forest plots where increasing specificities with decreasing sensitivities were observed. Later, the ROC plane and the Spearman correlation test also indicated the presence of the threshold effect. The variations in accuracy estimates among different studies could be due to a number of reasons other than the threshold, such as study population (anatomic tumor location, TNM staging), index test (differences in technology, assays), reference standard, and study design. Therefore, heterogeneity should be

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explored by relating study level co-variates to an accuracy measure by meta-regression techniques [27]. In the present study, meta-regression was performed to test the effect of sample type, sample size, anatomic tumor location, DNA methylation method, and methylated gene profiling. The results point to anatomic tumor location and gene profiling strategy as possible causes of heterogeneity. Stratified analysis by anatomic tumor location showed that salivary gene methylation had a higher diagnostic accuracy for discriminating oral (AUC = 0.88) and oropharyngeal (AUC = 0.87) tumors than overall HNC (AUC = 0.81). The explanation for these findings may be that tumors located in the oral cavity and oropharynx release more tumor cells directly into saliva. With respect to gene profiling strategy, subgroup analysis showed that single genes presented low sensitivity, but were highly specific to cancer tissue. The combination of salivary methylated genes had better diagnostic accuracy than single gene-based tests, with a dOR of 36.97 vs. 6.02 and AUC of 0.92 vs. 0.77, respectively, demonstrating that the use of salivary methylated gene panels as biomarkers may increase HNC detection accuracy without decreasing specificity. We also conducted subgroup analyses based on sample type, sample size, and DNA methylation method but no significant differences in diagnostic accuracy were observed. Future studies should be conducted to clarify the impact of these factors on the diagnostic potential of salivary DNA methylation.

The current meta-analysis is not free of limitations. Firstly, this meta-analysis included case–control studies, but none was multicenter. Moreover, no randomized controlled trials exist on this topic. Secondly, considerable heterogeneity was observed among the included studies. Although we examined the sources of heterogeneity in five variables, we were not able to explore other demographic and clinicopathological factors due to lack of information. Thirdly, studies involving saliva samples only evaluated some of the genes methylated in HNC tissue. The remaining genes should also be tested in saliva to determine their diagnostic potential. Lastly, confounding variables such as gender, age, lifestyle (tobacco and alcohol), and diet were not considered in most of the included studies. Due to these limitations, future research based on large-scale prospective diagnostic studies involving multiple health

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centers would contribute to further evaluating the clinical utility of salivary gene promoter methylation for HNC diagnosis. Furthermore, better comparison among future studies would benefit from standardization of analytic strategies and cut-off selection.

5. CONCLUSION

Our meta-analysis suggests that the detection of DNA promoter hypermethylation in saliva is a promising biomarker for HNC diagnosis, mainly in oral and oropharyngeal tumors. The use of salivary hypermethylated gene panels improves diagnostic accuracy with respect to single-gene analysis. This meta-analysis could provide valuable insights into methodology design for further research studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jpm11060568/s1, Figure S1: Quality assessment of the included studies according to Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) criteria, Figure S2: Forest plot of pooled dOR salivary DNA methylation for the diagnosis of HNC, Figure S3: Fagan's monogram evaluating the clinical utility of salivary DNA methylation for differentiating HNC patients, Figure S4: Representation of sensitivity against (1-specificity) in ROC space for each study of salivary methylation in the diagnosis of HNC, Figure S5: Deeks' funnel plot asymmetry test for the assessment of potential bias of included studies, Table S1: Results of meta-regression analysis, Table S2: Subgroup analysis of salivary DNA methylation for HNC detection based on different covariates.

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CHAPTER 3.4.2

Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-analysis

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OBJECTIVE 3.4.2

SALIVARY DNA METHYLATION AS AN EPIGENETIC BIOMARKER FOR HEAD AND NECK CANCER. PART II: A CANCER RISK META-ANALYSIS *

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ABSTRACT

Aberrant methylation of tumor suppressor genes has been reported as an important epigenetic silencer in head and neck cancer (HNC) pathogenesis. Here, we performed a comprehensive meta-analysis to evaluate the overall and specific impact of salivary gene promoter methylation on HNC risk. The methodological quality was assessed using the Newcastle–Ottawa scale (NOS). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate the strength of the association and Egger's and Begg's tests were applied to detect publication bias. The frequency of salivary DNA promoter methylation was significantly higher in HNC patients than in healthy controls (OR: 8.34 (95% CI = 6.10–11.39; $p < 0.01$). The pooled ORs showed a significant association between specific tumor-related genes and HNC risk: *p16* (3.75; 95% CI = 2.51–5.60), *MGMT* (5.72; 95% CI = 3.00–10.91), *DAPK* (5.34; 95% CI = 2.18–13.10), *TIMP3* (3.42; 95% CI = 1.99–5.88), and *RASSF1A* (7.69; 95% CI = 3.88–15.23). Overall, our meta-analysis provides precise evidence on the association between salivary DNA promoter hypermethylation and HNC risk. Thus, detection of promoter DNA methylation in saliva is a potential biomarker for predicting HNC risk.

Keywords

DNA methylation; epigenetics; head and neck cancer; saliva; biomarkers; liquid biopsy; meta-analysis.

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1. INTRODUCTION

The important role of epigenetic mechanisms in carcinogenesis has been widely reported. Identification of specific genes that are altered by aberrant epigenetic processes contributes to better understanding molecular pathogenesis in HNC [1]. As one of the most important epigenetic alterations, DNA hypermethylation may lead to transcriptional silencing of tumor suppressor genes and, thus, interfere in signaling pathways that control vital cell processes, such as DNA repair, apoptosis, cell proliferation, and cell-to-cell adhesion [2]. Gene promoter methylation is a common epigenetic event in early carcinogenesis, and therefore represents a promising biomarker for high-risk group stratification, early cancer detection, and prognosis prediction [3]. Numerous studies have evaluated DNA methylation as a biomarker in a wide variety of tumors [4,5,6,7]. Hypermethylation of tumor-related genes, such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*), E-cadherin (*CDH1*), death-associated protein kinase (*DAPK*), phosphatase and tensin homolog (*PTEN*), and O6-methylguanine-DNA methyltransferase (*MGMT*), have been reported in HNC [8]. Likewise, various studies have focused on the detection of DNA methylation in liquid biopsies in HNC [9,10,11]. Although evidence suggests a potential association between aberrant salivary DNA methylation patterns and HNC risk, no prior research assessing overall impact is available. Therefore, we conducted a systematic review and meta-analysis to gain better insight into the magnitude of the association between salivary DNA hypermethylation and HNC risk.

2. MATERIAL AND METHODS

2.1. Protocol and registration

This study was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines [12], and the protocol was registered with the International Prospective Register of Systematic Reviews (reference No. CRD42020199123).

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2.2. Search strategy, study selection and data extraction

The search strategy and data extraction were previously described in Part I [13].

2.3. Selection criteria

The inclusion criteria were as follows: (1) case-control studies; (2) studies based on salivary DNA hypermethylation biomarkers for HNC; and (3) sufficient data to calculate odds ratios (ORs) and corresponding 95% confidential intervals (CIs). The exclusion criteria were as follows: (1) reviews, letters, personal opinions, book chapters, case reports, conference abstracts, and meetings; (2) duplicate publications; (3) incomplete data; and (4) *in vitro* or *in vivo* animal experiments.

2.4. Assessment of study quality

Independent investigators evaluated methodological quality by applying the Newcastle–Ottawa scale (NOS) [14] to each study selected. Discrepancies were resolved by consensus. For the interpretation of meta-analytic data, the NOS scale was used to score the quality of non-randomized studies based on their design, content, and ease of use. Items were scored according to a "star system" and fell under three broad categories: study group selection, group comparability, and ascertainment of exposure/outcome for case-control or cohort studies. The maximum quality score for each item was one star, except for the comparability item, which had a maximum of two stars. The NOS score ranged from 0 to 9 stars, with 8–9 stars being high quality; 6–7 stars being medium quality; and <5 stars being low quality.

2.5. Statistical analysis

Statistical analysis was conducted using the meta package of free R software (v.3.4.4; https://www.r-project.org, accessed 30 November 2020). The pooled odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated to assess the strength of the association between salivary promoter methylation and HNC. To evaluate the statistical

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model applied to the meta-analytic database, heterogeneity was assessed on the basis of I-square (I^2) value and Cochran's Q statistic test-based Chi-squared test. Heterogeneity was considered significant when $I^2 > 50\%$ and/or presence of a $p < 0.10$ for the Cochran's Q test. If significant heterogeneity was detected, the DerSimonian and Laird random-effects model was applied to calculate the pooled OR with 95% CIs; otherwise, the Mantel–Haenszel fixed-effects model was used. Meta-regression and subgroup analyses were performed to explore the potential sources of heterogeneity among studies insofar as anatomic tumor location, sample type, sample size, DNA methylation method, and methylation gene profiling. Publication bias was assessed by Begg's and Egger's tests, and funnel plot inspection [15,16]. Begg's rank test examines the correlation between the effect sizes and their corresponding sampling variances. Egger's test regresses the standardized effect sizes on their precisions. In the presence of publication bias, both tests will be statistically significant. Moreover, publication bias was based on visual funnel-plot inspection, which shows the relationship between individual log ORs and their standard errors. The asymmetry of the funnel plot could indicate publication bias.

$p < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Study selection and characteristics of included studies

The main characteristics of the included studies have already been described in Part I [13].

3.2. Study quality

Bias risk and quality were assessed according to NOS (Table S1). With respect to the selection category, each of the included studies was considered adequate. Regarding comparability, 5 out of the remaining 18 studies matched for age or gender, and 2 studies matched for at least one additional risk factor. Therefore, the median NOS score in our meta-analysis was 7.33 stars.

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3.3. Association between salivary DNA promoter hypermethylation and HNC risk

A total of 7686 subjects, consisting of 4453 patients and 3233 controls, were included in this meta-analysis. As shown in Figure 1, the pooled analysis revealed a significant association between salivary DNA promoter hypermethylation and HNC with an OR of 8.34 (95% CI = 6.10–11.39; $p < 0.01$). A random-effects model was used because heterogeneity among the 18 studies (I^2 = 72%) was identified. The shape of the Begg's funnel plot did not reveal potential asymmetry (p = 0.271), although publication bias was detected by Egger's test (p = 0.002) (Figure S1).

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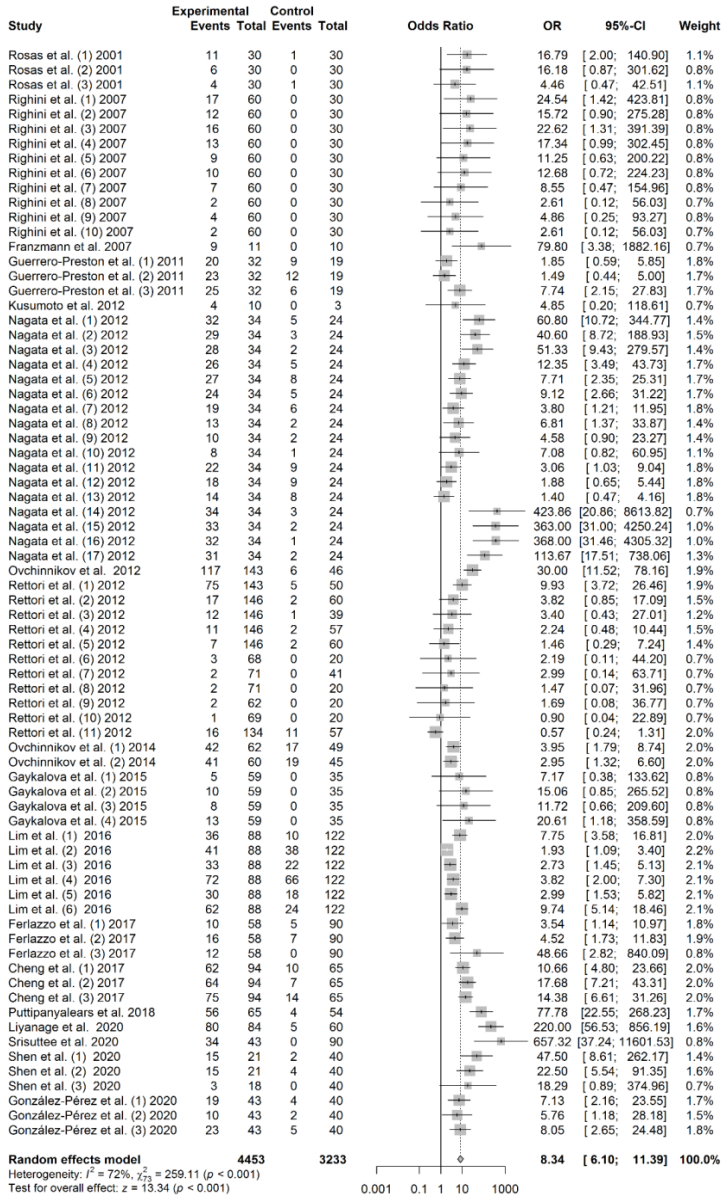

Figure 1. Forest plot for the association between salivary DNA promoter hypermethylation and the HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

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3.4. Meta-regression and subgroup analysis

Due to the presence of significant heterogeneity in the overall analysis, meta-regression and subgroup analysis were performed in order to reveal potential sources. The outcomes of meta-regression analysis showed that sample type ($p = 0.128$), sample size ($p = 0.349$), and DNA methylation method ($p = 0.275$) were not significant sources of heterogeneity. However, anatomic tumor location ($p = 0.002$) and gene profiling ($p < 0.001$) were, in fact, potential sources of heterogeneity in this study (Table S1—see Part I) [13]. As shown in Table S2, significant heterogeneity was found in all subgroups. With respect to sample type-based subgroup analysis, a significant association between promoter hypermethylation and HNC was found in oral rinse samples (OR: 9.42; 95% CI = 6.30–14.08) and saliva samples (OR: 6.33; 95% CI = 3.90–10.27). In tumor-based subgroup analysis, methylation rates were higher in specific head and neck locations compared to studies that made no differentiation. The pooled OR for oropharyngeal cancer was 13.26 (95% CI = 3.17–5.42) and for oral cancer was 13.07 (95% CI = 8.19–20.88), while for HNC it was 5.78 (95% CI = 3.86–8.67). A significant association between salivary promoter methylation and HNC was found by both MSP (OR: 9.06; 95% CI = 6.30–13.03) and qMSP (OR: 6.81; 95% CI = 3.70–12.54) techniques. With respect to the subgroups categorized by sample size, a significant association was found between salivary promoter methylation and HNC in studies with N < 100 (OR: 9.58; 95% CI = 6.44–14.27) and N > 100 (OR: 8.34; 95% CI = 6.10–11.39). In subgroup analysis based on the gene-profiling approach, salivary promoter hypermethylated gene panels had a significantly higher association to HNC risk (OR: 36.79; 95% CI = 16.81–81.32) than hypermethylated single genes (OR: 6.02; 95% CI = 4.46–8.13).

3.5. Association between *p16* promoter hypermethylation and HNC risk

A total of 410 cases and 399 controls from 9 studies were included to estimate the effect of *p16* promoter hypermethylation on HNC risk. As shown in Figure 2, a significant association was found between

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salivary p16 promoter hypermethylation and HNC risk (OR: 3.75; 95% CI = 2.51–5.60). The shape of the Begg's funnel plot did not reveal potential asymmetry (p = 1), although publication bias was detected by Egger's test (p = 0.040) (Figure S2).

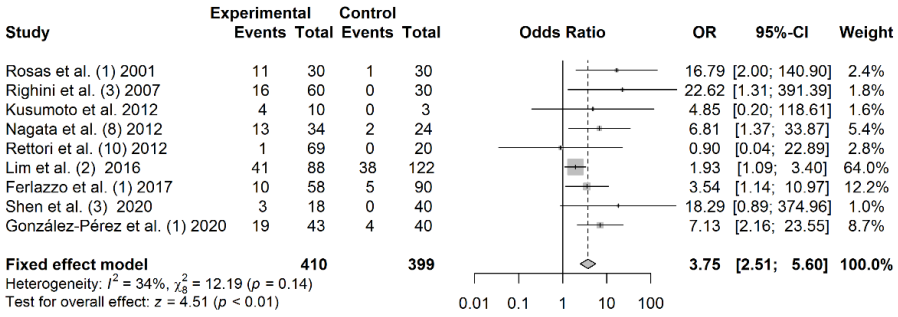

Figure 2. Forest plot for the association between *p16* promoter hypermethylation and HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

3.6. Association between *MGMT* promoter hypermethylation and HNC risk

A total of 328 cases and 231 controls from 5 studies were included to estimate the effect of *MGMT* promoter hypermethylation on HNC risk. As shown in Figure 3, salivary *MGMT* promoter hypermethylation was associated with an increased HNC risk (OR: 5.72; 95% CI = 3.00–10.91). Visual analysis of the funnel plot revealed a symmetrical distribution of the studies (Egger's test, p = 0.767; Begg's test, p = 0.624), indicating no evidence of publication bias (Figure S3).

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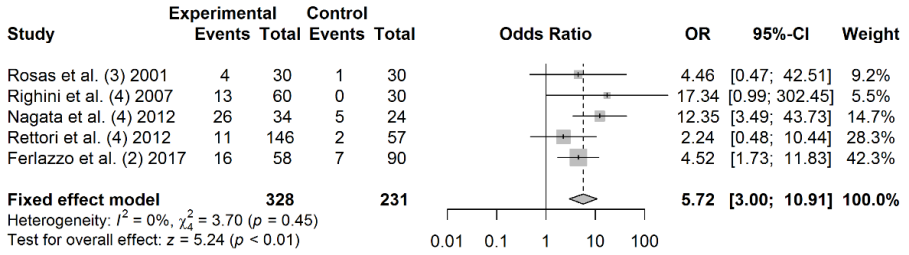

Figure 3. Forest plot for the association between *MGMT* promoter hypermethylation and HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

3.7. Association between *DAPK* promoter hypermethylation and HNC risk

A total of 270 cases and 123 controls from 4 studies were included to estimate the effect of *DAPK* promoter hypermethylation on HNC risk. As shown in Figure 4, the rate of salivary *DAPK* promoter hypermethylation was significantly higher in HNC patients compared to controls (OR: 5.34; 95% CI = 2.18–13.10). Visual examination of the funnel plot revealed a symmetrical distribution of the studies (Begg's test, p = 0.041; Egger's test, p = 0.187;), indicating no evidence of publication bias (Figure S4).

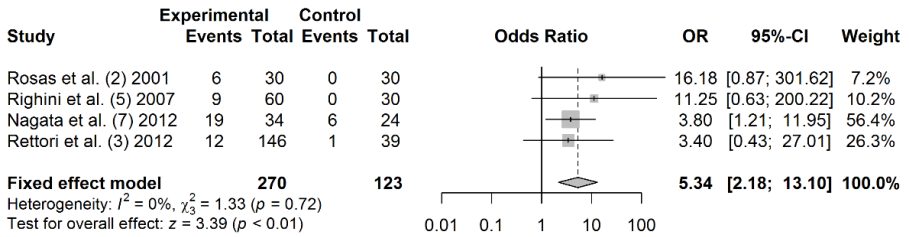

Figure 4. Forest plot for the association between *DAPK* promoter hypermethylation and HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

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3.8. Association between *TIMP3* promoter hypermethylation and HNC risk

A total of 328 cases and 236 controls from 4 studies were included to estimate the effect of *TIMP3* promoter hypermethylation on HNC risk. As shown in Figure 5, a significant association was found between salivary *TIMP3* promoter hypermethylation and HNC risk (OR: 3.42; 95% CI = 1.99–5.88). Visual inspection of the funnel plot revealed a symmetrical distribution of the studies (Begg's test, $p = 0.174$; Egger's test, $p = 0.419$), indicating no evidence of publication bias (Figure S5).

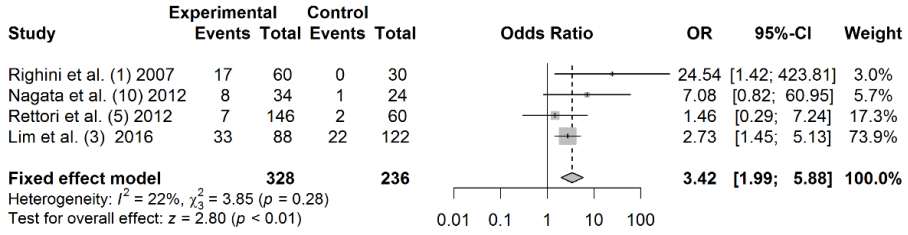

Figure 5. Forest plot for the association between *TIMP3* promoter hypermethylation and HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

3.9. Association between *RASSF1A* promoter hypermethylation and HNC risk

A total of 191 cases and 192 controls from 3 studies were included to estimate the effect of *RASSF1A* promoter hypermethylation on HNC risk. As shown in Figure 6, salivary *RASSF1A* promoter hypermethylation was associated with an increased HNC risk (OR: 7.69; 95% CI = 3.88–15.23). Visual examination of the funnel plot revealed a symmetrical distribution of the studies (Begg's test, $p = 0.601$; Egger's test, $p = 0.858$), indicating no evidence of publication bias (Figure S6).

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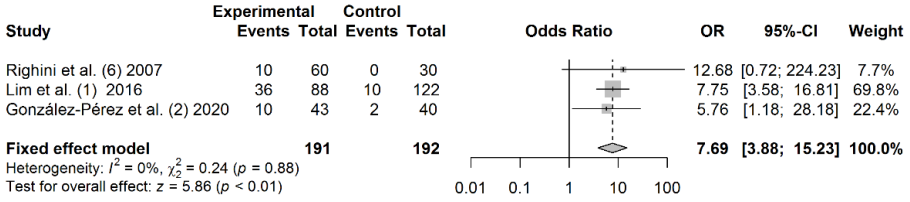

Figure 6. Forest plot for the association between *RASSF1A* promoter hypermethylation and the HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

3.10. Association between *APC* promoter hypermethylation and HNC risk

A total of 156 cases and 74 controls from 3 studies were included to estimate the effect of *APC* promoter hypermethylation on HNC risk. As shown in Figure 7, salivary *APC* promoter hypermethylation was not significantly associated with HNC (OR: 2.15; 95% CI = 0.84–5.51). Visual examination of the funnel plot revealed no potential asymmetry (Begg's test, p = 0.601; Egger's test, p = 0.609), indicating no evidence of publication bias (Figure S7).

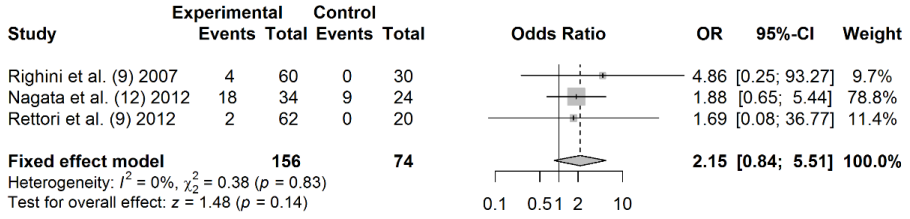

Figure 7. Forest plot for the association between *APC* promoter hypermethylation and HNC risk. The squares represent the ORs for individual studies. Bars represent the 95% CIs. The center of the diamond represents the summary effect size.

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4. DISCUSSION

Aberrant DNA hypermethylation has been recognized as an important epigenetic mechanism involved in head and neck carcinogenesis [1], suggesting its potential as a biomarker for evaluating cancer risk. Although prior studies have focused on the detection of promoter DNA hypermethylation in saliva from HNC patients [10,17], the evidence of a direct relationship is unclear and findings have been inconsistent.

To the best of our knowledge, this is the first meta-analysis evaluating the contribution of salivary promoter hypermethylation to HNC risk. The present comprehensive analysis included 18 studies comprising 4453 patients and 3233 controls. Overall, our results indicate that salivary promoter hypermethylation was significantly associated with an 8.34-fold increase in HNC risk.

As significant heterogeneity was observed among studies, meta-regression and subgroup analyses were carried out based on anatomic tumor location, sample type, sample size, DNA methylation method, and methylation gene profiling. The stratified analysis revealed that salivary DNA hypermethylation was associated with HNC risk in all subgroups. The association between salivary DNA promoter hypermethylation and HNC risk was stronger in oral rinses compared to saliva. This could be explained by the higher methylation proportion of oral exfoliated cells in oral rinse compared to saliva samples. Subgroup analysis of anatomic tumor location showed that the OR was higher in oral cancer and oropharyngeal cancer than overall HNC. These findings could be explained by the direct contact of saliva samples with tumors located in the oral cavity and oropharynx, which could result in an increased number of exfoliated tumoral cells during sample collection. Based on the methylation detection method subgroup, the frequency of salivary DNA promoter methylation was higher in MSP than in qMSP. This may be because MSP was the most commonly used technique for detecting aberrant DNA methylation in saliva samples (11 studies). In addition, the qualitative nature and lower specificity of MSP could lead to an overestimation of methylation data compared to qMSP methods [18]. However, quantitative approaches,

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such as qMSP or pyrosequencing, have shown better sensitivity than MSP [19]. With respect to sample size, a similar significant association was found between n < 100 and n > 100 subgroups. On the other hand, the gene profiling subgroup revealed that HNC risk was clearly higher when aberrant gene-specific DNA methylation was analyzed using gene panels rather than single gene analysis. This suggests that multiple tumor suppressor genes are epigenetically silenced in HNC pathogenesis, and, therefore, gene methylation panels should be used to better identify HNC risk.

We also explored the association between gene-specific promoter DNA methylation and HNC risk by analyzing the methylation frequency of genes reported in at least three studies. Thus, promoter hypermethylation of *p16, DAPK, TIMP3, MGMT*, and *RASSF1A* was significantly higher in HNC patients compared to controls, suggesting that the methylation of these tumor suppressor genes may play an important role in head and neck carcinogenesis. The *p16* gene acts as a negative cell cycle regulator that prevents the inactivation of retinoblastoma (Rb) protein by inhibiting the cyclin-dependent kinases and, therefore, cell cycle progression at G1/S phase [20]. Hypermethylation of *p16* promoter has been reported as a frequent epigenetic event in oral carcinogenesis [21,22]. In the present meta-analysis, methylation of *p16* promoter was significantly associated with a 3.75-fold increase in HNC risk, which is consistent with the study by Shi et al. (OR: 3.37) based on tissue and liquid biopsy methylation data [23]. In line with this, a more recent meta-analysis comprising 67 case-control studies reported an OR of 6.72. However, subgroup analysis in this study based on sample type revealed that OR was much higher in saliva (OR: 12.45) and blood (OR: 16.40) than in tissue (OR: 6.40) [24]. Overall, these findings indicate that hypermethylation of *p16* gene promoter in saliva could be a predictive biomarker for HNC risk. The *MGMT* gene is involved in the repair of O6-methylguanine in DNA sequences originating from the carcinogenic effects of alkylating agents [25]. The inactivation of *MGMT* promoter by aberrant hypermethylation has been associated with an increased frequency of GC > AT transition mutations in *TP53* and in *KRAS* oncogene, contributing to carcinogenesis and tumor progression [26,27]. In fact,

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our meta-analysis showed that methylation of *MGMT* promoter leads to a 5.72-fold increase in HNC risk. *DAPK* plays a critical role in the apoptotic process triggered by interferon-gamma (IFN-γ), tumor necrosis factor (TNF)-alpha, Fas ligand, and detachment from extracellular matrix [28]. Hypermethylation of *DAPK* gene promoter is a frequent alteration in HNC [29,30]. The results of the present meta-analysis show that individuals with salivary hypermethylation of *DAPK* gene promoter had a 5.34-fold higher HNC risk. A previous meta-analysis also showed that the frequency of *DAPK* promoter methylation was significantly higher in HNC vs. control groups (OR: 6.72) [31]. The *TIMP3* gene is a tissue inhibitor of matrix metalloproteinases, which acts as a potential anticancer agent by inducing apoptosis and inhibiting proliferation, angiogenesis, and metastasis [32]. The methylation of *TIMP3* promoter has been associated with HNC [33,34]. Interestingly, our meta-analysis revealed a significant association between salivary *TIMP3* promoter methylation and HNC with an OR of 3.42. The *RASSF1A* gene prevents tumorigenesis through multiple cellular process, such as cell cycle arrest, migration, microtubular stabilization, and apoptosis promotion [35]. Epigenetic inactivation of *RASSF1A* by hypermethylation has been observed in various cancers, including HNC [36]. Our data showed that methylation of *RASSF1A* promoter led to a 7.69-fold increase in HNC risk compared to the control group. In a previous study, Meng et al. evaluated the methylation prevalence of *RASSF1A* between cancerous tissues and controls, finding a significant association (OR: 2.93) between aberrant methylation of *RASSF1A* and HNC [37]. The *APC* gene acts as a negative regulator in the Wnt/beta-catenin signaling pathway and its dysfunction leads to increased β-catenin transcriptional activity, promoting the activation of downstream targets involved in tumorigenesis, such as cyclin D1 and Myc [38]. Hypermethylation of the *APC* promoter has been reported as a mechanism for APC-gene inactivation in oral carcinogenesis [39]. Our study did not reveal a significant association between salivary *APC* promoter hypermethylation and HNC, which could be explained by the low *APC*-gene methylation rates detected in saliva from HNC patients. Until now, few studies have reported *APC* hypermethylation in saliva from HNC

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patients [40,41,42]; however, this epigenetic alteration has been frequently observed in head and neck tumors [29,39,43,44]. It is important to note that hypermethylation of *p16, DAPK, TIMP3, MGMT,* and *RASSF1A* plays an important role in the carcinogenesis of various tumors, such as lung, breast, colorectal, renal, or gastric [45,46,47,48,49,50]. In line with this, several studies have focused on the association of cancer risk with the hypermethylation of these tumor suppressor genes [51,52,53,54,55], which highlights its potential for early diagnosis of the disease.

The present study has several strengths. It is the first meta-analysis highlighting the association between salivary DNA promoter hypermethylation and HNC. It explores the magnitude of the association both overall and by specific hypermethylated gene. In addition, it involved a comprehensive literature review without language restrictions. However, our study is not exempt from limitations. Firstly, all included research involved case-control retrospective studies, which could lead to selection bias. Some bias could also stem from the fact that cases and controls were not matched for demographic variables, such as age, sex, and lifestyle habits. Secondly, significant heterogeneity was found among studies. Despite performing subgroup analysis by anatomic tumor location, sample type, sample size, DNA methylation method, and methylation gene profiling, we were unable to elucidate the potential sources of this heterogeneity. Further subgroup analysis was hindered by the lack of original data regarding lifestyle habits or ethnicity. Thirdly, the association of salivary DNA promoter hypermethylation and clinicopathological variables (*i.e.,* TNM stage, histological grade) was not explored due to insufficient data. Therefore, well-designed prospective clinical studies with large sample sizes are necessary to validate the results of this meta-analysis.

5. CONCLUSION

Overall, the findings from this meta-analysis showed that salivary DNA promoter hypermethylation was associated with HNC risk. Salivary hypermethylation of *p16, MGMT, DAPK, TIMP3,* and

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RASSF1A showed an important role in HNC development. Thus, saliva could be used as a potential source of epigenetic biomarkers for predicting HNC. The development of HNC screening programs based on the combination of these 5-methylated genes in saliva could be useful for identifying high-risk patients and for detecting cancer before the occurrence of initial clinical symptoms. The clinical implementation of this salivary panel would represent the beginning of precision medicine for HNC. To attain this, prospective and multicenter studies should be carried out in order to validate the present results.

Supplementary materials: The following are available online at https://www.mdpi.com/article/10.3390/jpm11070606/s1, Figure S1: Funnel plot for studies (of 18 studies) on the association between salivary DNA hypermethylation and HNC, Figure S2: Funnel plot for studies (of 9 studies) on the association between salivary hypermethylation of *p16* gene promoter and HNC, Figure S3: Funnel plot for studies (of 5 studies) on the association between salivary hypermethylation of *MGMT* gene promoter and HNC, Figure S4: Funnel plot for studies (of 4 studies) on the association between salivary hypermethylation of *DAPK* gene promoter and HNC, Figure S5 Funnel plot for studies (of 4 studies) on the association between salivary hypermethylation of *TIMP3* gene promoter and HNC, Figure S6: Funnel plot for studies (of 3 studies) on the association between salivary hypermethylation of *RASSF1A* gene promoter and HNC, Figure S7: Funnel plot for studies (of 3 studies) on the association between salivary hypermethylation of *APC* gene promoter and HNC, Table S1: The Newcastle-Ottawa Scale (NOS) for assessing the quality of included studies, Table S2: Subgroup analysis of salivary DNA methylation for HNC detection based on different covariates.

Author Contributions: Conceptualization, Ó.R.-G. and M.M.S.-C.; methodology, Ó.R.-G. and M.M.S.-C.; software, C.M.-R. and Á.S.-B.; formal analysis, C.M.-R. and Á.S.-B.; investigation, Ó.R.-G., Á.D.-L. and M.M.S.-C.; data curation, Ó.R.-G., Á.D.-L. and M.M.S.-C.; Writing—Original draft preparation, Ó.R.-G. and M.M.S.-C.; Writing—Review and Editing, Á.D.-L. and R.L.-L.; visualization, Ó.R.-G. and M.A.S.; supervision, M.M.S.-C.; project administration,

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M.M.S.-C.; funding acquisition, M.M.S.-C. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 3.4.3

Genome-Wide DNA Methylation Profiling of Oral Tongue Squamous Cell Carcinoma

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OBJECTIVE 3.4.3

GENOME-WIDE DNA METHYLATION PROFILING OF ORAL TONGUE SQUAMOUS CELL CARCINOMA*

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ABSTRACT

Background: Epigenetic alterations such as aberrant DNA methylation are frequent events in cancer, however, the methylome landscape of oral tongue squamous cell carcinoma (OTSCC) yet remain poorly understand. OTSCC is the most common cancer of the oral cavity that is characterized by an aggressive behavior and poor prognosis so that to understand the underlying molecular is key to improve the management of the disease. This study aims to provide a comprehensive characterization of the DNA methylome of OTSCC and identify novel tumor-specific DNA methylation markers for early detection.

Material and methods: Genome-wide DNA methylation analysis on 12 oral tongue tissue samples including 6 OTSCC and 6 adjacent normal tissues was performed using Infinium MethylationEPIC array. Then, differential methylation analysis in specific promoter regions including cytosine-guanine dinucleotide (CpG) island (CpGIs) and CpG shore was performed between tumor and normal samples. Venn diagrams were used to identify methylation-regulated genes. Next, the methylation levels of hypermethylated genes were compared against methylation data from OTSCC-The Cancer Genome Atlas (TCGA) samples. Further, a list of selected tumor-specific hypermethylated CpG markers were evaluated in saliva samples. Methylation data were analyzed by unsupervised hierarchical clustering. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic value of DNA methylation markers using the pROC package.

Results: A total of 25,890 CpGs (20,505 hypomethylated and 5,385 hypermethylated) were differentially methylated (DMCpGs) between OTSCC and normal adjacent tissue. At the CpG context, the majority of the hypermethylated CpG sites (48%) were in the CpGIs, while hypomethylated CpG sites (86%) were primarily enriched at the open sea region. By analyzing DMCpGs overlapping promoter region a total of 1,000 tumor-specific DMCpGs (714 hypermethylated and 286 hypomethylated) spanning 807 genes were identified. Venn diagram

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analysis identified 40 hypermethylated genes that showed a downregulated expression in OTSCC-TCGA gene expression data, and 33 hypomethylated genes with an upregulated expression. The methylation pattern of 11 genes (*A2BP1*, *ACSS3*, *ALDH1A2*, *ANK1*, *CA3*, *GABRB3*, *GFRA1*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B*) that showed specific hypermethylation at least two CpGs sites was validated in OTSCC-TCGA methylation data. Of them, six genes (*A2BP1*, *ANK1*, *ALDH1A2*, *GFRA1*, *TTYH1*, and *PDE4B*) presented in saliva the same tumor-specific hypermethylated CpG markers. The site-specific CpG markers of these genes analyzed in saliva were able to distinguish OTSCC patients from healthy controls with high level of area under the ROC curve (AUC), sensitivity and specificity.

Conclusion: This is the first and largest genome-wide DNA methylation study on OTSCC samples that identifies a group of novel tumor-specific DNA methylation markers with potential for discriminating OTSCC from healthy individuals. The detection in saliva of these site-specific CpG methylation markers of *A2BP1*, *ANK1*, *ALDH1A2*, *GFRA1*, *TTYH1*, and *PDE4B* genes could be a promising strategy for screening and early detection of OTSCC. Further investigations are required to elucidate the biological function of these genes in OTSCC carcinogenesis and their clinical translation.

Keywords

Genome-wide methylation; DNA methylation; methylome; Infinium MethylationEPIC array; oral tongue squamous cell carcinoma; saliva.

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1. INTRODUCTION

Oral tongue squamous cell carcinoma (OTSCC) is the most common subtype of oral cavity cancer and is considered one of the most aggressive tumors due to its high potential for local invasiveness and a great propensity to metastasize cervical lymph nodes [1]. Consequently, OTSCC involved a poor prognosis even when is diagnosed at early stages, indicating a different molecular behavior compared to other head and neck cancers [2].

DNA methylation is a crucial epigenetic modification of the genome that is involved in regulating many cellular processes including embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, and chromosome stability [3]. Abnormal distribution of DNA methylation is one of the best-studied epigenetic changes in cancer, where global hypomethylation leading to activation of oncogenes and transposons is often accompanied by focal hypermethylation of the cytosine-guanine dinucleotide (CpG) islands (CpGIs) in the promoter regions of tumor suppressor genes leading to transcriptional silencing [4]. Several genome-wide DNA methylation studies have been developed in head and neck squamous cell carcinomas [5–9], however, there are few global DNA methylation analyses in OTSCC [10,11]. Since DNA methylation is an early event in cancer, comprehensive genome-wide DNA methylation studies represent an opportunity to better understand the underlying molecular mechanisms and the identification of potential DNA methylation markers for early cancer detection and better management of the disease.

Liquid biopsies have emerged as a novel strategy for DNA methylation profiling in cancer [12]. In this line, a recent meta-analysis has highlighted the potential of salivary DNA methylation for detecting head and neck cancer [13]. Saliva is the biological fluid of the oral cavity and oral exfoliated tumors cells released into saliva can reflect the methylation landscape of the tumor, representing an attractive tool for non-invasive detection and monitoring of DNA methylation alterations during treatment and recurrence of the disease.

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In this study, we conducted in OTSCC patients the first methylome-wide study using the MehtylationEPIC array which comprises over 850,000 CpG sites. After differential methylation analysis, a set of differentially methylated CpGs (DMCpGs) was identified. Then, we test the potential of tumor-specific methylated genes as salivary markers for the early detection of OTSCC.

2. MATERIAL AND METHODS

2.1. Study participants and sample collection

This observational study retrospectively included pretreatment tumor and normal formalin-fixed paraffin-embedded (FFPE) biopsies and saliva samples from 6 cases with early-stage OTSCC diagnosed between September 2020 and January 2021 at the Department of Oral and Maxillofacial Surgery from Complexo Hospitalario Universitario of A Coruña (CHUAC, SERGAS) in Galicia, Spain. Inclusion criteria included histologically confirmed OTSCC and early-stage pT1-pT2N0M0 according to the 8th edition of the Tumor-Node-Metastasis (TNM)-staging system promulgated by the American Joint Commission on Cancer (AJCC) and the International Union Against Cancer (UICC). This study was approved by the Galician Ethics Committee of Clinical Research (Ref. No. 2018/003) and carried out in accordance with principles outlined in the Declaration of Helsinki (World Medical Association, 2013). Written informed consent was obtained from all participants before enrollment in the study and sample collection.

Tumor and normal histologic sections were subsequently confirmed by an expert pathologist (R.A.R). Also, unstimulated saliva samples were collected using Danasaliva sample collection kit (Danagene, Barcelona, Spain) according to the manufacturer's instructions. The participants were asked to refrain from eating, drinking, smoking, and using oral hygiene products for at least 1 h prior to sample collection. After collection, saliva was centrifuged at 2600 xg for 15 min at room temperature, separating cellular pellet from cell-free salivary supernatant. Then, the cellular pellet was resuspended with

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Phosphate-Buffered Saline (PBS) and preserved at −20°C prior to assay. A description of the clinicopathological characteristics of patients is shown in Table S1.

2.2. DNA extraction and bisulfite modification

DNA extraction from FFPE tissue samples was performed using the AllPrep® DNA/RNA FFPE kit (Qiagen, Hilden, Germany) according to the manufacture's recommendations. Genomic DNA from tumor samples was eluted in 100 µL elution buffer AE and stored at -20 °C for further use. The QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from cellular pellets of saliva samples according to the manufacturer's instructions. Genomic DNA from cell pellets was eluted in 100 µL elution buffer AE and stored at -20 °C for further use. DNA concentration and quality were measured by Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Agilent's TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA) was used to assess the integrity of the extracted DNA. Before, bisulphite conversion the DNA from FFPE samples was undergone to Illumina FFPE QC kit for quality control (QC) as per the manufacturer's protocol. Then, 400 ng of extracted DNA of each sample was used for bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. After bisulphite conversion, only FFPE-isolated DNA was further subjected to restore with the Illumina Infinium HD FFPE Restoration kit as per the protocol. Genome-wide DNA methylation profiling was carried out using the Illumina Infinium MethylationEPIC BeadChip (EPIC array; Illumina, San Diego, CA, USA), which overspreads the DNA methylation profile across approximately 850,000 CpGs. A total of 4 µL of the bisulphite converted DNA was used as template for target preparation to hybridize on the BeadChip and was processed as per the manufacter`s intructions. To avoid batch effect, all samples were processed together. The Bead Chip was imaged on a HiScan System (Illumina, CA, USA) and intensity values (IDAT files) were extracted.

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2.3. Genome-wide DNA methylation analysis

Methylation array data were processed in the R statistical environment [14]. The R package RnBeads was used for quality control and preprocessing [15]. Firstly, a greedycut algorithm was used to filter out probes and/or samples. Next, probes overlapping with single nucleotide polymorphisms, probes in sex chromosomes, and probes whose sequence maps to multiple genomic locations (cross-reactive) were removed. Raw intensities obtained in the array were normalized using the BMIQ method. For each CpG site a specific β value was obtained that is defined as the ratio of fluorescent signal between methylated (M) probe relative to the sum of the M and unmethylated (UM) probes (β = M/(M+UM). The β values range from 0 (no methylation) to 1 (100% methylation of both alleles). Finally, hierarchical linear models were used with limma package to obtain the differences between groups [16]. P-values were corrected for multiple testing (False Discovery Rate, FDR) using Benjamini-Hochberg method and a threshold of $P < 0.05$ was selected for significance. A similar bioinformatic pipeline was applied to all the comparisons.

2.4. TCGA methylation analysis

Methylation data were obtained from The Cancer Genome Atlas (TCGA) database [17]. Infinium Human Methylation 450K BeadChip (HM450K) IDAT files from 36 early-stage OTSCC and 4 healthy controls were downloaded from TCGA database using the TCGAbiolinks R package. The IDAT files were subjected to preprocessing, normalization, and QC steps similar to our EPIC array files.

2.5. TCGA gene expression analysis

To identify the functional relevance of the differential methylated genes in OTSCC, we used normalized RNA-sequencing (RNA-seq) expression data from 36 early-stage OTSCC and 4 healthy controls from TCGA database [17] using the TCGAbiolinks R package. An FDR <0.05 and |log2 fold change (FC)| ≥ 1 was set as the criteria for

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screening differentially expressed genes. The genes differentially expressed between OTSCC and normal tissue TCGA samples were linked with the differentially methylated genes in our methylation analysis by Veen diagrams.

2.6. Saliva DNA methylation analysis

To identify salivary DNA methylation changes we obtained saliva DNA methylation data of 4 healthy controls (HM450K) from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession number GSE55734. In addition to the IDAT files from these 4 healthy controls, we also retrieved for salivary methylation analysis the EPIC array data from 2 saliva samples previously analyzed in our research group. An FDR <0.05 and *P*-value <0.05 were set as the criteria for screening DMCpG sites.

2.7. Gene ontology and functional pathway analysis

Gene Ontology (GO) enrichment analysis was performed to classify the differentially methylated genes into categories of cellular component, biological process, and molecular function [18]. In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed to detect the potential pathways of the differentially methylated genes [19].

2.8. Receiver operating characteristic analysis.

To assess the diagnostic value of candidate DNA methylation markers, receiver operating characteristic (ROC) analysis was performed using the pROC package in R [20]. The area under the ROC curve (AUC) was then calculated to assess the performance of each DNA methylation marker.

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3. RESULTS

3.1. Identification of tumor-specific differentially methylated CpG sites

After preprocessing the EPIC array data, a total of 785,613 methylation CpG sites provide a high-quality methylation signal. The comparison of the mean methylation values of all CpGs sites analyzed between tumor and normal samples showed a high correlation across all CpGs (Spearman, r^2= 0.99, $P < 2.2 \times 10^{-16}$), indicating a similar global DNA methylation pattern in both groups (Figure S1). Of them, a total of 25,890 tumor-specific DMCpGs spanning 7,210 genes with FDR <0.05 were identified between OTSCC and normal samples. These DMCpGs were distributed on all chromosomes (Figure 1A), and 5,385 (21%) were hypermethylated while 20,505 (79%) were hypomethylated (Figure 1B).

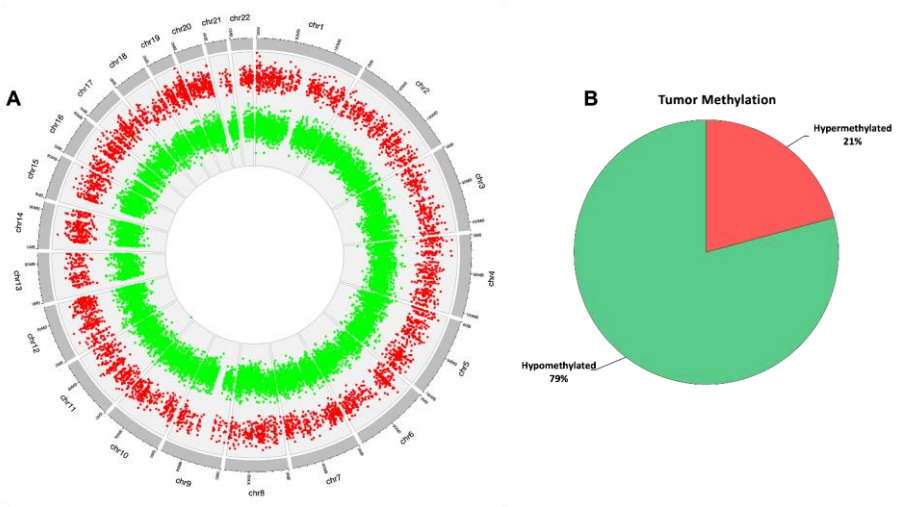

Figure 1. Tumor-specific DMCpGs. (A) Circos plot showing the hyper-(red) and hypomethyated (green) differentially methylated across the autosomes. (B) Graph showing percentages of hypermethylated and hypomethylated DMCpGs analyzed on the Infinium MethylationEPIC BeadChip platform. *Abbreviations:* DMCpGs, differentially methylated CpGs.

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In terms of the genomic distribution, DMCpGs were mostly distributed in the gene body (17,152 hypomethylated and 3,875 hypermethylated) followed by the 5´UTR (2,875 hypomethylated and 1,881 hypermethylated), TSS1500 (2,569 hypomethylated and 1,314 hypermethylated), TSS200 (533 hypomethylated and 1,218 hypermethylated), 1st exon (374 hypomethylated and 987 hypermethylated), 3´UTR (644 hypomethylated and 184 hypermethylated), and exonBnd (344 hypomethylated and 49 hypermethylated). In terms of the CpG context, DMCpGs were mostly distributed in the open sea (17,592 hypomethylated and 1,701 hypermethylated) followed by the island (250 hypomethylated and 2,565 hypermethylated), shore (1,371 hypomethylated and 940 hypermethylated), and shelf (1292 hypomethylated and 179 hypermethylated) (Figure 2). Unsupervised hierarchical clustering was performed and a heat-map using the methylation values of random 1,000 DMCpGs of which 44 were hypermethylated and 956 hypomethylated. The heat-map was two DNA methylation clusters indicating that DNA methylation profiling of OTSCC was significantly different from adjacent normal tissue (Figure S2).

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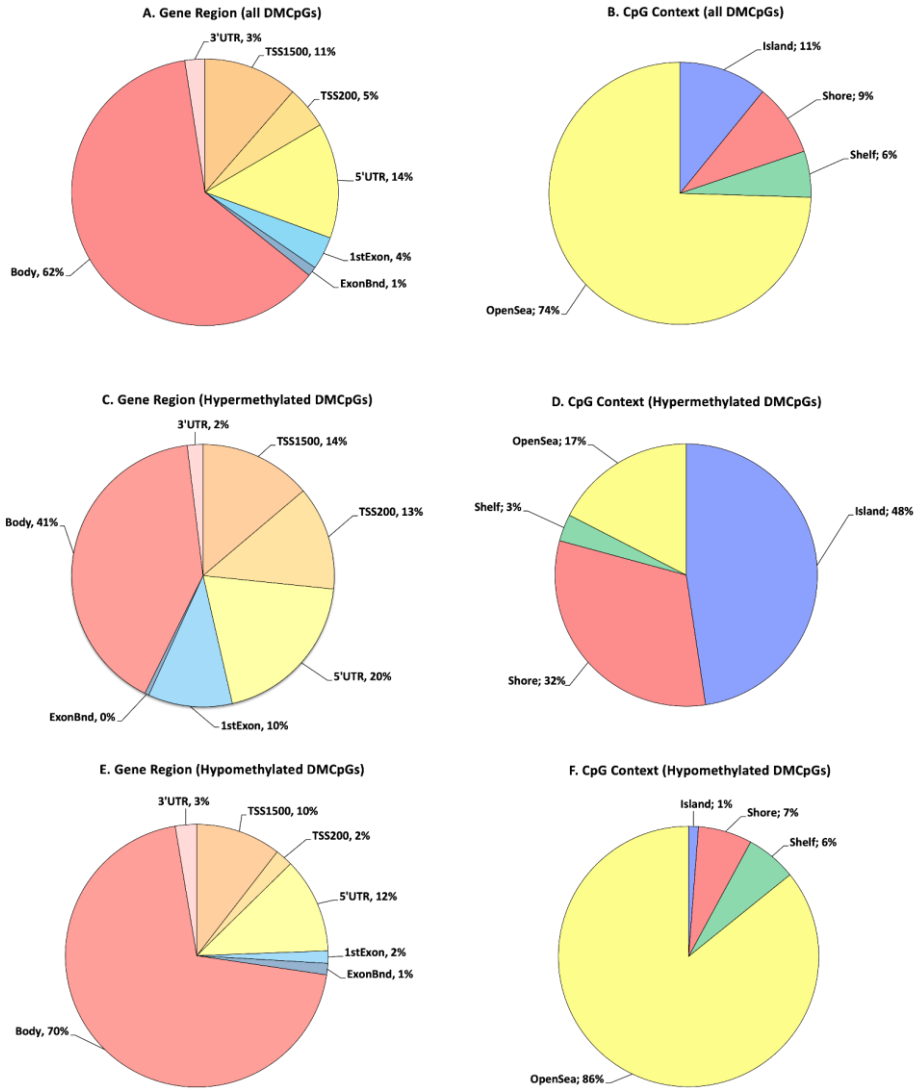

Figure 2. Genomic features of DMCpGs. (A, C, and E) Graphs showing percentages of hypermethylated and/or hypomethylated DMCpGs according to their functional genomic distribution. (B, D, and F) Graphs showing percentages of hypermethylated and/or hypomethylated DMCpGs according to their CpG content/neighborhood context. *Abbreviations:* DMCpGs, differentially methylated CpGs; UTR, untranslated region; TSS, transcription start site; ExonBnd, exon boundary.

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A genome-wide differential methylation analysis in specific promoter regions including CpGIs and CpG shore was performed between tumor and normal samples. A total of 2,700 tumor-specific DMCpGs spanning 1,597 genes were identified of which 2,216 were hypermethylated (82%) and 484 hypomethylated (18%) (Figure 3A). Unsupervised hierarchical clustering analysis and a heat-map using the methylation values of random 1,000 DMCpGs within/near to CpG island of which 714 were hypermethylated and 286 hypomethylated was produced. The heat-map showed two DNA methylation clusters indicating that DNA methylation profiling of OTSCC was significantly different from adjacent normal tissue at specific promoter regions (Figure 3B).

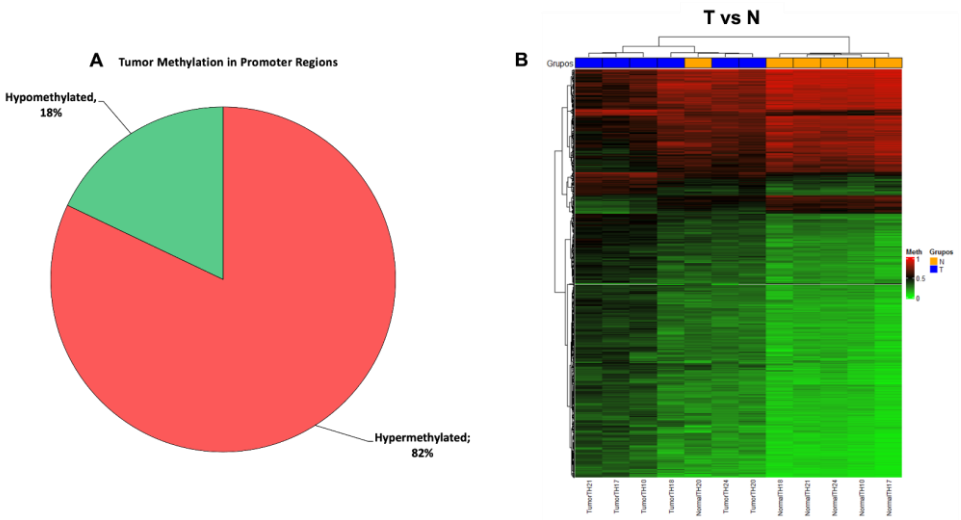

Figure 3. Tumor-specific DMCpGs in promoter regions including CpG island and CpG shore. (A) Graphs showing percentages of hypermethylated and hypomethylated DMCpGs. (B) Unsupervised hierarchical heatmap clustering of the random 714 hypermethylated DMCpGs and 286 hypomethylated DMCpGs between tumor and adjacent normal samples analyzed on the Infinium MethylationEPIC BeadChip platform. DNA methylation values were represented as colors, with red representing hypermethylated and green representing hypomethylated DMCpGs. The row represents individual CpGs and the column represents individual samples. *Abbreviations:* DMCpGs, differentially methylated CpGs; T, tumor; N, normal control.

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Enrichment analysis of the 807 genes containing DMCpGs revealed several important functional pathways and ontologies. The hypermethylated CpGs containing genes were enriched for several KEGG pathways, such as neuroactive ligand-receptor interaction, metabolic pathways, herpes simplex virus 1 infection, pathways in cancer, microRNAs in cancer, and PI3K-Akt signaling pathways among others (Figure 4A). GO for the molecular function was enriched particularly for regulation of transcription by RNA polymerase II, DNA-templated positive regulation of transcription, and signal transduction (Figure S3A). The hypomethylated CpGs containing genes were enriched in pathways such as metabolic pathways, neuroactive ligand-receptor interaction, herpes simplex virus 1 infection, human papillomavirus infection, MAPPK signaling pathway, or Wnt signaling pathway among others (Figure 4B). Furthermore, the most enriched biological processes of these genes were signal transduction, DNA-templated positive regulation of transcription, cell differentiation, and regulation of transcription by RNA polymerase II (Figure S3B).

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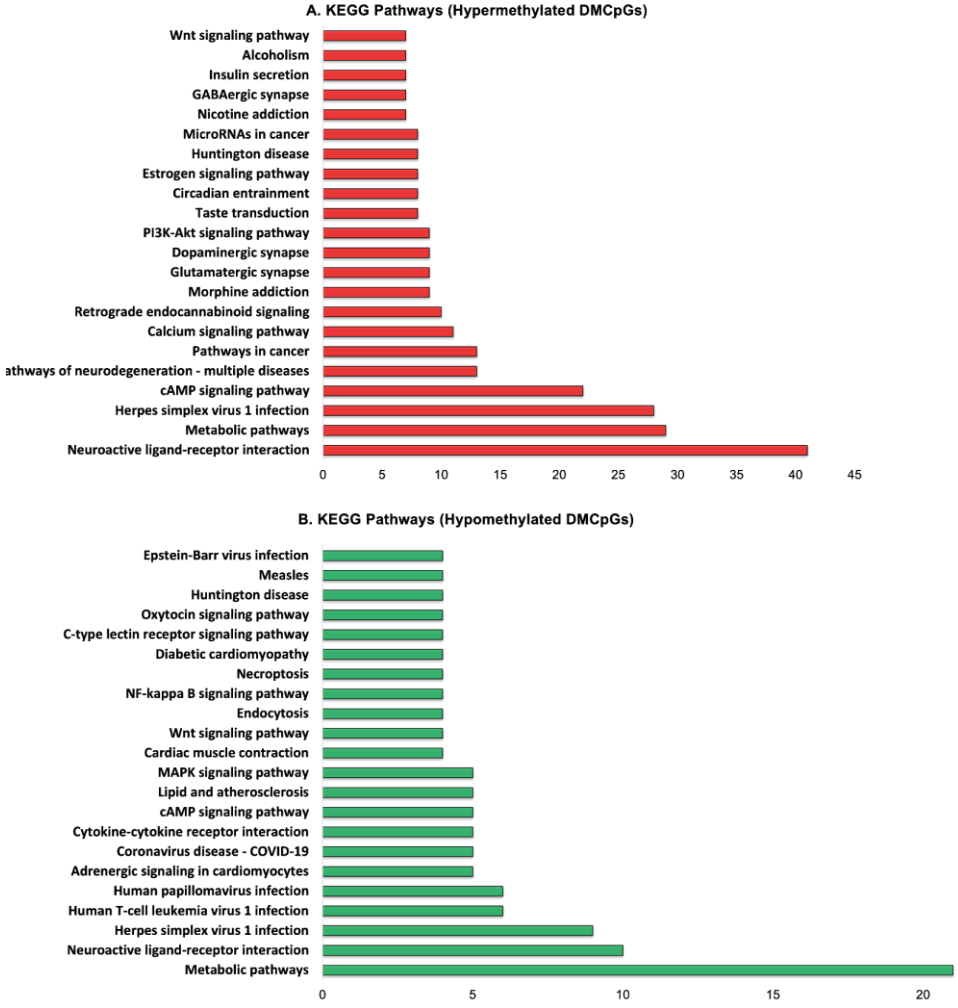

Figure 4. KEGG pathway enrichment analysis of the hyper- and hypomethylated DMCpGs. *Abbreviations:* DMCpGs, differentially methylated CpGs; KEGG, Kyoto Encyclopedia of Genes and Genomes.

3.2. Evaluation of the impact of differential methylation on associated gene expression

The expression of 19,947 genes was accessible from TCGA data, of which 2,494 genes were differentially expressed between OTSCC

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and normal tissue samples (FDR <0.05 and |logFC| ≥ 1). Specifically, the expression for 73/807 methylated genes was available from TCGA data. A total of 40 genes (*PDE4B*, *A2BP1*, *HS3ST1*, *TTYH1*, *GABRB3*, *ZBTB47*, *RBM24*, *CSDAP1*, *NDRG2*, *ZNF853*, *FRZB*, *FXYD1*, *NR3C2*, *ANK1*, *CA3*, *DLK1*, *GPX3*, *EPHX2*, *GDF10*, *SPEG*, *GYPC*, *ACTA1*, *DOK5*, *ACADL*, *PRKAA2*, *CHAD*, *SCGB3A1*, *ACSS3*, *CLDN11*, *CFRA1*, *LONRF2*, *ALDH1A2*, *ID4*, *ESRRG*, *CDNF*, *PHYH*, *WIF1*, *ALPK3*, *CHST9*, and *CLDN4*) hypermethylated in our assay showed a downregulated expression in tissue samples from TCGA cohort. By the contrary, 33 genes (*PRAME*, *ARTN*, *PHACTR3*, *SKA2*, *PRR11*, *GMNN*, *SORCS2*, *NR2C1*, *DDX60*, *SOX11*, *PTPRN*, *MCM5*, *CDH6*, *CABYR*, *TAP1*, *PSMB8*, *NMI*, *KIFC1*, *SNHG11*, *HSPA12A*, *CD70*, *C1orf59*, *ZNF530*, *PCDHGC5*, *NOX5*, *P2RY6*, *C1orf96*, *PSMB9*, *DLGAP5*, *ALPP*, *MED15*, *TROAP*, and *STAT2*) hypomethylated showed an upregulated expression (Figure 5).

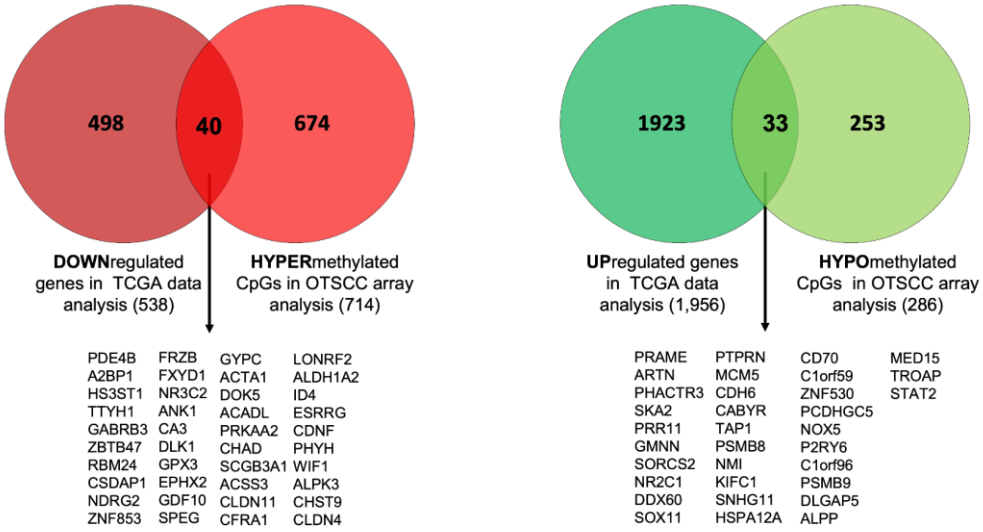

Figure 5. Venn diagrams showing overlap of genes with differentially hypermethylated CpGs in OTSCC array analysis and downregulated expression in TCGA database (A) and genes with differentially hypomethylated CpGs in OTSCC array with upregulated expression in TCGA database (B). *Abbreviations:* OTSCC, oral tongue squamous cell carcinoma; TCGA, The Cancer Genome Atlas; CpGs, cytosine-guanine dinucleotides.

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3.3. Validation of tumor-specific differentially methylated CpGs sites

We evaluated the overall overlap of the DMCpGs located at the promoter region of 40 hypermethylated and 33 hypomethylated genes in our discovery assay with the tumor-specific DMCpGs identified from 36 OTSCC and 4 normal tissue samples available in the TCGA database. Although limited by much lesser genome coverage in HM450K array data with almost half the number of CpGs from HM850K arrays, we identified 68/88 (77.27%) hypermethylated and 11/34 (32.35%) hypomethylated CpGs in TCGA HM450K data. Focused on the hypermethylation, 45 CpGs spinning 22 genes were differentially methylated between tumor and normal tissue of which 11 genes (*A2BP1*, *ACSS3*, *ALDH1A2*, *ANK1*, *CA3*, *GABRB3*, *GFRA1*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B*) were methylated at least 2 CpGs. The methylation pattern of these 11 genes observed in our discovery set was consistent with TCGA data, suggesting that these tumor-specific hypermethylated CpGs are frequent alterations in OTSSC and could be early events with a crucial role in carcinogenesis (Figure 6 and Table S2).

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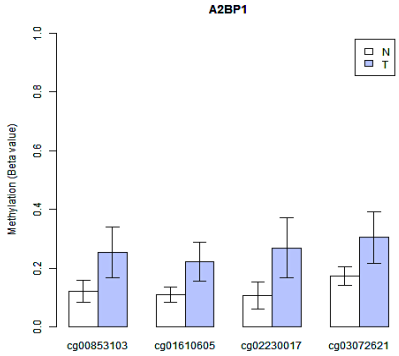

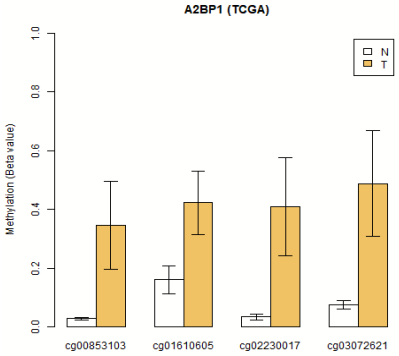

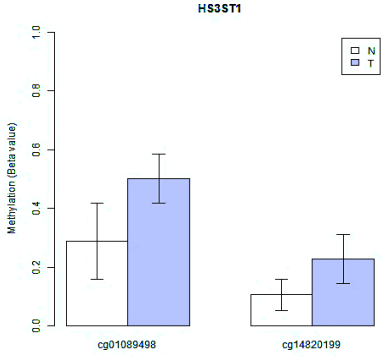

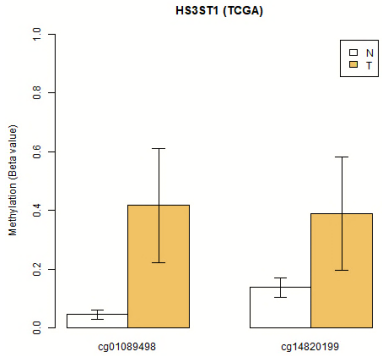

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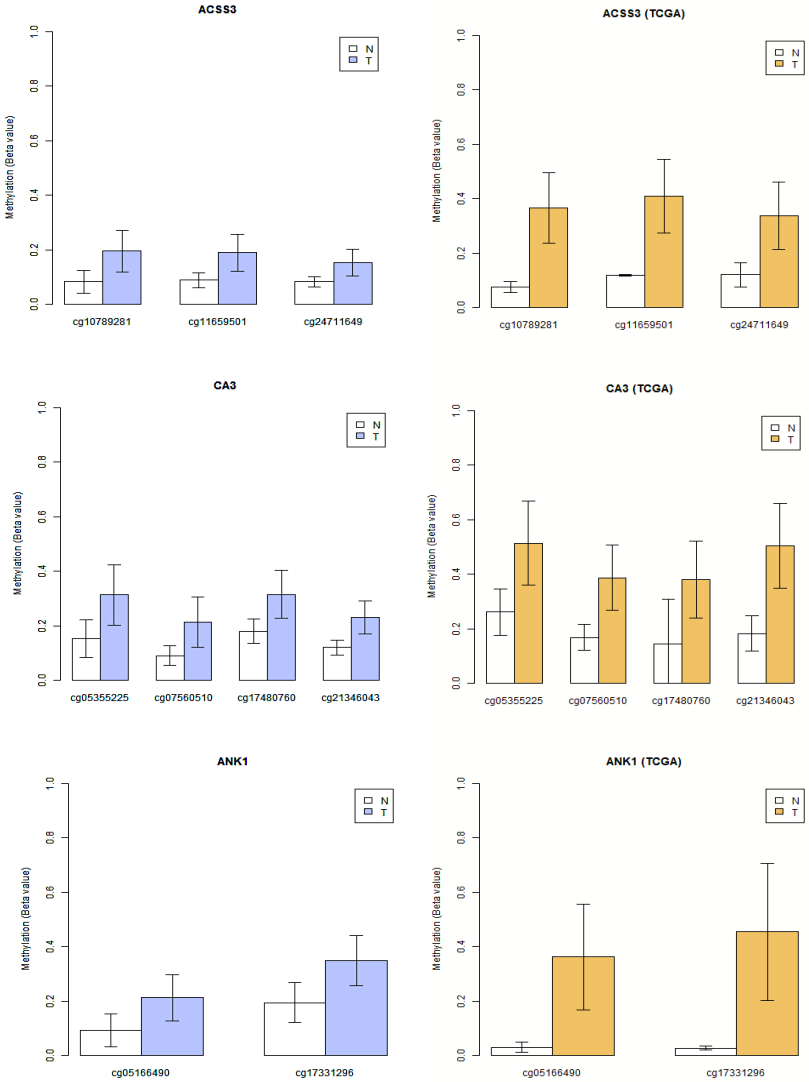

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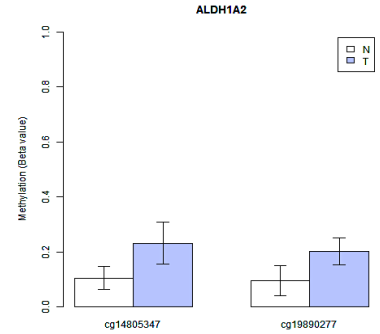

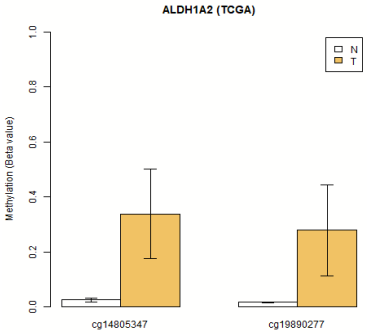

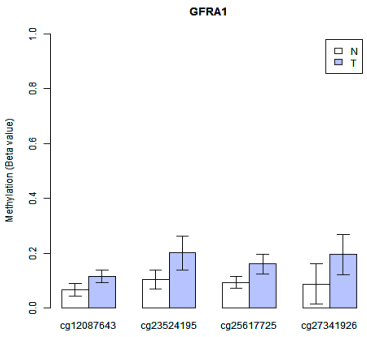

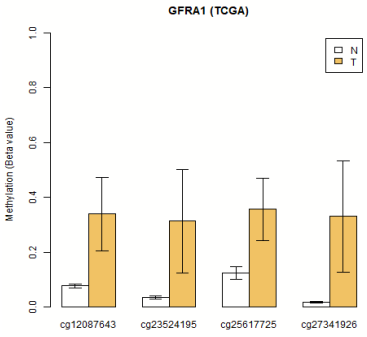

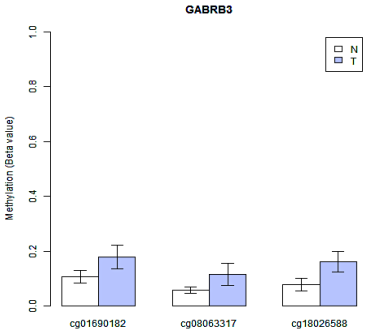

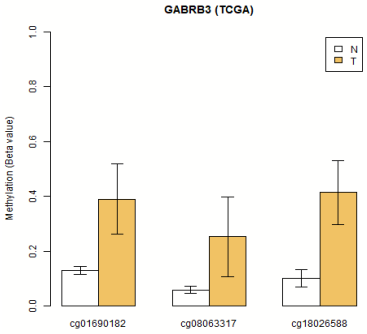

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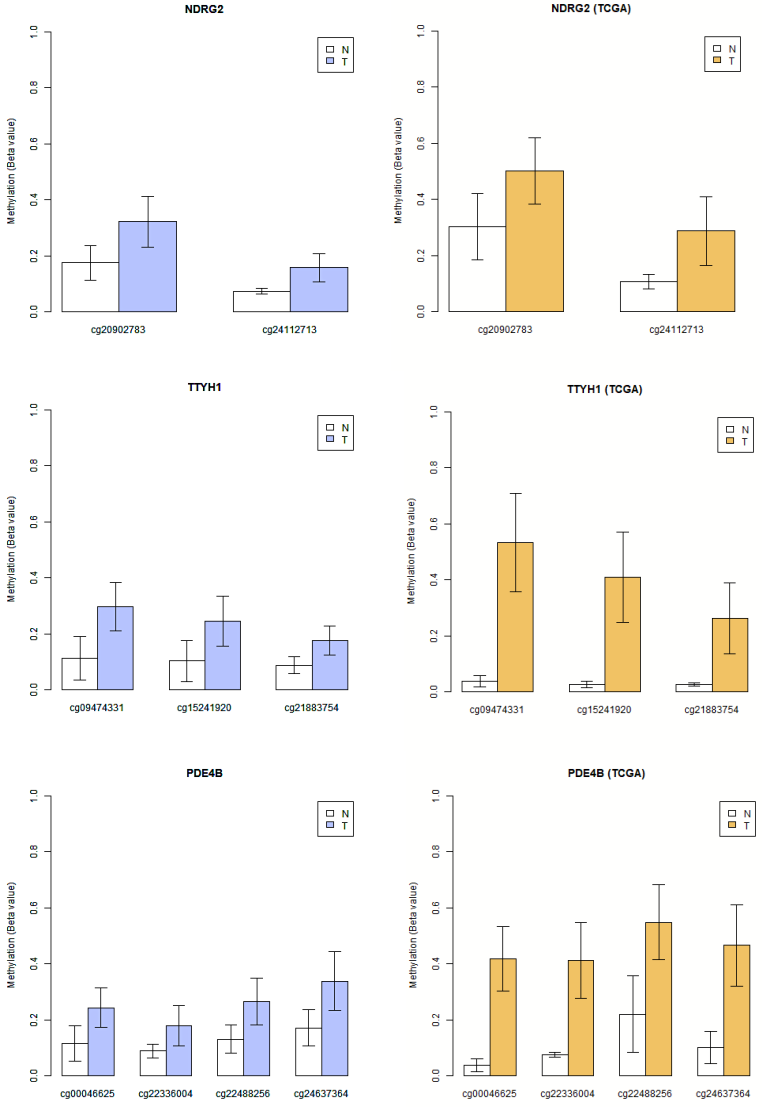

Figure 6. DNA methylation levels (median) of differentially hypermethylated CpGs in 11 genes in 6 OTSCC patients and normal adjacent tissues from our EPIC array and in 36 early-OTSCC and 4 healthy controls from the TCGA database. *Abbreviations:* OTSCC, oral tongue squamous cell carcinoma; TCGA, The Cancer Genome Atlas; N, normal; T, tumoral.

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3.4. Accuracy of tumor-specific DNA methylation markers

In addition, we performed ROC analysis and estimated AUC values of the 11 tumor-specific promoter hypermethylated genes (Figure 7). Furthermore, ROC analysis in TCGA dataset revealed the following AUC values for each marker: *A2BP1* (0.991), *ACSS3* (0.991), *ANK1* (0.935), *CA3* (0.954), *ALDH1A2* (0.954), *GFRA1* (0.935), *GABRB3* (0.991), *HS3ST1* (0.944), *NDRG2* (0.954), *TTYH1* (1), and *PDE4B* (1) (Figure S4). Overall, these DNA methylation markers showed high sensitivity and specificity values (Table S3) indicating the potential of methylation profiling of these gene promoters for discriminating early-OTSCC from healthy individuals.

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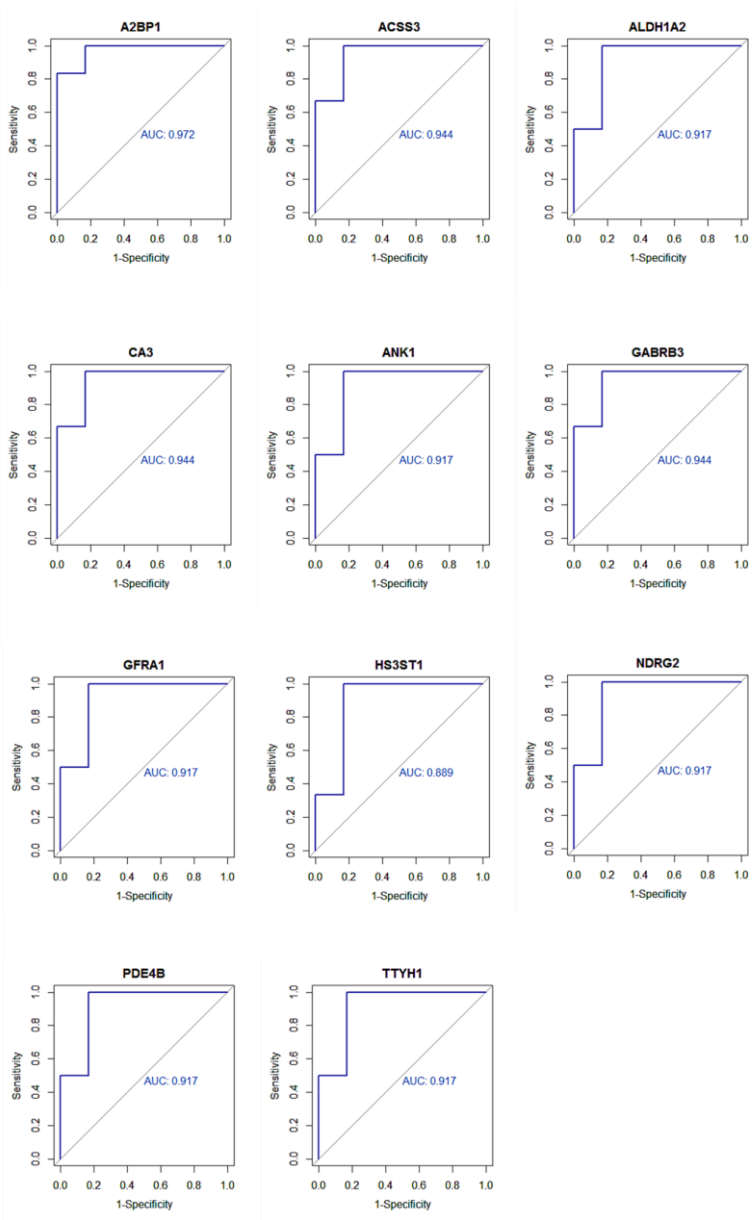

Figure 7. ROC curves of the 11 selected tumor DNA methylation markers for discriminating OTSCC patients from healthy controls. ROC curve and AUC values were estimated for *A2BP1*, *ACSS3*, *ANK1*, *CA3*, *ALDH1A2*, *GFRA1*, *GABRB3*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B* using R software. *Abbreviations:* ROC, receiver operating characteristic; AUC are under the ROC curve.

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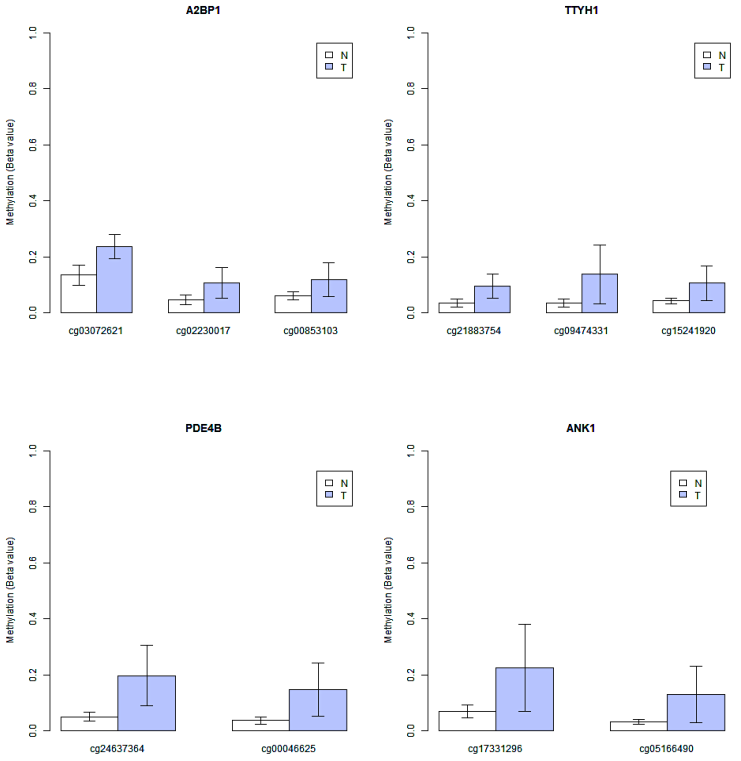
3.5. Accuracy of tumor-specific DNA methylation markers in saliva

To test the potential of tumor-specific CpG methylation markers of the *A2BP1*, *ACSS3*, *ALDH1A2*, *ANK1*, *CA3*, *GABRB3*, *GFRA1*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B* as salivary biomarkers for early detection of OTSCC, we analyzed salivary cellular pellet of 4 OTSCC patients and 6 healthy controls. Differential methylation levels between saliva from OTSCC patients and healthy individuals were observed for specific-site methylated CpGs in *A2BP1* (P = 0.005), *ALDH1A2* (P = 0.033), *ANK1* (P = 0.033), *GFRA1* (P = 0.033), *PDE4B* (P = 0.012), and *TTYH1* (P = 0.009) genes (Figure 8). The sensitivity observed for the individual markers ranged from 75% to 100% and the specificity ranged from 83% to 100% (Figure 9 and Table S4).



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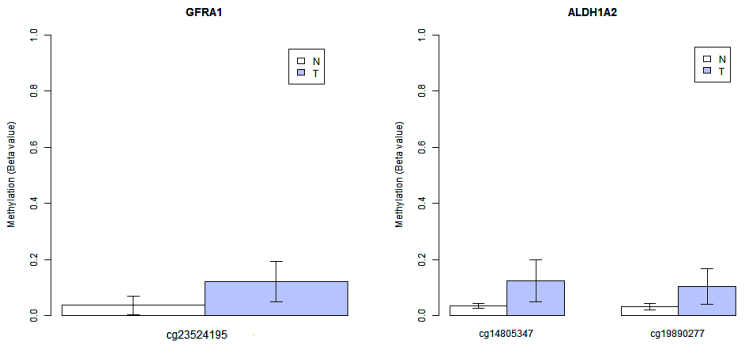

Figure 8. Saliva DNA methylation levels (median) of significant hypermethylated CpGs corresponding to 6 genes in saliva samples from 4 OTSCC patients compared to 6 healthy individuals. *Abbreviations:* OTSCC, oral tongue squamous cell carcinoma; CpGs, cytosine-guanine dinucleotides; N, normal; T, tumoral.

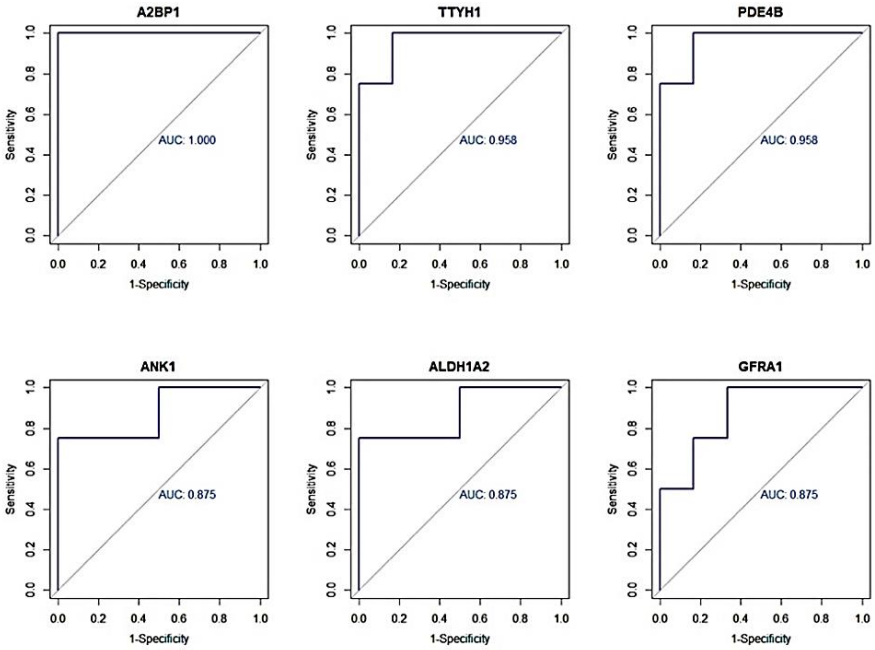

Figure 9. ROC curves of the 6 salivary DNA methylation markers for discriminating OTSCC patients from healthy controls. ROC curve and AUC values were estimated for *A2BP1*, *ANK1*, *ALDH1A2*, *GFRA1*, *TTYH1*, and *PDE4B* using R software. *Abbreviations:* ROC, receiver operating characteristic; AUC are under the ROC curve.

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4. DISCUSSION

To the best of our knowledge, this is the first genome-wide DNA methylation study that investigates aberrant DNA methylation at 850,000 CpG sites using the EPIC array. Our study showed an OTSCC specific methylation pattern consisting of 25,890 CpGs that were differentially methylated compared to adjacent normal tissues. We found that OTSCC had more CpGs hypomethylated (79%) than hypermethylated (21%), which is consistent with previously published studies [8,21]. Most of the hypermethylated CpG sites (48%) were located in the CpGIs, while hypomethylated CpG sites (86%) were primarily enriched in the open sea region. In agreement with our results, a similar distribution of DMCpGs has been observed in OSCC using the HM450K array [8].

Aberrant DNA promoter hypermethylation has been associated with the transcriptional silencing of different tumor suppressor genes in oral cancer [22]. In this study, we focused on the CpGs that overlapped with the promoter region identifying a methylation pattern of 2,700 tumor-specific CpGs mapped 807 unique genes differentially methylated in OTSCC compared to normal tissue. For forty of the 807 genes that presented hypermethylated CpGs in their promoter region, we observed a downregulated expression in the available TCGA OTSCC database, suggesting that they may play an important role in OTSCC carcinogenesis. To further explore the consistency of our results, we investigated the methylation pattern of the differentially methylated CpGs of these 40 genes using genome-wide DNA methylation data from the publicly available TCGA head and neck squamous cell carcinoma database. Due to the high heterogeneity of head and neck tumors, we have considered only data from 36 OTSCC patients in the TCGA dataset. For this approach, we compared data from two different microarray platforms (TCGA-HM450K vs. EPIC) finding 45 CpGs sites located at 22 genes available in both databases. After analysis, we have observed in 11 genes (*A2BP1*, *ACSS3*, *ALDH1A2*, *ANK1*, *CA3*, *GABRB3*, *GFRA1*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B*) a consistent methylation pattern at different CpG sites with TCGA array data. These genes could be functionally important for

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OTSCC cells and disruption of their control by aberrant DNA methylation could act as a driver event in the OTSCC development. Furthermore, these hypermethylated 11 genes showed a high and comparable diagnostic accuracy both in our discovery cohort as well as in TCGA-derived samples, indicating the potential clinical utility of these tumor-specific methylated markers for early OTSCC detection.

This study has identified novel convincing site-specific DNA methylation changes in genes that have not previously been described in OTSCC. However, DNA methylation alterations of these genes have been associated with an important role in tumorigenesis in other tumor types. The acyl-CoA synthetase short-chain family member 3 (*ACSS3*) is a gene located on chromosome 12q21.31 that plays an important role in cancer cell metabolism especially under metabolic stress [23]. Downregulation of the *ACSS3* due to gene promoter hypermethylation has been associated with prostate cancer [24]. The aldehyde dehydrogenase 1 subfamily A2 (*ALDH1A2*) is a gene located on chromosome 15q21.3 that encodes for the protein *ALDH1A2*, which catalyzes the cellular synthesis of the vitamin A metabolite retinoic acid which regulates multiple biological processes during embryonic development and in adult tissue remodeling and homeostasis [25]. Methylation of *ALDH1A2* and subsequent low expression mRNA levels have been associated with an unfavorable prognosis in head and neck cancer [26]. In addition to head and neck cancer, methylation-associated silencing of the *ALDH1A2* gene has been also observed in prostate [27], ovarian [28], and cervical [29] cancers. The ankyrin 1 (*ANK1*) is a gene located on chromosome 8p11.21 that encodes for the protein ankyrin 1 that acts as a linker between the integral membrane proteins and spectrin-based cytoskeleton. *ANK1* inactivation via promoter hypermethylation has been involved in lung carcinogenesis [30]. In oral cancer, the loss of *ANK1* and *miR-486-3p* expression has been significantly correlated with *ANK1* promoter hypermethylation. Interestingly, the re-expression of *ANK1* and *miR-486-3p* by inhibiting DNA methylation with 5-aza-2′-deoxycytidine reduced the expression of *DDR1* oncoprotein, decreasing proliferation and enhancing apoptosis in OSCC cells [31]. The carbonic anhydrase III (*CA3*) is a gene located on chromosome 8q21.2 and it belongs to a multigene family that

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encodes carbonic anhydrase isozymes that catalyze the reversible hydration of carbon dioxide to form carbonic acid. Loss of expression of *CA3* has been observed both in head and neck squamous cell lines and oral cancer samples and it could be explained by aberrant methylation in its promoter region [32]. Also, hypermethylation of *CA3* has been reported in bladder cancer [33]. The NDRG family member 2 (*NDRG2*) gene is located on chromosome 14q11.21 and it belongs to the N-myc downregulated gene family that has predominantly tumor-suppressor effects. *NDRG2* encodes a cytoplasmic protein that may play a role in cell growth cell, differentiation and apoptosis, neuronal plasticity, and stress response [34]. *NDRG2* promoter hypermethylation has been associated with the transcriptional *NDRG2* down-regulation in numerous malignancies including meningiomas [35], glioma [36–38], liver cancer [39,40], colorectal cancer [41,42], gastrointestinal cancer [43,44], and oral squamous cell carcinoma [45]. Interestingly, the downregulation of *NDRG2* could be modulating the activation of PI3K/Akt signaling in oral carcinogenesis. In this line, the re-expression of *NDRG2* in oral cancer cell lines has been associated with a decrease of the level of phosphorylated Akt at Serine 473 (p-Akt) that resulted in a cell growth suppression. Moreover, positive p-Akt immunostaining was significantly associated with downregulated *NDRG2* expression, mainly in moderate and poor differentiated oral cancer tumors [45]. The GDNF family receptor alpha 1 (*GFRA1*) gene is located on chromosome 10q25.3 and encodes neurotrophic factor and neurturin that are potent neurotrophic factors involved in the control of neuron survival and differentiation and has an important role in the progression and metastasis of human cancer [46–48]. Aberrant methylation of *GFRA1* has been associated with low *GFRA1* expression levels in rectal cancer [49], lung cancer [50], and gastric cancer [51]. The gamma-aminobutyric acid type A receptor subunit beta3 (*GABRB3*) is a gene located on chromosome 15q12 which encoded protein acts as a receptor for gamma-aminobutyric acid, a major inhibitory neurotransmitter of the mammalian nervous system. Promoter methylation of *GABRB3* has been associated with low concurrent mutation frequency in head and neck cancer, indicating promoter methylation as the main mechanism of inactivation for this tumor suppressor [52]. The RNA binding fox-1

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homolog 1 (*A2BP1*) located on chromosome 16p13.3 regulates tissue-specific alternative splicing and tweety family member 1 (*TTYH1*) located on chromosome 19q13.42 encodes a member of the tweety family of proteins that functions as chloride anion channels. The phosphodiesterase 4B (*PDE4B*) located on chromosome 1p31.3 regulates the cellular concentrations of cyclic nucleotides and plays a role in signal transduction and the heparan sulfate-glucosamine 3-sulfotransferase 1 (*HS3ST1*) located on chromosome 4p15.33 encodes enzyme with multiple biological activities. However, no reports have been made about aberrant methylation of these genes (*A2BP1, TTYH1, PDE4B,* and *H3ST1*) in cancer. Overall, these findings evidence the methylation of various of these genes in different tumor types, however, future research should be addressed to elucidate their exact biological function in OTSCC carcinogenesis.

In the present study, we have also explored the possibility of detecting aberrant promoter hypermethylation in saliva from OTSCC patients. Several groups have examined the potential utility of DNA methylation in saliva for oral cancer diagnosis [53–57], however the most of these studies have analyzed the promoter hypermethylation of common tumor suppressor genes such as *MGMT*, *p16*, *DAPK1*, or *RASSF1A*. Using a tissue-informative approach, we detected in saliva the site-specific DMCpGs markers in six genes (*A2BP1, ANK1, ALDH1A2, GFRA1, TTYH1,* and *PDE4B*) that showed high sensitivity (values ranging from 75% to 100%) and specificity (values ranging 85% from to 100%) for discriminating OTSCC patients from healthy individuals. Although the promoter DNA hypermethylation of these genes had not been reported so far in saliva, some of these genes have been involved with oral carcinogenesis [45]. Notably, the high diagnostic performance of these tumor-specific methylation genes in saliva shows their potential clinical value for non-invasive early OTSCC detection.

One of the strengths of this study was the broad coverage from the EPIC array that allowed to perform a massive characterization of the methylome in OTSCC. In addition, from a clinical and histopathological point of view, our sample characteristics are strongly homogenous. The consistent results observed for the comparison

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between the tumor-specific methylated pattern in our cohort with TCGA OTSCC methylation data can also be viewed as a strength. However, our study is not exempt from weakness. The methylation analysis was carried out in a small sample size of patients with OTSCC. Another limitation is the lack of transcriptome profiles of patients with OTSCC. Although we have evaluated the transcriptional silencing of the selected methylated markers using the TCGA database, functional validation of these targets should be carried out in a large OTSCC cohort to confirm the impact of DNA methylation on gene expression.

4. CONCLUSION

In summary, our study is the first and largest applying methylome-profiling approach to OTSCC samples that identified novel tumor-specific DNA methylation markers with high diagnostic accuracy for early detection of oral cancer in saliva. Further investigations are required to elucidate the biological function of these genes in OTSCC carcinogenesis and their clinical translation.

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GENERAL DISCUSSION

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DISCUSSION

The aim of this thesis was to provide novel knowledge regarding the value of different liquid biopsies such as saliva and blood for improving the diagnosis, characterization, and prognosis of head and neck and colorectal cancers. For that, we applied a comprehensive analytic strategy to characterize both genetic and epigenetic markers and also performed robust meta-analyses to clarify the real clinical value of numerous potential biomarkers previously described by different authors. Of note, based on the facts that during the carcinogenesis process different components appear in fluid samples related to CRC and OTSCC, we have performed a discovery study that allowed the identification in the saliva of a miRNA signature (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p,* and *miR-491-5p*) in CRC patients and tumor-specific DNA methylation markers (*ALDH1A2, ANK1 GFRA1, PDE4B, A2BP1*, and *TTYH1*) in OTSCC patients, both with promising diagnostic value. In addition, we have successfully used fluid samples as a tool for detecting somatic mutations characterized in tumor tissue from advanced HNC. We also found the presence of additional somatic mutations in plasma cfDNA which reflect the clinical potential of cfDNA to capture the tumor heterogeneity and evolution. Notably, we were also able to detect in salivary total DNA additional mutations, suggesting these preliminary results a complementary value of saliva analyses for detecting tumor alterations in oral cavity cancer. All these results are further discussed below.

Currently, a large proportion of cancers are still diagnosed at the advanced stage due to a wide variety of personal, socioeconomic, and medical reasons [1]. Besides, while the survival rates for certain cancers have improved, overall, these rates remain quite low. Hence, there is an urgent need for further improvement of cancer survival, being one of the main goals of the World Cancer Declaration issued by the Union for International Cancer Control. Therefore, any new diagnostic tool that can potentially improve cancer diagnosis and disease monitoring may

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implies an improvement in the patients' survival and is worth considering. Liquid biopsies have emerged as a novel tool in medical oncology, providing a new horizon for improving clinical decision-making. As we previously commented within the present thesis, tumors release several biomolecules into body fluids that could be used as biomarkers for diagnosis, prognosis, and therapy selection. Nowadays, analyses of tissue biopsies still remain the gold standard for diagnosis, however, these analyses hardly reflect tumoral heterogeneity. Importantly, liquid biopsies can also provide information on the molecular landscape during tumor evolution allowing to evaluate the response of the tumor to given therapies and the detection of resistance mechanisms [2]. For this reason, with the present work we aimed to generate new data which support the use of liquid biopsies-based biomarkers in the context of CRC and HNC.

Among the different body fluids, saliva has aroused great interest as a promising liquid biopsy in oncological research. Several studies have highlighted the usefulness of saliva for identifying biomarkers, noting that it represents a great opportunity to improve diagnosis and monitoring of the disease [3]. As a liquid biopsy, saliva presents many advantages over blood and other biofluids as the collection is easy, safe, non-invasive, cost-effective, and enables self-sampling. The advances in saliva-omics have allowed investigating the salivary proteome, transcriptome, metabolome, genome, epigenome, and microbiome [4]. Thus, numerous studies have identified different classes of biomarkers in saliva which could be a great impact in the area of cancer diagnosis. To provide more evidence about the potential of salivary biomarkers for cancer detection we evaluated the diagnostic capacity of saliva for detecting tumors distant to the oral cavity using a meta-analytic approach [5]. This study represents the first work that analyzes the utility of salivary biomarkers for non-oral cancers diagnosis taking into account the different "omic" fields and tumor types. As a result of this study, we could state that salivary biomarkers showed good diagnostic accuracy. However, we also concluded that further research is still needed in the different "omics" to consider saliva as a liquid biopsy for routine clinical usage. Different classes of biomolecules have been identified as salivary biomarkers with significant differences among

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cancer and control groups; however, these biomarkers were not validated in longitudinal prospective and multicenter studies with larger sample sizes. Moreover, robust studies correlating and/or comparing salivary biomarkers with those present in other body fluids such as plasma or serum will be also important for deciphering if saliva can be a substitute of plasma or serum or if it has an additional value with respect to blood. There is also the urgent need for developing specific and standardized protocols regarding pre-sampling conditions, sample collection, storage, preparation, and posterior molecular analysis to obtain reproducible quantifiable data among the studies. Although proteomics leads the current investments in salivary biomarker development, high-throughput techniques such as NGS are currently important techniques used in this field, increasing knowledge on miRNA and mRNA biomarkers, among the other RNAs present in saliva. Despite many efforts directed to identify new salivary markers, as we have mentioned, the validation of previously discovered saliva markers is still required until the salivary analysis has a real clinical impact on cancer diagnosis and therapeutic monitoring.

Over the last few years, ongoing research has highlighted the significant clinical potential of cell-free miRNAs in a variety of human diseases, including cancer. Although there is work to do in the investigation of extracellular miRNAs until they could be used in clinical practice, their analysis shows many advantages. One of these advantages is that miRNAs present robust stability and resistance to RNase degradation in body fluids, reinforcing the high value as tumor biomarkers. Thus, in human cancer, aberrant miRNA profiles could reflect the tumor staging and allow subtypes stratification [6]. Furthermore, miRNA expression has been evaluated in cancer with the goal of providing biomarkers for the early diagnosis [7], prognosis [8], and therapy selection and monitoring [9,10]. In this sense, cell-free miRNAs have been identified in different liquid biopsies including plasma, serum, urine, or saliva as potential diagnostic and prognostic cancer biomarkers. For instance, in prostate and bladder cancers numerous studies have reported the utility of urine-circulating miRNAs as potential biomarkers [11,12]. Moreover, urinary miRNAs have been purposed as biomarkers for breast, ovarian, hepatocellular, and pancreatic cancer [13–16]. In

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plasma and serum, several miRNAs have been found significantly differentially expressed in patients compared with healthy controls in cancers located at oral cavity [17,18], breast [19,20], prostate, [21,22], gastric [23,24], esophageal [25,26], colorectal [27,28], pancreatic [29,30], lung [31,32], and hepatocellular carcinoma [33,34]. In addition to blood and urine, several studies have also demonstrated the potential use of saliva for detecting miRNAs associated with local and systemic release from oral cancer cells [35,36]. Also, salivary miRNAs have been identified in distant tumors such as esophageal [37], pancreatic [38], or prostate [39] cancers. In this context, we carried out a comprehensive study based on the fact that colorectal carcinogenesis is a process associated with altered miRNAs expression, and this alteration could be detected also in saliva samples. Therefore, we decided to address for the first time a global characterization of the salivary miRNA population in CRC in comparison with healthy controls and patients with benign disease to gain knowledge on salivary miRNAs composition in the three experimental groups and identify specific expression patterns in the pathologic group. After this study, we identified a 5-miRNA signature (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p,* and *miR-491-5p*) with value for CRC diagnosis [40]. Notably, when we explored the biological function of these miRNAs, we found that they were implicated in many cancer-related pathways such as ECM-receptor interaction, p53, and PI3K-Akt signaling pathways, and cell cycle. All of them are relevant pathways with a role in CRC biology. For example, *miR-766* is known to participate in CRC, promoting its overexpression cell proliferation by repressing *SOX6* [41]. Besides, *miR-186-5p* was found overexpressed in CRC tissues compared to non-cancer samples and this expression was linked to the promotion of cell proliferation, migration, and colony formation by inhibiting tumor suppressor *FAM134B* [42].

On the other hand, although the salivary miRNAs included in our miRNA signature have been related to CRC in previous studies, we do not know the origin of these miRNAs in the saliva of our CRC cohort. Different studies pointed out that miRNAs could come directly from the tumor or be released by salivary gland cells in response to the presence of secondary messengers into the oral cavity. An *in vitro* study

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demonstrated that circulating breast tumor-derived exosomes could interact with salivary gland cells and induce proteomic and transcriptomic changes in exosomes derived from salivary gland cells [43]. Moreover, there are several murine models in melanoma, lung, and pancreatic cancer that support the hypothesis that tumor-exosomes travel from remote tumor locations to the oral cavity through blood circulation, where these biomarkers or their molecular content can be detected in saliva [44–46]. For example, using a pancreatic cancer mouse model, Lau et al. [45] investigated the origin of transcriptomic biomarkers present in saliva. They found that the salivary transcriptomic profile is different after inhibition of exosome secretion at the tumor site, providing a mechanistic rationale about the connection between discriminatory salivary biomarkers and remote tumors. Although there are no animal models that demonstrate specifically the origin of salivary miRNAs in HNC, scientific evidence indicates that tumor-derived exosomes could function as the shuttle between the remote tumor and the oral cavity leading to the identification of disease-associated miRNAs. Interestingly, this hypothesis also could be applied to oral cavity tumors. In oral cancer, there is a direct release of miRNAs into saliva due to the direct contact with oral cancer lesions. In addition, miRNAs can also be released into the bloodstream like in remote cancers and, therefore, reach salivary glands in exosomes that could modify the salivary miRNA composition. Numerous studies have identified altered expression of cell-free miRNAs in saliva and blood from OSCC patients highlighting their promising value as diagnostic biomarkers. In this context, we developed a meta-analytic approach to provide more evidence about the potential utility of saliva and blood miRNAs for detecting oral cancer [47]. Twenty-five study units from 16 articles with 2,562 subjects were included in this meta-analysis. We found a pooled sensitivity and specificity of blood and salivary miRNAs for the diagnosis of OSCC of 0.78 and 0.82, respectively with an AUC of 0.91. Although these findings demonstrated the potential utility of cell-free miRNAs detected in liquid biopsies for oral cancer diagnosis, it is important to remark that the studies included in the meta-analysis applied diverse cut-off values and different normalization methods for miRNA quantification, which could substantially affect the

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results of our analysis and reports of potential clinical efficacy. In the same line, a recent meta-analysis evaluated the diagnostic accuracy of salivary and blood miRNAs separately reporting sensitivities of 0.70 and 0.79, respectively, and a specificity of 0.82, which reinforces the potential value of salivary and blood miRNAs for oral cancer diagnosis [48].

Despite growing research of liquid biopsies based on miRNAs, there are still several challenges that remain to be overcome before their clinical implementation. Although several dysregulated cell-free miRNAs have been found in liquid biopsies there is an urgent need to validate cell-free miRNA signatures with the highest sensitivity and specificity for cancer detection. In addition, it is necessary to determine the most suitable biofluid for analyzing the miRNA profile in cancer patients. Comprehensive studies would allow us to identify the most commonly altered miRNAs present in saliva and blood, and to discover specific molecular patterns in both biofluids. Nowadays, only a few studies have evaluated the expression profiles of cell-free miRNAs in saliva compared to serum or plasma [49–51]. In serum and saliva, Alemar et al. [49] evaluated the expression levels of six pancreatic ductal adenocarcinoma-associated miRNAs (*miR-21, miR-34a, miR-155, miR-196a, miR-200b,* and *miR-376a*). Only significant differences between cancer patients and controls were observed for serum *miR-21* and *miR-34a*. Conversely, in saliva only *miR-196a* was significantly higher in the control group, showing a heterogeneous and almost undetectable expression [49]. However, in OSCC, miR-31 salivary levels revealed a strong correlation with *miR-31* serum levels. Besides, saliva presented a greater amount of *miR-31* than plasma, indicating that saliva could be more sensitive than plasma for detecting *miR-31* expression levels. [50]. Recently, expression levels of *miR-21* were analyzed in plasma and saliva of CRC patients. Although no significant individual correlation of *miR-21* expression levels in the plasma and saliva was observed, saliva showed higher sensitivity and specificity than plasma for CRC detection [51]. Overall, these studies show the promising potential of salivary miRNAs for cancer diagnosis, however, these findings should be taken with great caution, since they need to be validated in future research.

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Importantly, up today we don't know exactly how reproducible and consistent the cell-free miRNAs levels are intra- and inter-individuals. In fact, lifestyle habits, circadian rhythm, or comorbidities cause inter-individual variation in cell-free miRNA levels which might limit their clinical utility as biomarkers [52]. Thus, miRNA expression levels are sensitive to various factors unrelated to the disease such as physical activity [53], diet [54], or smoking [55]. Also, different oral conditions such as untreated cavities, gingival, or periodontal disease could influence miRNA expression levels both in blood and saliva [56,57]. It is important to note that we have considered the influence of various of these covariables in the design of our salivary miRNA experiment. Thus, to avoid the impact of circadian rhythm and diet in the miRNA expression levels we have collected saliva samples from all participants in fasting and at the same time range. In addition, all participants underwent an oral exploration for excluding saliva samples from subjects with oral lesions and/or inflammatory diseases that could influence miRNA expression levels. Unfortunately, the analyses of these potential confounders are not usually included in biomarker studies, so future investigations on cell-free miRNAs should take in mind these covariables in its design with the aim to minimize the number of confounding factors that can influence miRNA analysis. In fact, although numerous miRNAs have been examined with the same clinical purpose, there is not a coincidence between single miRNAs or miRNA panels reported by many studies, and even between studies that analyzed the same miRNAs, there are sometimes opposite results. These heterogeneous results could be explained by the selection strategy of patients (sample sizes, tumor stages, socio-demographic, and clinicopathological characteristics), the samples collection (type of body fluid, collection tubes, and protocols), the sample processing, and the different approaches for the miRNAs isolation, quantification, and data analysis including normalization (endogenous or exogenous normalizers), and the use of different cutoff values for the same miRNAs. For example, the harmonization of circulating exosome extraction constitutes a key element to reach a higher reproducibility in studies characterizing exosome-derived miRNAs. Therefore, a consensus of the scientific community should be carried out to

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standardize pre-analytical and post-analytical protocols in order to translate laboratory findings into tangible clinical application. Other important limitation is the lack of standardized guidelines for normalizing data. Normalization is the cornerstone for accurate quantification of RNA levels using RT-qPCR, and therefore, a reliable biological interpretation of the results. The normalization strategies most commonly used for miRNAs expression studies are based on the global mean expression [58], exogenous (spiked-in) miRNAs, and endogenous controls [59]. Particularly, the normalization based on endogenous controls such as miRNA, snRNA, and snoRNA is the most widely used approach [60]. However, currently, there is no consensus on which miRNA is best for normalization. Researchers select reference genes based on other studies [37,38,51,61,62] or searching stable miRNAs in their own samples [35], following the strategy described by Vandesompele et al. [63], where stable genes across the samples (among other factors) make them suitable for their usage as a normalizer. Moreover, the development of different algorithms such as NormFinder, geNorm, BestKeeper, and comparative Cq allows the identification of the most stable genes in a group of samples for being used as normalizers [64].

Nowadays, various studies have applied a normalization strategy based on multiple reference genes due to its combination reduces the effects of the analytical variability compared to single gene-based normalization [65,66]. To normalize our microarray data, we applied the global mean expression approach. This method has been reported as the most appropriate strategy to normalize a large number of miRNAs and is based on the mean of all expressed miRNAs [58]. After this normalization, we selected two endogenous controls (*miR-1274B* and *miR-193b-3p*) as candidate reference genes because they were homogeneously expressed between groups (patients with CRC, adenomas, and healthy individuals). Moreover, the NormFinder algorithm revealed that *miR-193b-3p* was the most stable control to normalize RT-qPCR analyses, showing further high stability among salivary samples. According to the literature, the reference miRNAs most commonly used for the normalization of expression studies in saliva are *miR-16* and *miR-191* [35,37,50,67–69], but also two

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snoRNAs (*SNORD68* and *SNORD96A*) [36,70] and one snRNA (*U6*) [38,51,62,71–74]. However, none of them seem to be robust enough to be considered as a standard for studies focused on saliva. Importantly, with our study, we provided one additional miRNA with value as normalizer in studies that analyze saliva samples in CRC patients.

On the other hand, nowadays, it seems unlikely to use a single miRNA as a diagnostic, prognostic, or predictive cancer biomarker. In this sense, the combination of multiple miRNAs or even the combination of miRNA panels with analytes from other omics would also contribute to the development of multi-omic panels for improving precision oncology. In our study, we demonstrated that the combination of our salivary 5-miRNA (*miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p,* and *miR-491-5p*) signature with the traditional biomarker CEA yielded an improvement of the detection rate of CRC. Of note, an ideal clinical model must have both high sensitivity and specificity and suitable cost-effectiveness. It is important to remark that although several miRNA-panels for both colorectal and oral cancer have been reported, until now, cost-benefit analysis has not yet been evaluated.

Taking into account the wide variety of multi-omics components in liquid biopsies we have also explored the potential value of cfDNA in plasma and total DNA in saliva for detecting somatic mutations in advanced HNC. Today, anti-EGFR monoclonal antibody cetuximab is the only targeted therapy approved by the USFDA in HNC for the treatment of locally advanced disease. The overexpression of *EGFR* is observed in 80-90% of HNSCC and is associated with poor survival [75]. The combination of radiotherapy plus cetuximab has demonstrated a significant improvement in locoregional control and survival compared to radiotherapy alone for the treatment of locoregionally advanced disease [76]. However, there is no evidence about the benefit of radiotherapy plus cetuximab compared to standard of care chemoradiotherapy, limiting the use of radiotherapy plus cetuximab in unfit patients for cisplatin based-chemoradiotherapy [77]. Despite the multimodal treatment, the patients with locally advanced disease have a high risk of local recurrence and distant metastasis leading to 5-year OS <50% [78]. In patients with recurrent or metastatic

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HNC, the first line of treatment includes the immune checkpoint inhibitor pembrolizumab [79]. Both the combination of chemotherapy plus pembrolizumab or pembrolizumab alone have shown a better survival compared to chemotherapy plus cetuximab [80]. This latter is considered the second line of treatment for patients who are not candidates for first-line immunotherapy [81]. Unfortunately, around 85-90% of recurrent or metastatic HNC have not a response to PD-1–directed immune-check-point inhibitor or in case of a response this is followed by disease progression, and finally death [78]. Therefore, it is necessary to know the molecular mechanisms underlying the resistance to chemotherapy, which allow the development of more effective therapeutic strategies in the treatment of HNC. In addition, the lack of predictive and monitoring markers to guide treatment decisions in HNC justifies the search for non-invasive biomarkers that allow the selection of personalized treatment strategies. Based on this clear clinical need for finding biomarkers to predict the therapeutic response and for the early identification of resistance, our research group is developing a project (FIS: PI20/01449) directed to molecularly characterize locally advanced and metastatic/recurrent HNSCC using longitudinal fluid samples. Our purpose is to identify the molecular alterations associated with primary and secondary resistance to the therapy. We hypothesize that there is a dynamic mutational spectrum in the different phases of HNSCC, from diagnosis to progression. This mutational variability leads to inter- and intra-tumoral heterogeneity, which results in treatment failure, leading to regional recurrence or disease progression in distant organs. To understand what happens, the mutational profile of a cohort of HNSCC patients is being analyzed at baseline and during treatment (surgery and/or chemoradiotherapy) in tissue, blood, and saliva by targeted NGS gene panels. As a proof-of-concept, we have evaluated the performance of the TST170 panel for detecting pathogenic gene variants in tumor and plasma cfDNA from advanced HNC patients. Overall, the results of this study evidenced the potential of this technology for detecting tumor somatic mutations in cfDNA from HNC patients. Thus, we detected somatic variants (SNVs and InDels) in a total of 19 genes in tumor and/or cfDNA, of which *TP53*, *NOTCH1*, and *EP300* are described as common driver genes in HPV-

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ve HNC [82]. Besides, an overall concordance rate of 58.3% was observed between the somatic mutations detected on tissue and cfDNA from plasma, demonstrating the value of cfDNA to characterize the tumor molecular landscape. But even more important is the fact that additional variants were found analyzing the cfDNA, which can represent the molecular characteristics of the metastatic lesions. Despite these interesting results, larger panels should be used to better capture the tumor heterogeneity and determine the real potential of cfDNA for reflecting the tumor mutational profile. It is also important to remark that discordances between the somatic genomic alterations detected in solid samples and cfDNA could be associated with several factors, comprising MAF and tumor heterogeneity. Biological factors including tumor anatomic location, tumor size, and vascularity of the tumor can determine the release of tumor DNA into blood, and therefore, influence the detectability of tumor DNA. In our assay, we have observed that the concordance between tumor and plasma cfDNA was higher in the hypopharynx tumor compared to oral cavity tumors. Previously, Wang et al. [83] demonstrated that plasma analyses are more sensitive than saliva for detecting ctDNA in the advanced oropharynx (91% vs. 47%), larynx (86% vs.70%), and hypopharynx (100% vs. 67%) tumors. On the contrary, saliva showed a high sensitivity for detecting early oral cavity tumors compared to ctDNA from plasma (100% vs. 80%). Another factor that could be involved in poor concordance is the presence of a low MAF of genomic alterations that fall below the detection cut-off of the assay used. In addition, the release of genomic DNA from white cells can lead to a diluting effect on the tumor fraction of cfDNA. Interestingly, tumor fraction can be also influenced by the type of cancer and stage. Regarding tumor type, Bettegwada et al. [84] found that ctDNA was detectable in >75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and HNC, but in less than 50% of primary brain, renal, prostate, or thyroid cancers. However, in patients with localized tumors, ctDNA was detected in 73%, 57%, 48%, and 50% of patients with CRC, gastroesophageal cancer, pancreatic cancer, and breast adenocarcinoma, respectively. Moreover, based on tumor stage, ctDNA was detected in 47% of

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patients with stage I, in 59% of patients with stage II, in 69% of patients with stage III, and 82% of patients with stage IV independently of tumor type which indicates a higher frequency of ctDNA detection in metastatic compared to localized disease. Since we have evaluated only the concordance between primary tumor and cfDNA, the comparison between the metastasis and cfDNA could increase the concordance rate due to the possibility to detect variants that are characterizing the metastatic lesions.

It is also important to highlight that we successfully applied a targeted panel on plasma cfDNA which is normally used for analyses on paraffin tissues. The possibility of analyzing both tumor tissue and cfDNA in a single assay represents an advantage for the clinical implementation of cfDNA analyses. For example, for the early detection of actionable alterations which could be only detected in cfDNA as consequence of the tumoral heterogeneity and/or their metastatic origin. Importantly, as we previously mentioned, due to the direct contact of saliva with tumor lesions present in the oral cavity, the combined analysis of tumor tissue and saliva could provide more information regarding the tumor genomic characteristics than plasma analyses in oral cancer patients. Interestingly, we analyzed by ddPCR the potential of salivary total DNA for detecting the somatic mutations previously identified by NGS. We validated one somatic mutation in saliva indicating the possibility of detecting somatic variants in saliva from patients with oral cavity tumors. It is important to consider that saliva includes a high proportion of non-tumor DNA which could decrease the accuracy of tumor DNA detection. Various studies have reported that tumor DNA in the saliva is often present at frequencies <1% [83,85] which implies the use of highly sensitive platforms for rare mutant fragment detection, such as the ddPCR. This is in line with the MAF observed in our experiment, which reinforced the utility of ddPCR for the monitoring of specific somatic mutations on saliva during the treatment and disease follow-up.

Importantly, as part of our work focused on salivary DNA characterization, we have hypothesized that scfDNA may be composed of ccfDNA from blood and non-ccfDNA released from local and proximal cells into the oral cavity (local source) as a result of multiple

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processes such as apoptosis, necrosis, and active release, which can be associated with EVs [86]. Moreover, scfDNA could represent an enriched fraction of tumor DNA with less background of wild-type DNA from non-tumoral cells, which could increase the accuracy of mutant fragment detection. Some studies [87,88] have demonstrated the feasibility to detect ctDNA in saliva from patients with tumors distant to the oral cavity. However, the potential of salivary cfDNA is underexplored in HNC. Therefore, the analysis of stDNA through highly sensitive techniques such as NGS represents an attractive and novel approach for revealing the genetic tumor profile of HNC and it could be used as a non-invasive tool for tracking the molecular tumor alterations in real-time. Of note, quantitative and qualitative analysis of scfDNA could have great potential for early detection, diagnosis, prognosis, monitoring treatment response, prediction of recurrence, detection of minimal residual disease, and therapy selection. Taking into account that scientific evidence about scfDNA is limited [89], we carried out an exploratory study in order to provide knowledge about the characteristics of scfDNA in oral cavity tumors. Our experiment was focused on the quantity and integrity assessment of scfDNA based on the hypothesis that scfDNA fragments from OCSCC patients may be longer than those of healthy individuals due to tumor necrosis. The results of our study indicated a significantly higher percentage of long scfDNA fragments in oral cavity cancer compared to healthy controls, which could be explained by the tumor necrotic activity. However, scfDNA of high molecular weight was also observed in healthy individuals indicating a non-necrotic origin. Based on these results, we can state that non-tumoral scfDNA is innately a mixture of long and short DNA fragments. To better understand the origin of the scfDNA fragments, the future characterization of the DNA content of salivary exosomes will be of great value. It is also important to remark that not all scfDNA are similarly sensitive to DNase activity that could be influenced by the oral microbiome [90]. Different bacteria have shown an important DNase activity in periodontal disease. Importantly, this DNase activity secreted by periodontal bacteria has displayed a vital role in the breakdown of neutrophil extracellular traps DNA which would enable bacteria to evade the host immune defense [90,91]. In this

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context, we hypothesized that the oral microbiome environment present in the oral cancer patients can modulate the DNAse activity in saliva, and therefore affect the scfDNA integrity. As a continuation of this pilot study which characterized the scfDNA, we will quantify the tumoral fraction of scfDNA to evaluate its potential for detecting tumor genetic alterations in HNC compared to total salivary DNA.

In addition to the presence of genetic alterations in tissue and liquid biopsy, we also focused our attention on epigenetic alterations characterizing oral tumors. DNA methylation is one of the most frequently evaluated epigenetic alterations in cancer. Moreover, since aberrant DNA methylation is an early event in cancer development its detection in saliva has emerged as an attractive non-invasive strategy for early diagnosis of HNC. In this context, to provide comprehensive evidence about the impact of salivary DNA methylation in HNC we developed two meta-analyses [92,93]. This double-meta-analytic strategy was addressed both to analyze the impact of salivary DNA methylation on HNC risk and the potential of salivary DNA methylation as a diagnostic biomarker. The cancer risk meta-analytic approach showed that salivary DNA hypermethylation was associated with HNC risk, evidencing the involvement of this epigenetic alteration in HNC carcinogenesis [93]. For this analysis, a total of 18 studies comprising 4,453 patients and 3,233 controls were included. We found that salivary promoter hypermethylation was significantly associated with an 8.34-fold increase in HNC risk. Moreover, subgroup analysis revealed a stronger association between salivary promoter hypermethylation and oral (OR = 13.26) and oropharyngeal (OR = 13.07) cancer risk. On the other hand, the diagnostic meta-analytic approach demonstrated the potential of salivary DNA methylation for detecting HNC. Eighty-four study units from18 articles involving 4,758 HNC patients and 3,605 healthy individuals were included in this meta-analysis. We found a pooled sensitivity and specificity of salivary promoter hypermethylation of 0.39 and 0.87, respectively with an AUC of 0.81. Although the pooled sensitivity was low, subgroup analysis revealed that the combination of methylation genes improved the sensitivity (0.73) and specificity (0.88) yielding to high diagnostic

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efficiency for detecting HNC in saliva [92]. Overall, these findings demonstrate the potential clinical utility of salivary DNA methylation for screening and early detection of HNC. However, most of the methylation studies in HNC englobe tumors from different anatomic locations and do not characterize the specific methylation changes of the different tumor subsites of the head and neck region. In this context, we developed an epigenome-wide DNA methylation analysis in OTSCC. OTSCC is the most common type of oral cancer characterized by an aggressive behavior that entails a poor prognosis so that to elucidate in depth the methylome landscape of these tumors represents an opportunity to identify potential DNA methylation markers for screening and early cancer detection. Therefore, in order to provide more insights about molecular mechanisms underlying OTSCC carcinogenesis we performed for the first time the largest characterization of the methylome landscape in OTSCC in which aberrant DNA methylation at 850,000 CpG sites was investigated. Among a total of 25,890 DNA methylation candidate markers, we identified a set of 11 tumor-specific hypermethylated genes (*A2BP1, ACSS3, ANK1, CA3, ALDH1A2, GFRA1, GABRB3, HS3ST1, NDRG2, TTYH1,* and *PDE4B*) which showed a downregulated expression in the TCGA database, suggesting the promoter hypermethylation as the possible mechanism of silencing of these genes in OTSCC carcinogenesis. Then, we demonstrated the capacity of saliva for detecting tumor-specific DNA methylation markers, which highlights its potential clinical value as a diagnostic fluid sample. Thus, we have identified in saliva tumor-specific DNA methylation markers in the promoter region of six genes including *ALDH1A2, A2BP1, ANK1, GFRA1, TTYH1,* and *PDE4B,* which showed a high diagnostic value for the early detection of OTSCC. Although the exact biological function of these genes in OTSCC remains unknown, various of them have been described as methylated in cancer. For example, the *ANK1* inactivation via promoter hypermethylation has been described in oral cancer. Interestingly, the re-expression of *ANK1* and *miR-486-3p* by inhibiting DNA methylation with 5-aza-2′-deoxycytidine reduced the expression of *DDR1* oncoprotein, decreasing proliferation and enhancing apoptosis in OSCC cells [94]. Also, the methylation-

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associated silencing of the *ALDH1A2* gene has been observed in a wide variety of malignancies such as HNC [95], prostate cancer [96], ovarian cancer [97], and cervical cancer [98]. Furthermore, the DNA methylation markers identified in our study could be useful for the development of screening test in saliva due to its non-invasiveness nature, easiness, and cost-effectiveness. However, this utility should be further validated in a larger cohort of patients, which is now being recruited by our group.

In summary, the present thesis shows the potential clinical value of different saliva and blood genetic/epigenetic biomarkers in CRC and HNC. Actually, we demonstrated the high value of saliva as liquid biopsy both in local and distant tumors to the oral cavity, opening new horizons in the salivary research field. Besides, we focused our work on different types of markers developing a multi-omic approach, including both genetic and epigenetic alterations, to identify non-invasive biomarkers. In fact, the application of multi-omic panels will improve the sensitivity and specificity of biomarkers and will have a positive impact to perform more precise oncology in CRC and HNC. Of note, our work is the result of an important effort to clarify the real clinical value of numerous biomarkers already described in the literature for both tumors, through the development of robust meta-analysis. Therefore, this thesis is pioneer describing new genetic and epigenetic signatures and using saliva for both remote and oral cavity tumors but also provides a critical revision of previous knowledge, which is essential to have a clear vision about the validation status of numerous potential biomarkers described as candidates for the management of these two tumors. It is also important to keep in mind that, in the long-term, ongoing technological and bioinformatic advances will provide to the medical community large accurate molecular profiles of each patient yielding to a new health system. To achieve this objective, fundamental efforts as those made in the present thesis, should be addressed to develop clinically relevant experimental research with reliability and reproducibility results, taking into account the lack of concordance between the labs and the inability to validate studies reported by other labs. Of note, biomarker development

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guidelines should be considered in order to reach an effective clinical implementation.

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CONCLUSIONS, CONCLUSIONES, CONCLUSIÓNS

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CONCLUSIONS

In answer to the raised objectives, the main conclusions extracted from the research conducted in the current PhD thesis are summarized below:

1. Derived from the meta-analysis focused on the accuracy of saliva for the diagnosis of non-oral malignant tumors, salivary biomarkers show "good" diagnostic capability for detecting different tumors remote to the oral cavity, including breast, lung, pancreatic, ovarian, gastric, and esophageal cancers. However, the methodological design and the substantial heterogeneity in the outcome measures may influence the accuracy of saliva biomarkers for cancer diagnosis. These summary findings show the growing importance of salivary biomarkers in cancer and can help researchers design more rigorous diagnostic validation studies.

2. Characterization of the profile of miRNAs in the saliva of CRC patients constitutes a valuable tool for the identification of tumor markers. Following this strategy, we identified a panel of 5 miRNAs (*miR-185-5p, miR-29a-3p, miR-29c-3p, miR-491-5p,* and *miR-766-3p*) with potential value for the diagnosis of CRC, alone and/or in combination with CEA levels, and also for discriminating benign digestive disease.

3. Derived from the meta-analysis directed to analyze the accuracy of circulating miRNAs for the diagnosis of OSCC we determined that saliva and blood miRNAs show a "good" diagnostic performance for miRNAs could be weakened by methodological design and the considerable heterogeneity in the diagnostic values reported.

4. The total concentration of scfDNA has limited diagnostic value for oral cancer detection, however scfDNA integrity indexes (ALU115/60 and ALU247/60) showed potential for discriminating OCSCC patients from healthy controls.

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5. Targeted NGS analysis of tumor and cfDNA with the TST170 panel allows the detection of somatic genetic variants in patients with advanced HNC. Besides, the analysis of tumor-derived gene variants by ddPCR in both plasma and saliva represents a valuable tool for the detection and monitoring of predictive biomarkers in HNC management.

6. After the meta-analysis which interrogated the diagnostic accuracy of salivary DNA methylation in HNC, we concluded that gene promoter hypermethylation can be potentially used to detect HNC, especially in oral or oropharyngeal cancer. However, we found limitations in the methodological design and heterogeneity in the diagnostic values reported in the literature.

7. Based on the meta-analysis directed to determine the impact of salivary DNA methylation on HNC risk, we demonstrated that hypermethylation in the promoter region of genes related to tumor biology is associated with an 8.34-fold increase in HNC risk. In addition, the combined analysis of *p16*, *MGMT*, *DAPK*, *TIMP3*, and *RASSF1A* methylation in saliva could be useful for predicting the risk of developing HNC, mainly in the oral and oropharyngeal tumors. However, weaknesses in the methodological design, the heterogeneity, and the lack of adjustment for potentially confounding effects were found in the studies analyzed.

8. The characterization of the methylome landscape in tissue and liquid biopsies represents a valuable strategy for unrevealing the epigenetic features of OTSCC. Thus, by this strategy we identified in saliva a group of novel tumor-specific methylation markers in the 6-gene promoter region (*ALDH1A2*, *A2BP1*, *ANK1*, *GFRA1*, *PDE4B*, and *TTYH1*), with a high potential value for early detection of OTSCC.

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CONCLUSIONES

En respuesta a los objetivos planteados, a continuación, se resumen las principales conclusiones extraídas de la investigación realizada en esta tesis doctoral:

1. Derivado del metanálisis centrado en la precisión de la saliva para el diagnóstico de tumores malignos no orales, los biomarcadores salivales muestran una "buena" capacidad diagnóstica para la detección de diferentes tumores alejados de la cavidad oral, incluidos el cáncer de mama, el cáncer de pulmón, el cáncer de páncreas, el cáncer de ovario, el cáncer gástrico y el cáncer de esófago. Sin embargo, el diseño metodológico y la heterogeneidad sustancial en los resultados podrían influir en la precisión de los biomarcadores salivales para el diagnóstico del cáncer. En resumen, estos hallazgos demuestran la creciente importancia de los biomarcadores salivales en el cáncer, y pueden ayudar a los investigadores a diseñar estudios de validación diagnóstica más rigurosos.

2. La caracterización del perfil de miRNAs en la saliva de pacientes con CRC es una herramienta valiosa para la identificación de marcadores tumorales. Siguiendo esta estrategia, identificamos un panel de 5 miRNAs (*miR-185-5p, miR-29a-3p, miR-29c-3p, miR-491-5p,* y *miR-766-3p*) con valor potencial para el diagnóstico de CRC, solo y/o en combinación con los niveles de CEA, y también para la discriminación de enfermedades benignas digestivas.

3. Derivado del metanálisis destinado a analizar la precisión de los miRNAs circulantes para el diagnóstico del OSCC determinamos que los miRNAs en saliva y sangre muestran una "buena" capacidad diagnóstica para discriminar pacientes con OSCC de individuos sanos. Sin embargo, la solidez de la evidencia en la precisión diagnóstica de los miRNAs en la saliva y la sangre podría

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verse debilitada por el diseño metodológico y la considerable heterogeneidad en los valores de diagnóstico descritos.

4. La concentración total de scfDNA tiene un valor diagnóstico limitado para la detección de cáncer oral, sin embargo, los índices de integridad del scfDNA (ALU115/60 y ALU247/60) mostraron potencial para discriminar pacientes con OCSCC de controles sanos.

5. El análisis de secuenciación de nueva generación dirigido en tumor y cfDNA con el panel TST170 permite la detección de variantes genéticas somáticas en pacientes con HNC avanzado. Además, el análisis de variantes genéticas derivadas del tumor mediante ddPCR, tanto en plasma como en saliva, representa una herramienta valiosa para la detección y seguimiento de biomarcadores predictivos en HNC.

6. Después de un metanálisis que cuestionó la precisión diagnóstica de la metilación del ADN en la saliva de pacientes con HNC llegamos a la conclusión de que la hipermetilación en la región promotora del gen podría potencialmente usarse para detectar HNC, especialmente cáncer oral u orofaríngeo. A pesar de ello, encontramos limitaciones en el diseño metodológico y heterogeneidad en los valores diagnósticos descritos en la literatura.

7. En base al metanálisis destinado a determinar el impacto de la metilación del ADN en la saliva sobre el riesgo de HNC, mostramos que la hipermetilación en la región promotora de genes relacionados con la biología del tumor se asocia con un riesgo 8,34 veces mayor del HNC. Además, el análisis combinado de la metilación de los genes *p16*, *MGMT*, *DAPK*, *TIMP3* y *RASSF1* en la saliva podría ser útil para predecir el riesgo de desarrollar HNC, principalmente de tumores orales y orofaríngeos. Sin embargo, se encontraron debilidades en el diseño metodológico, heterogeneidad y falta de ajuste para variables potencialmente confusoras.

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8. La caracterización del metiloma en tumor y biopsias líquidas representa una estrategia valiosa para descifrar las características epigenéticas del OTSCC. Así, mediante esta estrategia identificamos en saliva un grupo de marcadores de metilación específicos del tumor en la región promotora de 6 genes (*ALDH12*, *A2BP1*, *ANK1*, *GFRA1*, *PDE4B* y *TTYH1*), con alto valor potencial para la detección precoz del OTSCC.

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CONCLUSIÓNS

En resposta aos obxectivos plantexados, as conclusións principais extraídas da investigación realizada na presente tese doutoral resúmense a continuación:

1. Derivado da metanálise centrada na precisión da saliva para o diagnóstico de tumores malignos non-orais, os biomarcadores salivares mostran unha "boa" capacidade diagnóstica para a detección de diferentes tumores remotos á cavidade oral, incluíndo o cancro de mama, o cancro de pulmón, o cancro de páncreas, o cancro de ovario, o cancro gástrico, e o cancro de esófago. Non obstante, o deseño metodolóxico e a heteroxeneidade substancial nos resultados poderían influír na precisión dos biomarcadores salivares para o diagnóstico do cancro. En resumo, estes achados demostran a crecente importancia dos biomarcadores salivares no cancro e poden axudar aos investigadores no deseño de estudos de validación diagnóstica máis rigorosos.

2. A caracterización do perfil de miRNAs na saliva de pacientes con CRC constitúe unha ferramenta valiosa para a identificación de marcadores tumorais. Seguindo esta estratexia, identificamos un panel de 5 miRNAs (*miR-185-5p, miR-29a-3p, miR-29c-3p, miR-491-5p,* e *miR-766-3p*) con potencial valor para o diagnóstico de CRC, só e/ou en combinación cos niveis de CEA, e tamén para a discriminación de enfermidade benigna dixestiva.

3. Derivado da metanálise encamiñada a analizar a exactitude dos miRNAs circulantes para o diagnóstico de OSCC determinamos que os miRNAs en saliva e sangue mostran una "boa" capacidade diagnóstica para discriminar pacientes con OSCC de individuos sans. No obstante, a forza da evidencia na precisión diagnóstica dos miRNAs en saliva e sangue podería estar debilitada polo deseño

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metodolóxico e a considerábel heteroxeneidade nos valores de diagnóstico descritos.

4. A concentración total de scfDNA ten un limitado valor diagnóstico para a detección de cancro oral, non obstante, os índices de integridade de scfDNA (ALU115/60 e ALU247/60) mostraron potencial para discriminar pacientes con OCSCC de controis sans.

5. A análise de secuenciación de nova xeración dirixida en tumor e cfDNA co panel TST170 permite a detección de variantes xenéticas somáticas en pacientes con HNC avanzado. Ademais, a análise de variantes xenéticas derivadas do tumor por ddPCR, tanto no plasma como na saliva, representa unha ferramenta valiosa para a detección e a monitorización de biomarcadores preditivos no HNC.

6. Despois da metanálise que interrogou a precisión diagnóstica da metilación do ADN na saliva de pacientes con HNC, concluímos que a hipermetilación na rexión promotora do xen pode ser potencialmente usada para detectar HNC, especialmente cancro oral ou de orofarinxe. Malia a isto, atopamos limitacións no deseño metodolóxico e heteroxeneidade nos valores de diagnóstico descritos na literatura.

7. Baseado na metanálise orientada a determinar o impacto da metilación do ADN na saliva no risco de desenrolar HNC, demostramos que a hipermetilación na rexión promotora de xenes relacionados coa bioloxía do tumor está asociada con 8.34 veces máis risco de HNC. Ademais, a análise combinada da metilación dos xenes *p16, MGMT, DAPK, TIMP3,* e *RASSF1* na saliva podría ser útil para predicir o risco de desenrolar HNC, principalmente de tumores orais e de orofarinxe. Non obstante, atopáronse debilidades no deseño metodolóxico, heteroxeneidade, e falta de axuste para variables potencialmente confusas.

8. A caracterización do metiloma no tumor e nas biopsias líquidas representa unha estratexia valiosa para descifrar as características

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epixenéticas do OTSCC. Así, mediante esta estratexia identificamos en saliva un grupo de marcadores de metilación específicos do tumor na rexión promotora de 6 xenes (*ALDH12*, *A2BP1*, *ANK1*, *GFRA1*, *PDE4B*, e *TTYH1*), cun alto valor potencial para a detección temperá de OTSCC.

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SUPPLEMENTARY MATERIALS

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OBJECTIVE 1

SALIVARY BIOMARKERS FOR CANCER DIAGNOSIS: A META-ANALYSIS

Figure S1. Flow chart of studies selection in this meta-analysis.

Figure S2. Quality assessments of included studies by using the QUADAS-2 tool.

Figure S3. SROC curves for subgroup analysis based on the different class of biomarkers

Figure S4. SROC curves for subgroup analysis based on saliva fractions.

Figure S5. SROC curves for subgroup analysis based on type of saliva.

Figure S6. SROC curves for subgroup analysis based on sample size.

Figure S7. SROC curves for subgroup analysis based on type of controls. HC, healthy controls; BC, benign controls; NCCs, non-cancer controls.

Figure S8. Deeks' funnel plot asymmetry test for publication bias.

Table S1. Meta-regression of salivary biomarkers associated with saliva fraction, type of saliva, anatomic tumor location, sample size, class of biomarker and type of controls.

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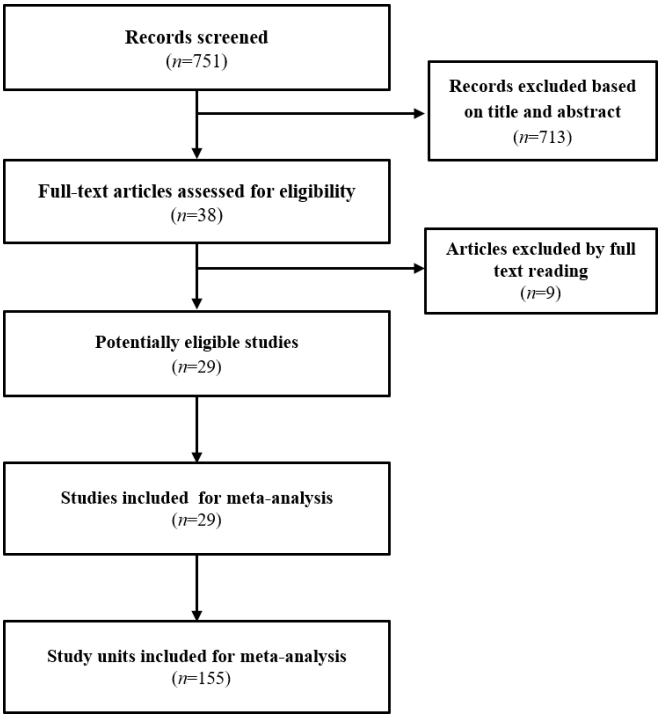

Figure S1. Flow chart of studies selection in this meta-analysis.

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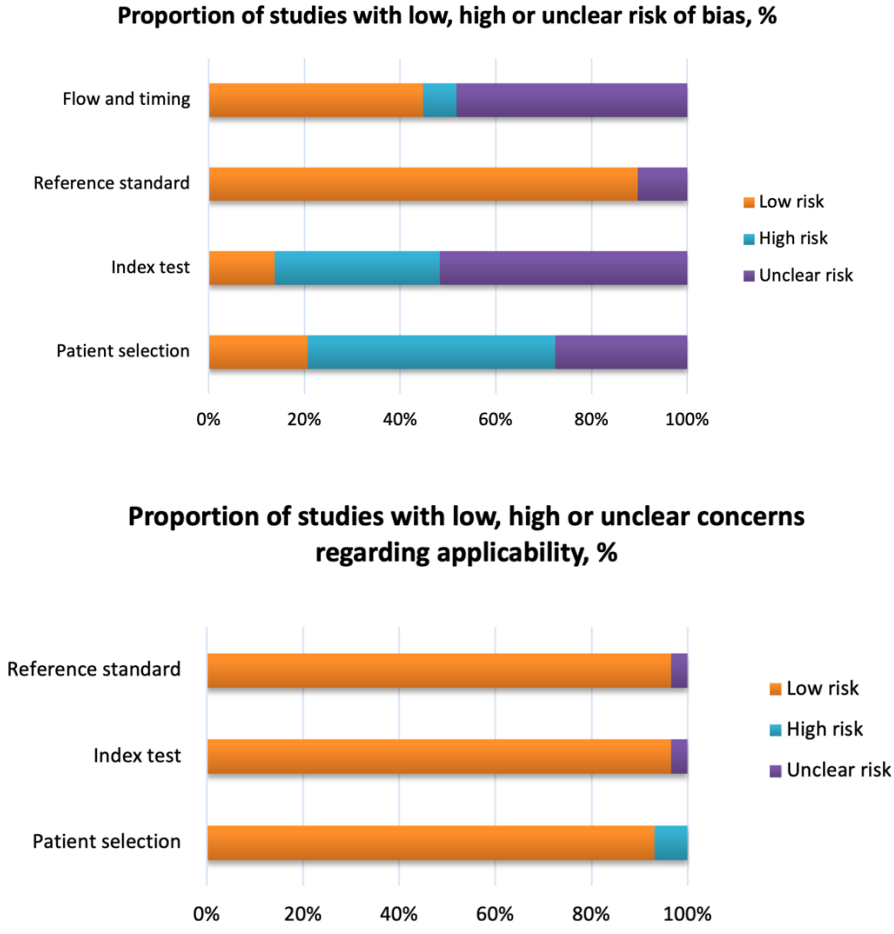

Figure S2. Quality assessments of included studies by using the QUADAS-2 tool.

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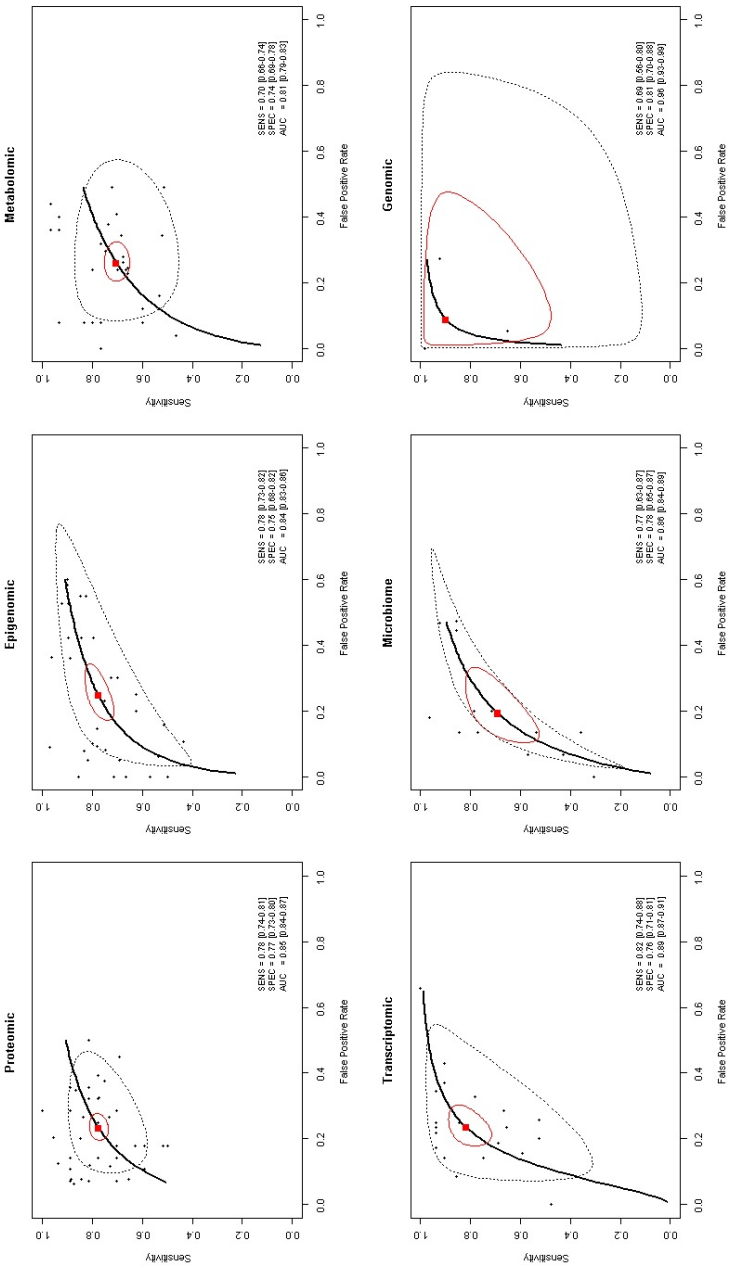

Figure S3. SROC curves for subgroup analysis based on the different class of biomarkers.

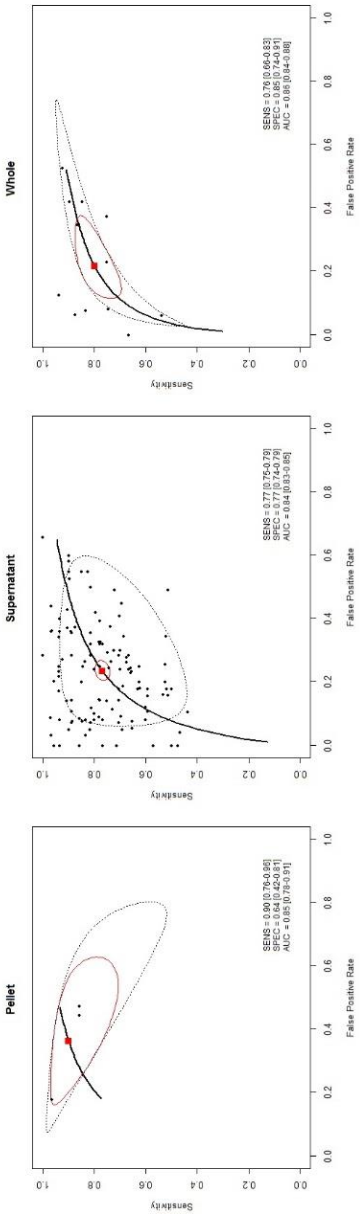

Figure S4. SROC curves for subgroup analysis based on saliva fractions.

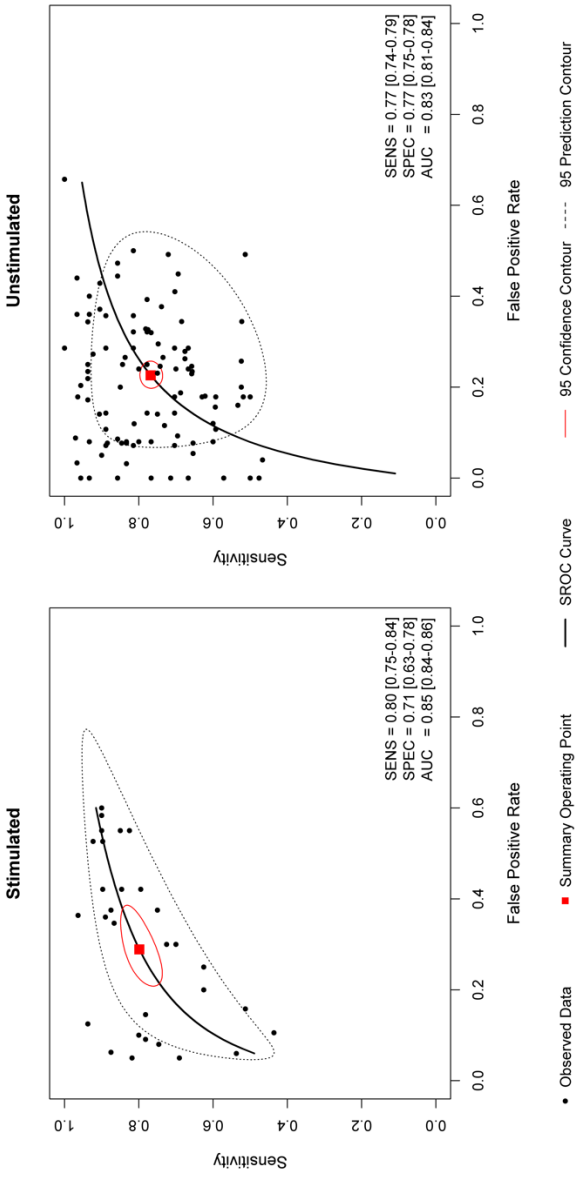

Figure S5. SROC curves for subgroup analysis based on type of saliva.

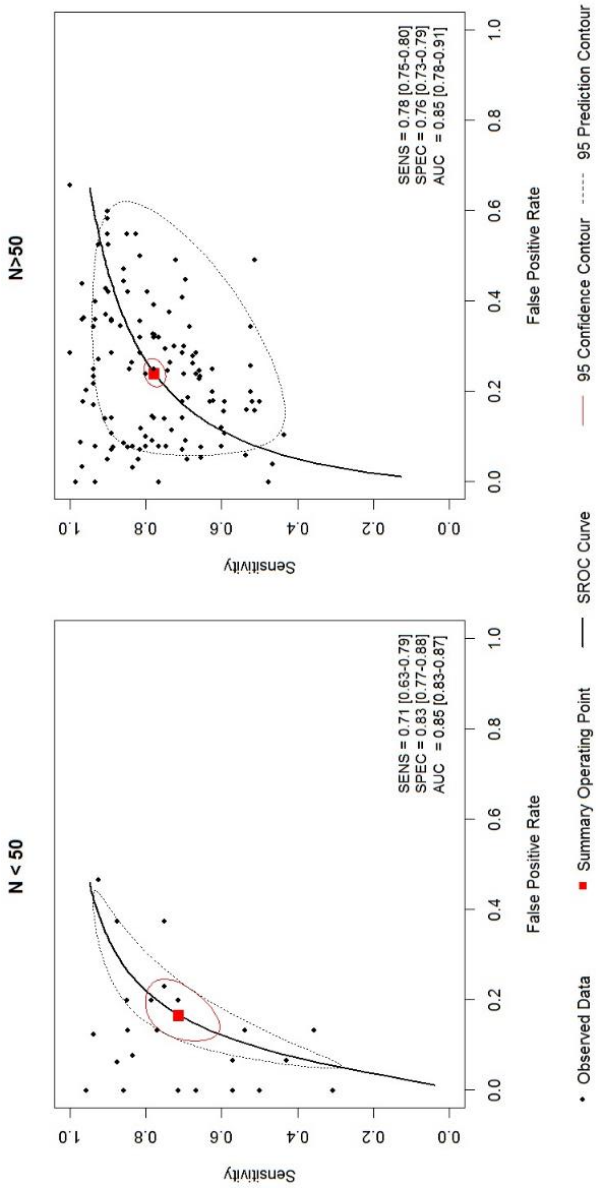

Figure S6. SROC curves for subgroup analysis based on sample size.

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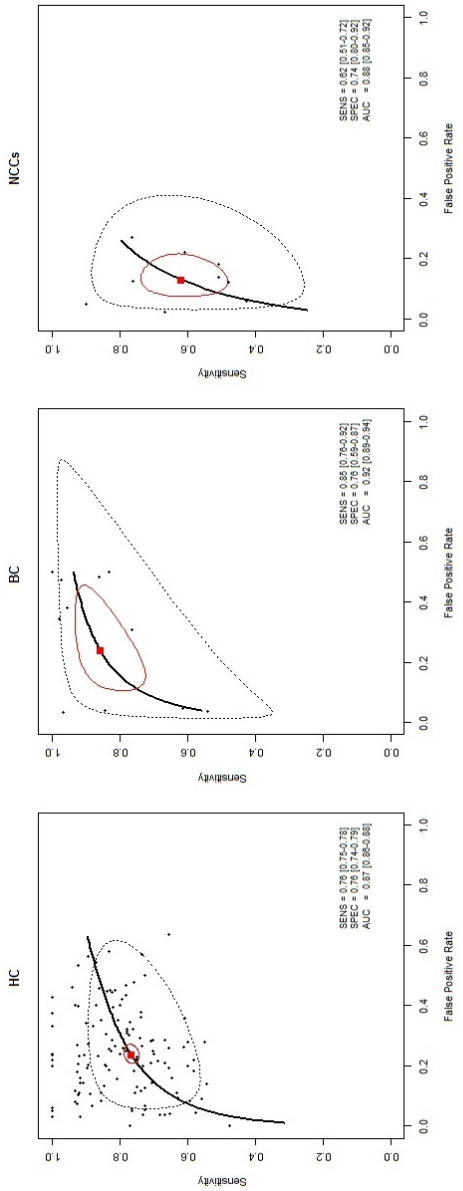

Figure S7. SROC curves for subgroup analysis based on type of controls. *Abbreviations*: HC, healthy controls; BC, benign controls; NCCs, non-cancer controls.

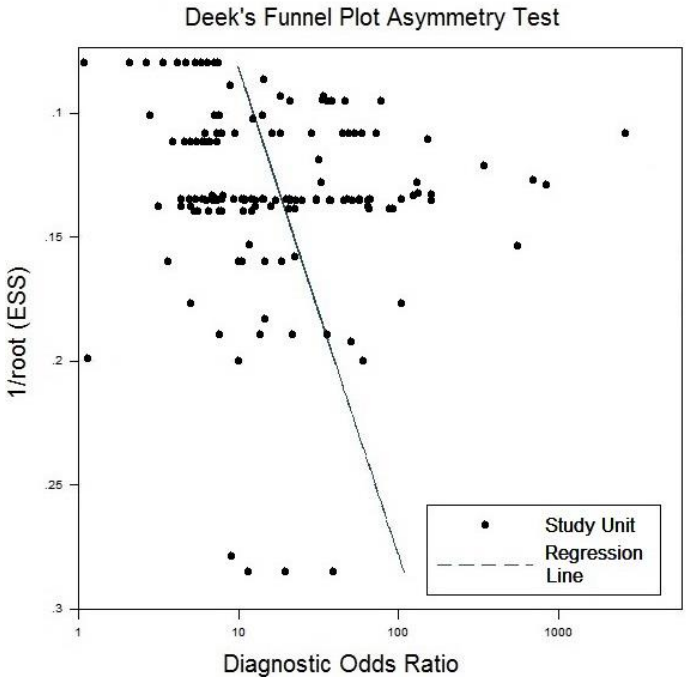

Figure S8. Deeks' funnel plot asymmetry test for publication bias.

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Table S1. Meta-regression of salivary biomarkers associated with saliva fraction, type of saliva, anatomic tumor location, sample size, class of biomarker and type of controls.

Var	Coeff.	*P*-value	RDOR	(95% CI)
Cte.	3.523	0.002	-	-
S	-0.040	0.594	-	-
Saliva fraction	0.292	0.429	1.34	(0.65 to 2.78)
Type of saliva	-0.304	0.244	0.74	(0.44 to 1.23)
Anatomic tumor location	-0.189	0.002	0.83	(0.73 to 0.93)
Sample size (n>50)	-0.545	0.301	0.58	(0.21 to 1.64)
Class of biomarker	-0.147	0.063	0.86	(0.74 to 1.01)
Type of control	0.114	0.484	1.12	(0.81 to 1.54)

No. studies = 143
Filter OFF
Add 1/2 to all cells of the studies with zero

Abbreviations: Coeff., coefficient; RDOR, relative diagnostic odds ratios; CI, confidence interval

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OBJECTIVE 2

A NOVEL SALIVA-BASED MIRNA SIGNATURE FOR COLORECTAL CANCER DIAGNOSIS

1. SUPPLEMENTARY METHODS:

1.1 Saliva collection and processing
1.2 Saliva RNA extraction
1.3 Salivary miRNA discovery profiling with TaqMan Low Density Arrays
1.4 MiRNA expression analysis using RT-qPCR
1.5 Selection of internal controls for quantification of salivary miRNAs

Table S1. Clinicopathological characteristics of study participants.

Table S2. Candidate reference genes based on expression stability calculated by NormFinder and Coefficient of Variation score.

Table S3. Discriminatory power of salivary miRNAs for the detection of CRC.

Table S4. Association of salivary miRNAs expression levels with clinical variables.

Table S5. Univariate Cox regression analysis for clinicopathological parameters and salivary miRNAs in stage IV CRC patients included in the study.

Table S6. Multivariate Cox regression analysis for clinicopathological parameters and salivary miRNAs in stage IV CRC patients included in the study.

Table S7. Significantly enriched signaling pathways in our five miRNAs salivary panel ($P < 0.05$).

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Figure S1. Study design for CRC salivary miRNAs discovery and validation. *Abbreviations:* HC, healthy controls; A, adenomas; CRC, colorectal cancer.

Figure S2. Cq values of the selected reference gene. No significant difference was found between the groups ($P > 0.05$, Mann Whitney U test). *Abbreviations:* HC, healthy controls; CRC, colorectal cancer.

Figure S3. Venn diagram analysis showing overlapping and non-overlapping of differentially expressed miRNAs. (A) HC and CRC patients. (B) HC and non-metastatic CRC patients. (C) HC and metastatic CRC patients. *Abbreviations:* HC, healthy controls; CRC, colorectal cancer.

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1. SUPPLEMENTARY METHODS:

1.1. Saliva collection and processing

Saliva samples were collected and processed as described previously Majem et al. (Majem et al. 2017). Unstimulated whole saliva samples were collected from the participants between 9 and 10 am, before any therapeutic procedures. Subjects refrained from eating, drinking and oral hygiene procedures for at least 1 hour before the collection. Subjects rinsed their mouth with distilled water to minimize contamination of the salivary samples. Five min after the oral rinsing, the participant starts to spit into a 50-mL Falcon tube kept on ice. As a minimum, five milliliters of saliva were collected from each participant. Immediately after collection, salivary samples were centrifuged at 2,600 x g for 15 min at 4°C to remove cellular components. Saliva supernatant was then separated from the pellet and 1 µL per milliliter of supernatant saliva of RNase inhibitor (SUPERase-In, AM2694, Ambion, Life Technologies) was added. All samples were aliquoted in 1,200 µL and stored at –80°C prior to assay. Salivary samples were randomized for RNA extractions, RT reactions and RTqPCR analyses in all cases.

1.2. Saliva RNA extraction

At first, frozen saliva samples were thawed thoroughly on ice and centrifuged 10,000 x g at 4°C for 5 min to eliminate cell debris saliva. Then, cell-free saliva was separated into 2 eppendorf of 250 µL-saliva which were lysed in 750 µL of Trizol LS Reagent (10296-028, Ambion, Life Technologies). Thereafter, 200 µL chloroform was added to the denatured saliva and mixed by vortex for 30 s, followed by incubation for 5 min at room temperature. The addition of chloroform causes phase separation where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Total RNA was eluted from the spin column membrane in 60 µL pre-heated RNA-free water (50ºC) and DNase treatment (DNase, Rocher) was used to remove contaminating DNA during RNA extraction. After

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RNA precipitation, the final RNA was suspended in 10 µL pre-heated RNA-free water (50ºC), then incubated for 5 min at 55ºC and RNA samples were stored at -80ºC for further analyses.

1.3. Salivary miRNA discovery profiling with TaqMan Low Density Arrays

This miRNA microarray assay was performed using 3 µL (1-350 ng) of total RNA from each sample, according to the manufacture's protocol. From each sample, 3 µL (1-350 ng) of total RNA were reverse-transcribed using MegaplexTM RT primer pool (pool A v2.1 and pool B v3.0, Applied Biosystems) and TaqMan® MicroRNA reverse transcription kit (Applied Biosystems). In order to increase the sensitivity, cDNA was preamplified using MegaplexTM PreAmp primer pool (pool A v2.1 and pool B v3.0, Applied Biosystems) and TaqMan® Preamp Master Mix (Applied Biosystems). Then, samples were mixed with TaqMan Universal PCR Master Mix, No AmpErase® UNG, 2X (Applied Biosystems), and applied to the TLDA cards. The 8 ports on each array card were loaded with 100 µL reaction followed by brief centrifugation and sealing. RT-qPCR was performed at 7900HT Fast Thermocycler System. After real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) raw data were exported to the ExpressionSuite software (v.1.1, Applied Biosystem) for performing global normalization and quality control. Threshold and baseline were automatically calculated for each assay, and a global normalization was performed as recommended for large scale miRNA expression profiling (Mestdagh et al. 2009). MiRNAs with significantly different levels ($P < 0.05$) between CRC and healthy controls samples, a 1.5-fold change (up or down) and positive expression in at least 70% of tumor samples was considered as candidates to be confirmed in a larger cohort of samples.

1.4. MiRNA expression analysis using RT-qPCR

A total volume of 3 µL of each sample was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied

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Biosystems, Thermo Fischer, Baltics, UAB) in a total volume of 15 µL with the following thermal-cycling conditions: 16 ºC for 30 min, 42ºC for 30 min, 85 ºC for 5 min and then storing at 4 ºC. Then, 2.5 µL of cDNA was pre-amplified using TaqMan® PreAmp Master Mix (Applied Biosystems, Thermo Fischer, Foster City, CA, USA) with the following thermal-cycling conditions: 95ºC for 10 min, 55ºC for 2 min, 72ºC for 2 min, 12 cycles at 95 ºC for 15 s and 60 ºC for 4 min, then 99.9ºC for 10 min. Then samples were stored at -20ºC. RT-qPCR analyses were conducted in 96-well plates (Applied Biosystems) in a reaction volume of 15 µL using TaqMan® Gene Expression Master Mix (Applied Biosystems, Thermo Fischer). All RT-qPCR reactions were run in the StepOne Plus system (Applied Biosystems) with the following cycling conditions: 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. All reactions were performed in duplicates for each sample, and a no-template control was used as a negative control. Cycle quantification (Cq) values were calculated using StepOne Software v2.3 (Applied Biosystems) for target miRNAs and endogenous normalizers.

1.5. Selection of internal controls for quantification of salivary miRNAs

For validating the selected miRNAs, miRNA profiling data of the discovery cohort was used to choose an appropriate endogenous control based on the following criteria: (a) Relative quantification close to 1:1 (where the healthy group is the reference biological group); (b) miRNA detected in more than 90% of all replicates of all samples; (c) amplification score above 1.24 and (d) no differential expression between cancer and control samples ($P > 0.05$). As *U6* small nuclear RNA, which is normally used as a reference gene for miRNAs expression analyses, was not uniformly expressed in samples after microarray analysis, 2 miRNAs (*miR-1274B* and *miR-193b-3p*) were selected as candidate reference genes because there were homogeneously expressed between groups in terms of Cq values between groups (*miR-1274B* ($P = 0.279$) and *miR-193b-3p* ($P = 0.584$) and their levels appear to be stable across the whole cohort. The stability

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of selected reference genes was calculated using two different algorithms, NormFinder for R (version 5, 2015-01-05; https://moma.dk/normfinder-software) and Coefficient of Variation score. *MiR-193b-3p* expression levels showed the lowest intragroup and intergroup variability (Supplementary Table S2). In order to further validate the stability of *miR-193b-3p* and *miR-1274B*, we analyzed the expression patterns in 45 saliva samples (30 CRC and 15 HC). Using the Cq values of each validated reference gene, there was no evidence for differential expression of *miR-193b-3p* whereas salivary expression levels of *miR-1274B* were significantly different between CRC patients and healthy individuals (P = 0.0276). Therefore, *miR-193b-3p* was chosen as an internal control to perform all the RT-qPCR analyses due to its high stability among samples (Figure S2).

References

Majem B, Li F, Sun J, Wong DT. RNA Sequencing analysis of salivary extracellular RNA. *Methods Mol Biol* **2017**;*1537*:17−36.

Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* ***2009***;*10*:R64.

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Table S1. Clinicopathological characteristics of study participants.

	Discovery Phase		Validation Phase		
	HC (n = 10)	CRC (n = 14)	HC (n = 37)	CRC (n = 51)	A (n =19)
Age (years)					
Mean ± SD	71.90 ± 12.39	67.21 ± 11.27	62.81 ± 12.19	66.27 ± 11.16	63.95 ± 8.13
Median	76.50	65.50	62	66	60
Range	53-87	50-85	38-86	46-85	52-80
Gender					
Male	3 (30%)	10 (71.4%)	19 (51.4%)	33 (64.7%)	14 (73.7%)
Female	7 (70%)	4 (28.6%)	18 (48.6%)	18 (35.3%)	5 (26.3%)
TNM status					
I	–	2 (14.3%)	–	6 (11.8%)	–
II	–	2 (14.3%)	–	3 (5.9%)	–
III	–	3 (21.4%)	–	21 (41.2%)	–
IV	–	7 (50%)	–	21 (41.2%)	–
Tumor (T)					
Tx	–	3 (21.4%)	–	5 (9.8%)	–
T1	–	–	–	–	–
T2	–	2 (14.3%)	–	6 (11.8%)	–
T3	–	5 (35.7%)	–	25 (49.0%)	–
T4	–	4 (28.6%)	–	15 (29.4%)	–
Regional lymph node status (N)					
N0	–	4 (28.6%)	–	11 (21.6%)	–
N1	–	5 (35.7%)	–	23 (45.1%)	–
N2	–	2 (14.3%)	–	12 (23.5%)	–
Nx	–	3 (21.4%)	–	5 (9.8%)	–
Distant metastasis status (M)					
M0	–	7 (50%)	–	30 (58.8%)	–
M1	–	7 (50%)	–	21 (41.2%)	–
Tumor location					
Cecum	–		–	2 (3.9%)	–
Colon	–	2 (14.3%)	–	11 (21.6%)	14 (73.7%)
Sigma	–	6 (42.9%)	–	15 (29.4%)	3 (15.8%)
Rectum-sigma	–	–	–	3 (5.9%)	-
Rectum	–	6 (42.9%)	–	20 (39.2%)	2 (10.5%)
Histological types					
Adenocarcinoma	–	14 (100%)	–	48 (94.1%)	-
Mucinous adenocarcinoma	–	–	–	1 (2%)	-
Signet ring cell	–	–	–	2 (3.9%)	-
Tubular adenoma	–	–	–	–	12 (63.2%)
Tubulovillous adenoma	–	–	–	–	7 (36.8%)
Unknown	–	–	–	–	–
Grading					
Low grade	–	13 (92.9%)	–	39 (76.5%)	15 (78.9%)
High grade	–	1 (7.1%)	–	4 (7.8%)	4 (12.5%)
Unknown	–	–	–	8 (15.7%)	–
Smoking status					
Non-smoker	7 (70%)	5 (35.7%)	20 (54.1%)	22 (43.1%)	6 (31.6%)
Ex-smoker	2 (20%)	5 (35.7%)	13 (35.1%)	21 (41.2%)	7 (36.8%)
Smoker	1 (10%)	4 (28.6%)	4 (10.8%)	8 (15.7%)	6 (31.6%)
Alcohol intake					
Never	7 (70%)	11 (78.6%)	22 (59.5%)	31 (60.8%)	10 (52.6%)
Current	3 (30%)	3 (21.4%)	15 (40.5%)	20 (39.2%)	9 (47.4%)
BMI					
Normal	–	–	–	4 (7.8%)	–
Pre-obesity	–	9 (64.3%)	–	27 (52.9%)	–
Obesity	–	3 (21.4%)	–	14 (27.5%)	–
Unknown	–	2 (14.3%)	–	6 (11.8%)	–
CEA (ng/ml)					
Mean ± SD	–	24.59 ± 36.65	–	146.37 ± 530.70	–
Median	–	6.05	–	4.00	–
Range	–	1.20-131.90	–	0.40-3330.90	–

Abbreviations: HC, healthy controls; A, adenomas; CRC, colorectal cancer; BMI, body mass index; CEA, carcinoembryonic antigen; SD, standard deviation.

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Table S2. Candidate reference genes based on expression stability calculated by NormFinder and Coefficient of Variation score.

	RQ	Detection*	Cq mean (tumor/control)	Cq median (tumor/control)	*P* Value	NormFinder stability value	CV	SD
miR-193b-3p	1:1.017	100%	27/27.71	26.71/27.49	0.5387	0.05	0.11	3.20
miR-1274B	1:1.046	95.83%	24.01/25.23	23.86/25.65	0.1450	0.06	0.14	3.54

*Percentage of samples where the miRNA was detected. *Abbreviations:* RQ, relative quantification; CV, coefficient of variation; SD, standard deviation.

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Table S3. Discriminatory power of salivary miRNAs for the detection of CRC.

CRC vs. HC	AUC	95% CI	S (%)	95% CI	Sp (%)	95% CI	cut-off point	*P* Value
miR-186-5p	0.6550	0.5416 to 0.7684	64.71	50.07 to 77.57	54.05	36.92 to 70.51	1.096	0.013
miR-29a-3p	0.6306	0.5139 to 0.7474	64.71	50.07 to 77.57	56.76	39.49 to 72.90	0.9864	0.037
miR-29c-3p	0.6592	0.5452 to 0.7733	64.71	50.07 to 77.57	62.16	44.76 to 77.54	1.184	0.011
miR-766-3p	0.6308	0.5132 to 0.7484	64	49.19 to 77.08	62.16	44.76 to 77.54	1.477	0.037
miR-491-5p	0.6324	0.5150 to 0.7497	70.59	56.17 to 82.51	50	32.92 to 67.08	1.126	0.036
5-miRNA panel	0.7539	0.6524 to 0.8554	72	57.51 to 83.77	66.67	49.03 to 81.44	0.5132	<0.001

Abbreviations: CRC, colorectal cancer; HC, healthy controls; AUC, area under the ROC curve; Se, sensitivity; Sp, specificity; CI, confidence interval.

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Table S4. Association of salivary miRNAs expression levels with clinical variables.

Characteristics		miR-186-5p		miR-29a-3p		miR-29c-3p		miR-491-5p		miR-766-3p	
	Total (%)	Mean ± SD	*P* Value	Mean ± SD	*P* Value	Mean ± SD	*P* Value	Mean ± SD	*P* Value	Mean ± SD	*P* Value
Gender											
Male	33 (64.7)	3.81 ± 3.04	**0.016**	3.36 ± 3.05	**0.030**	3.12 ± 2.62	**0.026**	2.94 ± 2.23	0.056	3.19 ± 2.58	0.055
Female	18 (35.3)	1.98 ± 2.55		2.14 ± 3.07		2.24 ± 3.46		2.48 ± 4.16		2.12 ± 2.52	
Age (years)											
≥ 65	28 (54.9)	2.75 ± 2.88	0.167	2.29 ± 2.38	0.140	2.20 ± 2.06	0.191	2.03 ± 1.81	0.072	2.39 ± 2.59	0.085
< 65	23 (45)	3.66 ± 3.09		3.71 ± 3.67		3.55 ± 3.66		3.69 ± 3.88		3.29 ± 2.56	
Metastasis											
Negative	30 (58.8)	3.24 ± 3.01	0.688	3.02 ± 3.09	0.688	2.90 ± 3.05	0.730	3.05 ± 3.54	0.646	3.05 ± 2.94	0.716
Positive	21 (41.2)	3.05 ± 3.02		2.80 ± 3.14		2.68 ± 2.84		2.40 ± 2.09		2.46 ± 2.02	
Nodes											
Negative	11 (21.6)	2.99 ± 2.91	0.652	3.10 ± 3.51	0.652	3.23 ± 4.10	0.690	2.71 ± 2.72	0.728	2.71 ± 2.37	0.990
Positive	35 (68.6)	3.37 ± 3.17		3.06 ± 3.13		2.87 ± 2.70		2.99 ± 3.30		2.93 ± 2.83	
Grading											
Low grade	39 (76.5)	3.46 ± 3.04	0.702	3.19 ± 3.10	0.531	3.06 ± 2.99	0.531	3.00 ± 3.20	0.856	2.96 ± 2.69	0.605
High grade	4 (7.8)	2.48 ± 1.92		1.77 ± 1.60		1.76 ± 1.35		2.52 ± 2.18		2.86 ± 0.98	
TNM stage											
I	6 (11.8)	2.38 ± 2.06	0.839	2.82 ± 3.76	0.889	3.48 ± 5.21	0.939	2.25 ± 2.26	0.739	2.35 ± 1.98	0.839
II	3 (5.9)	3.88 ± 4.33		3.60 ± 4.14		2.65 ± 2.78		4.09 ± 4.04		4.05 ± 3.36	
III	21 (41.2)	3.40 ± 3.15		3.00 ± 2.92		2.77 ± 2.41		3.13 ± 3.86		3.12 ± 3.20	
IV	21 (41.2)	3.05 ± 3.02		2.80 ± 3.14		2.68 ± 2.84		2.40 ± 2.09		2.46 ± 2.02	
Early (I-II)	9 (17.6)	2.88 ± 2.86	0.780	3.08 ± 3.64	0.799	3.20 ± 4.37	0.743	2.86 ± 2.85	0.971	2.92 ± 2.45	0.728
Late (III-IV)	42 (82.4)	3.22 ± 3.05		2.90 ± 3.00		2.73 ± 2.60		2.76 ± 3.09		2.78 ± 2.65	
Tumor location											
Left	40 (78.4)	3.16 ± 2.90	0.732	3.01 ± 3.08	0.602	2.87 ± 2.96	0.585	2.86 ± 3.18	0.713	2.86 ± 2.63	0.979
Right	9 (17.6)	3.40 ± 3.66		2.79 ± 3.47		2.77 ± 3.23		2.43 ± 2.41		2.93 ± 2.71	
CEA (ng/mL)											
≥5	21 (44.7)	2.53 ± 2.84	0.441	2.38 ± 2.37	0.983	2.13 ± 1.98	0.748	2.83 ± 3.85	0.847	2.45 ± 2.54	0.559
<5	26 (55.3)	3.39 ± 3.05		3.16 ± 3.56		3.20 ± 3.57		2.78 ± 2.48		2.75 ± 2.32	
BMI											
Normal	4 (7.8)	5.39 ± 4.38	0.447	5.09 ± 4.21	0.464	4.50 ± 3.48	0.496	6.80 ± 7.66	0.346	4.21 ± 4.66	0.756
Pre-obesity	27 (52.9)	3.27 ± 3.05		2.98 ± 3.07		2.95 ± 3.20		2.66 ± 2.26		2.91 ± 2.64	
Obesity	14 (27.5)	2.36 ± 2.38		2.10 ± 2.73		2.12 ± 2.47		2.10 ± 2.02		2.37 ± 2.17	
Alcohol intake											
Never	31 (60.8)	2.70 ± 2.93	0.086	2.70 ± 3.42	0.067	2.64 ± 3.36	0.086	2.82 ± 3.61	0.263	2.69 ± 2.96	0.153
Current	20 (39.2)	3.87 ± 3.00		3.28 ± 2.51		3.07 ± 2.20		2.72 ± 1.83		3.00 ± 1.888	
Smoking status											
Non-smoker	22 (43.1)	2.24 ± 2.39	0.052	1.80 ± 1.88	0.078	1.78 ± 1.73	0.082	1.69 ± 1.43	0.058	2.08 ± 1.70	0.116
Ex-smoker	21 (41.2)	4.54 ± 3.45		4.18 ± 3.66		3.73 ± 3.08		4.13 ± 4.07		3.91 ± 3.30	
Smoker	8 (15.7)	2.08 ± 1.71		2.75 ± 3.21		3.23 ± 4.43		2.23 ± 1.40		1.80 ± 1.05	

Abbreviations: BMI, body mass index; SD, standard deviation; CEA, carcinoembryonic antigen.

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Table S5. Univariate Cox regression analysis for clinicopathological parameters and salivary miRNAs in stage IV CRC patients included in the study.

	PFS		OS	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Gender (male vs. female)	0.65 (0.20−2.07)	0.46	0.79 (0.20−3.05)	0.73
Age (<65 vs. ≥65 years)	1.45 (0.52−4.05)	0.47	1.12 (0.35−3.60)	0.84
Lymph node metastasis (no vs. yes)	0.48 (0.06−3.77)	0.47	0.03 (0.01−52.64)	0.14
Liver metastasis (no vs. yes)	0.74 (0.16−3.37)	0.72	1.19 (0.15−9.49)	0.86
CEA serum levels (<5 vs. ≥5 ng/mL)	4.44 (1.3−14.54)	**0.008**	5.22 (1.13−24.1)	**0.018**
5miRNAs-panel model (good vs. bad prognosis)	4.73 (1.31−17.01)	**0.009**	4.13 (0.89−19.15)	**0.049**

HR represent the risk of the bad prognosis group vs. the good prognosis group. *Abbreviations*: PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

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Table S6. Multivariate Cox regression analysis for clinicopathological parameters and salivary miRNAs in stage IV CRC patients included in the study.

	PFS	
	HR (95% CI)	*P* Value
CEA serum levels (<5 vs. ≥5 ng/mL)	2.61 (0.39−17.2)	0.31
5miRNAs-panel model (good vs. bad prognosis)	4.73 (1.3−17.01)	**0.017**
	OS	
	HR (95% CI)	*P* Value
CEA serum levels (<5 vs. ≥5 ng/mL)	5.22 (1.13−24.09)	**0.034**
5miRNAs-panel model (good vs. bad prognosis)	1.41 (0.15−12.77)	0.75

HR represent the risk of the bad prognosis group vs. the good prognosis group. *Abbreviations:* PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

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Table S7. Significantly enriched signaling pathways in our five miRNAs salivary panel ($P < 0.05$).

KEGG pathway maps	Enriched pathways (nº of miRNAs implicated)	Adjusted *P* Values
Environmental Information Processing		
Signaling molecules and interaction	ECM-receptor interaction (2)	<1.00E-325
Signal transduction	PI3K-Akt signaling pathway (2)	0.00175845
	Hippo signaling pathway (2)	0.01932169
	FoxO signaling pathway (2)	0.03226584
Metabolism		
Lipid metabolism	Fatty acid biosynthesis (3)	<1.00E-325
	Fatty acid metabolism (3)	<1.00E-325
	Steroid biosynthesis (4)	1.92E-08
	Fatty acid elongation (3)	0.00012716
Amino acid metabolism	Lysine degradation (5)	<1.00E-325
Cellular Processes		
Cell growth and death	p53 signaling pathway (4)	5.17E-09
	Cell cycle (2)	0.00013274
Cellular community - eukaryotes	Focal adhesion (3)	3.02E-05
	Adherens junction (2)	0.00497083
Human Diseases		
Cancer: overview	Viral carcinogenesis (4)	<1.00E-325
	Proteoglycans in cancer (3)	0.00227843
	Pathways in cancer (2)	0.04253708
Neurodegenerative disease	Prion diseases (1)	2.22E-16
	Huntington disease (2)	0.00876501
Infectious disease: parasitic	Amoebiasis (2)	2.23E-05
Cancer: specific types	Colorectal cancer (3)	0.0001118
	Chronic myeloid leukemia (3)	0.00426377
	Small cell lung cancer (2)	0.00475059
	Endometrial cancer (2)	0.02146469
	Glioma (1)	0.03492608
	Renal cell carcinoma (2)	0.04238292
Infectious disease: bacterial	Bacterial invasion of epithelial cells (2)	0.00686747
Genetic Information Processing		
Folding, sorting and degradation	Protein processing in endoplasmic reticulum (2)	6.77E-05
Organismal Systems		
Digestive system	Protein digestion and absorption (2)	0.04884862

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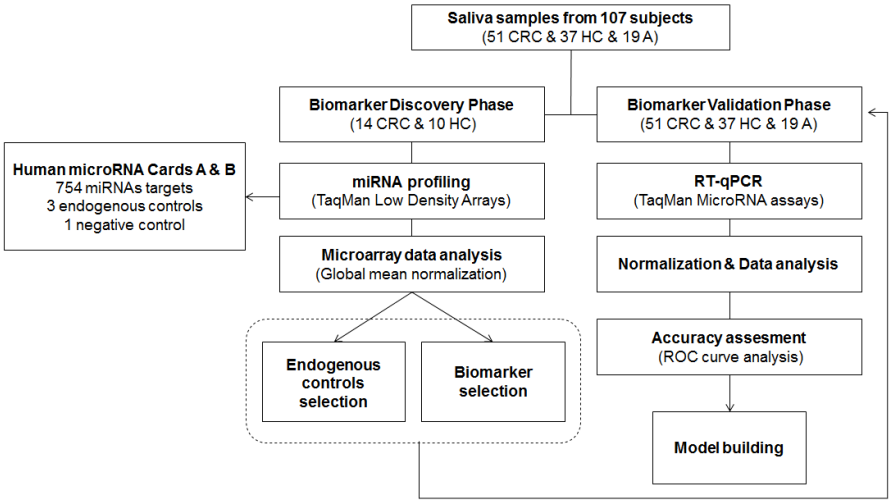

Figure S1. Study design for CRC salivary miRNAs discovery and validation.
Abbreviations: **HC, healthy controls; A, adenomas; CRC, colorectal cancer.**

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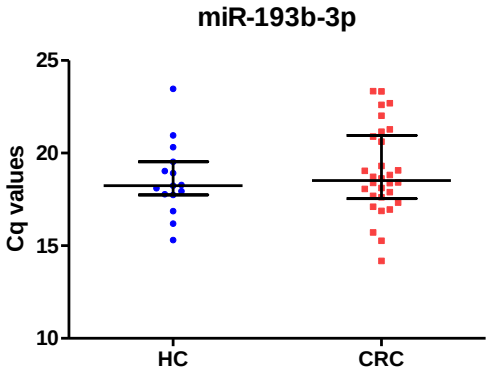

Figure S2. Cq values of the selected reference gene. No significant difference was found between the groups (*P* > 0.05, Mann Whitney U test). *Abbreviations:* HC, healthy controls; CRC, colorectal cancer.

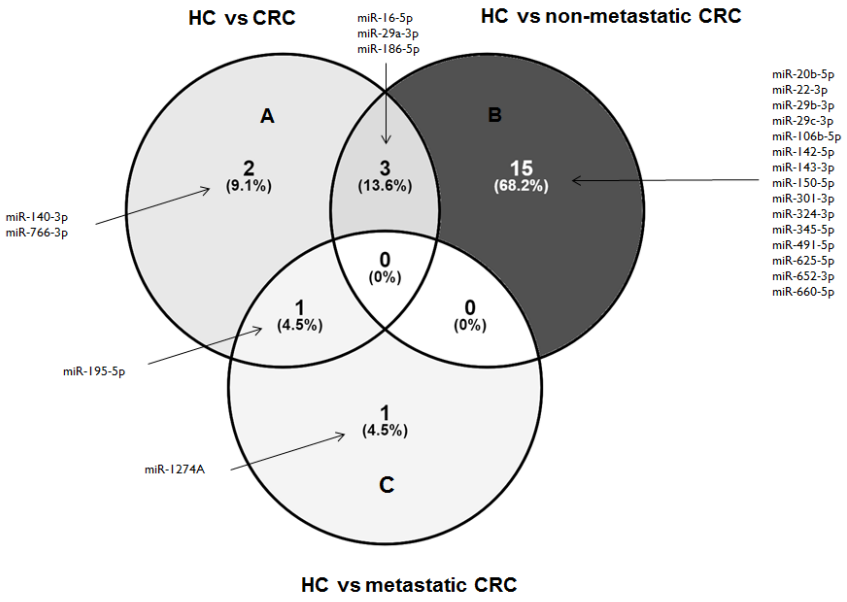

Figure S3. Venn diagram analysis showing overlapping and non-overlapping of differentially expressed miRNAs. (A) HC and CRC patients. (B) HC and non-metastatic CRC patients. (C) HC and metastatic CRC patients. *Abbreviations:* HC, healthy controls; CRC, colorectal cancer.

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OBJECTIVE 3.1

MIRNAS IN LIQUID BIOPSY FOR ORAL SQUAMOUS CELL CARCINOMA DIAGNOSIS: SYSTEMATIC REVIEW AND META-ANALYSIS

Figure S1. Quality assessment of the included studies according to Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) criteria. (A) Proportion of studies with low, high, or unclear risk of bias. (B) Proportion of studies with low, high, or unclear concerns regarding applicability.

Figure S2. SROC space for the assessment of the threshold effect of miRNAs in liquid biopsy. *Abbreviations:* miRNA, microRNAs; SROC, summary receiver operator characteristic.

Figure S3. Deeks' funnel plot asymmetry test for the assessment of potential bias of included studies.

Table S1. Results of meta-regression analysis

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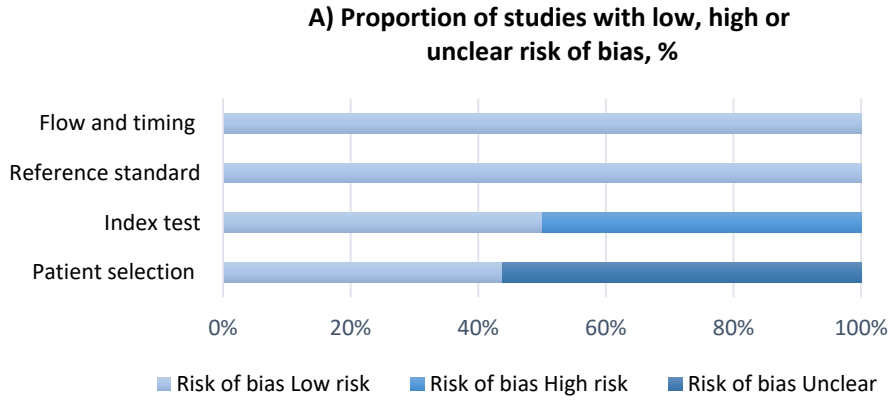

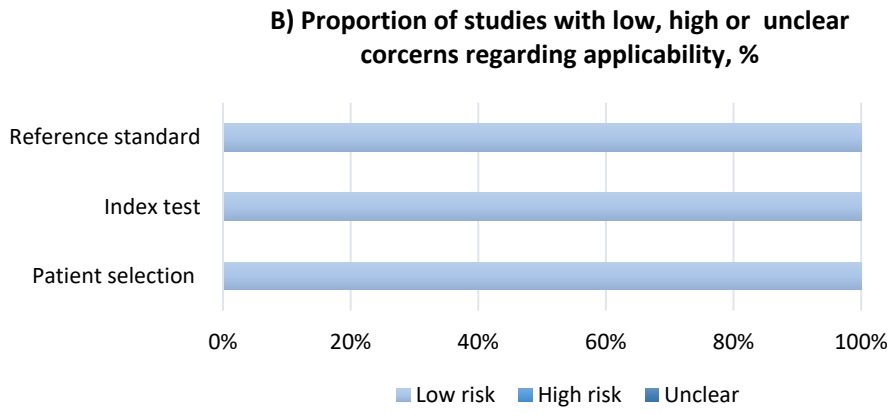

Figure S1. Quality assessment of the included studies according to Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) criteria. (A) Proportion of studies with low, high, or unclear risk of bias. (B) Proportion of studies with low, high, or unclear concerns regarding applicability.

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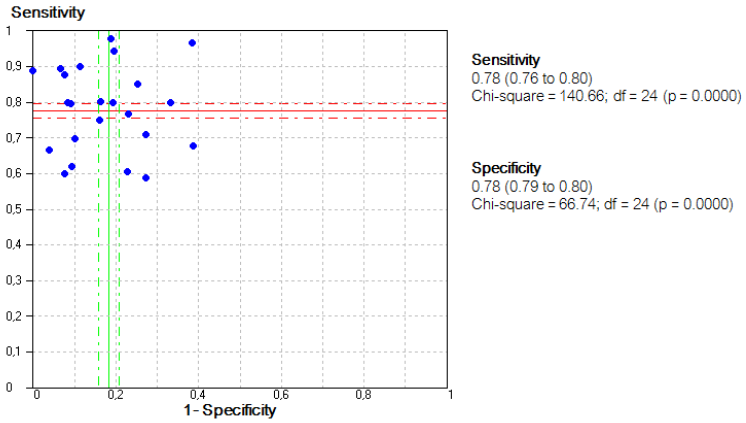

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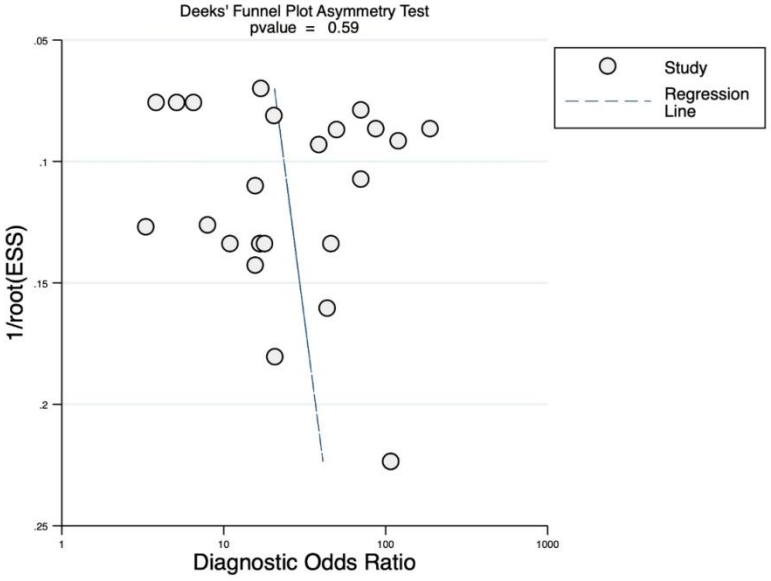

Figure S3. Deeks' funnel plot asymmetry test for the assessment of potential bias of included studies.

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Table S1. Results of meta-regression analysis.

Var	Coeff.	*p*-value	RDOR	[95%Cl]
Cte.	2.895	0.0052	-	-
Ethnicity	0.163	0.8694	1.18	(0.15;9.11)
Sample size	0.166	0.8146	1.18	(0.27;5.09)
MiRNA expression	-0.434	0.5404	0.65	(0.15;2.77)
MiRNA profiling	-0.132	0.8564	0.88	(0.19;3.95)

No. studies = 25
Filter OFF
Add ½ to all cells of the studies with zero
Ethnicity: Asian vs. others; sample size: n < 100 vs. n ≥ 100; miRNA expression: upregulated vs. downregulated; miRNA profiling: single vs. combined.

Abbreviations: miRNA, microRNAs; CI, confidence interval; Coeff., coefficient; Std. Err., standard error; RDOR, relative diagnostic odds ratios.

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OBJECTIVE 3.2

SOMATIC MUTATIONS IN TUMOR AND PLASMA OF LOCOREGIONAL RECURRENT AND/OR METASTATIC NECK CANCER USING A NEXT-GENERATION SEQUENCING PANEL

Table S1. Clinicopathological characteristics.

Table S2. List of genes analyzed in DNA by the TST170 panel.

Table S3. Somatic mutations detected by NGS.

Table S4. Absolute and relative quantification of mutant and wild-type DNA in tumor, plasma, and saliva samples by ddPCR.

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Table S1. Clinicopathological characteristics.

	Patient No. 2	Patient No. 3	Patient No. 6
Age (years)	64	78	68
Gender	Male	Male	Male
Smoking status	Current smoker (>10 cig/day)	Non-smoker	Current smoker (>10 cig/day)
Alcohol intake	Yes (Moderate)	Yes (Moderate)	Yes (Heavy)
OPMD	No	No	No
Tumor location	Tongue	Maxillary gingiva	Hypopharynx
Tumor size	6 cm	2.7 cm	3 cm
Tumor recurrence	-	Locoregional	-
Metastasis Location	Lung	Vertebra	Lung
TNM Stage	T3N2M1	T0N3M1	T3N0M1
Grade of differentiation	G2	G3	G2
HPV-status	Negative	Negative	Negative

Abbreviations: OPMD, oral potentially malignant disorders; HPV, human papillomavirus.

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Table S2. List of genes analyzed in DNA by the TST170 panel.

AKT1	*CREBBP*	*FGFR2*	*MSH3*	*RAD51D*
AKT2	*CSF1R*	*FGFR3*	*MSH6*	*RAD54L*
AKT3	*CTNNB1*	*FGFR4*	*MTOR*	*RB1*
ALK	*DDR2*	*FLT1*	*MUTYH*	*RET*
APC	*DNMT3A*	*FLT3*	*MYC*	*RICTOR*
AR	*EGFR*	*FOXL2*	*MYCL1*	*ROS1*
ARID1A	*EP300*	*GEN1*	*MYCN*	*RPS6KB1*
ATM	*ERBB2*	*GNA11*	*MYD88*	*SLX4*
ATR	*ERBB3*	*GNAQ*	*NBN*	*SMAD4*
BAP1	*ERBB4*	*GNAS*	*NF1*	*SMARCB1*
BARD1	*ERCC1*	*HNF1A*	*NOTCH1*	*SMO*
BCL2	*ERCC2*	*HRAS*	*NOTCH2*	*SRC*
BCL6	*ERG*	*IDH1*	*NOTCH3*	*STK11*
BRAF	*ESR1*	*IDH2*	*NPM1*	*TERT*
BRCA1	*EZH2*	*INPP4B*	*NRAS*	*TET2*
BRCA2	*FAM175A*	*JAK2*	*NRG1*	*TP53*
BRIP1	*FANCI*	*JAK3*	*PALB2*	*TSC1*
BTK	*FANCL*	*KDR*	*PDGFRA*	*TSC2*
CARD11	*FBXW7*	*KIT*	*PDGFRB*	*VHL*
CCND1	*FGF1*	*KMT2A(MLL)*	*PIK3CA*	*XRCC2*
CCND2	*FGF2*	*KRAS*	*PIK3CB*	
CCNE1	*FGF3*	*MAP2K1*	*PIK3CD*	
CD79A	*FGF4*	*MAP2K2*	*PIK3CG*	
CD79B	*FGF5*	*MCL1*	*PIK3R1*	
CDH1	*FGF6*	*MDM2*	*PMS2*	
CDK12	*FGF7*	*MDM4*	*PPP2R2A*	
CDK4	*FGF8*	*MET*	*PTCH1*	
CDK6	*FGF9*	*MLH1*	*PTEN*	
CDKN2A	*FGF10*	*MLLT3*	*PTPN11*	
CEBPA	*FGF14*	*MPL*	*RAD51*	
CHEK1	*FGF23*	*MRE11A*	*RAD51B*	
CHEK2	*FGFR1*	*MSH2*	*RAD51C*	

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Table S3. Somatic mutations detected by NGS.

	Patient	Gene	SNP effect	AF in tumor	AF plasma
Tumor	No.2	FGFR1 c.1119G>T	Missense	0.011	0.0005914
	No.2	GNAQ c.303C>A	Stop-gained	0.046	0.009489
	No.2	TP53 c.723delA	Frameshift	0.391	0.0007113
	No.2	RAD51C c.725A>T	Missense	0.506	0.0008349
	No.3	ARID1A c.2291C>T	Missense	0.013	0.00308
	No.3	ARID1A c.5548delT	Frameshift	0.012	0.007476
	No.3	MYC c.482C>T	Missense	0.096	0.005205
	No.3	NOTCH1 c.6208C>T	Missense	0.055	0.003768
	No.3	CDK12 c.4382delT	Frameshift	0.011	0.008152
	No.6	GNAQ c.303C>A	Stop-gained	0.018	0.00541
Plasma	No.2	FOXL2 c.25_101delT	Frameshift	0.002154	0.271
	No.2	TP53 c.485T>A	Missense	0.0008673	0.054
	No.3	FOXL2 c.25_101delT	Frameshift	0.001089	0.21
	No.3	BRAF c.1208delA	Frameshift	0.009126	0.012
	No.3	CHEK2 c.478A>G	Missense	0.004062	0.017
	No.3	EP300 c.6970delG	Frameshift	0.008962	0.011
	No.6	FOXL2 c.25_101delT	Frameshift	0.004564	0.034
	No.6	TET2 c.2218C>T	Stop-gained	0.006923	0.012
	No.6	KMT2A c.2318delA	Frameshift	0.007584	0.011
Tumor and plasma	No.2	MCL1 c.55delG	Frameshift	0.04	0.014
	No.2	KMT2A c.2318delA	Frameshift	0.033	0.016
	No.2	SLX4 c.1406delT	Frameshift	0.027	0.016
	No.2	EP300 c.6970delG	Frameshift	0.018	0.013
	No.3	ARID1A c.2296del	Frameshift	0.014	0.013
	No.3	MCL1 c.55delG	Frameshift	0.015	0.021
	No.3	DNMT3A c.1010 C>T	Missense	0.063	0.287
	No.3	KMT2A c.2318delA	Frameshift	0.011	0.012
	No.3	SLX4 c.1406delT	Frameshift	0.024	0.012
	No.6	ARID1A c.2296del	Frameshift	0.011	0.013
	No.6	MCL1 c.55delG	Frameshift	0.021	0.012
	No.6	SLX4 c.1406delT	Frameshift	0.011	0.017
	No.6	TP53 c.339delA	Frameshift	0.562	0.034
	No.6	EP300 c.4241A>G	Missense	0.327	0.018

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Table S4. Absolute and relative quantification of mutant and wild-type DNA in tumor, plasma, and saliva samples by ddPCR.

	Sample ID	MT-DNA concentration		WT-DNA concentration		FAmut(%)
		Sample (copies/uL)	Sample corr (copies/uL)	Sample (copies/uL)	Sample corr (copies/uL)	
Tumor	Patient No.3 (NOTCH1 C.6208 C>T)	6.7	6.09	80.5	73.18	8.3
	Patient No.6 (EP300 c.4241 A>G)	29.9	27.18	69.2	62.9	43.2
	Patient No.2 (TP53 c.723del)	34.2	31.09	58.2	52.90	58.7
Plasma	Patient No.3 (NOTCH1 c.6208 C>T)	0.23	0.21	13.1	11.91	1.7
	Patient No.6 (EP300 c.4241 A>G)	0.44	0.4	60.4	54.9	0.72
	Patient No.2 (TP53 c.485T>A)	1.12	1.02	41.9	38.09	2.7
Saliva	Patient No.2 (TP53 c.485T>A)	0.19	0.17	261	237.2	0.07

Abbreviations: MT-DNA, mutant DNA; WT-DNA, wild-type DNA; FAmut, fractional abundance of mutant copies.

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OBJECTIVE 3.3

SALIVARY DNA METHYLATION AS AN EPIGENETIC BIOMARKER FOR HEAD AND NECK CANCER. PART I: A DIAGNOSTIC ACCURACY META-ANALYSIS

Figure S1. Quality assessment of the included studies according to Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) criteria.

Figure S2. Forest plot of pooled dOR salivary DNA methylation for the diagnosis of HNC.

Figure S3. Fagan's monogram evaluating the clinical utility of salivary DNA methylation for differentiating HNC patients.

Figure S4. Representation of sensitivity against (1-specificity) in ROC space for each study of salivary methylation in the diagnosis of HNC.

Figure S5. Deeks' funnel plot asymmetry test for the assessment of potential bias of included studies.

Table S1. Results of meta-regression analysis.

Table S2. Subgroup analysis of salivary DNA methylation for HNC detection based on different covariates.

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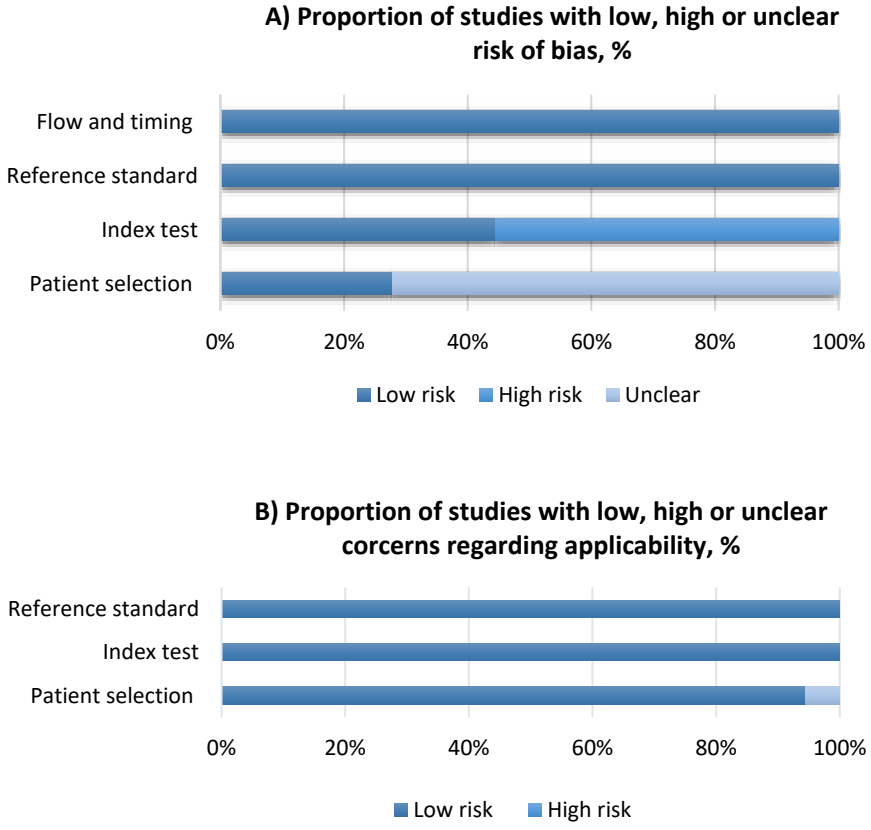

Figure S1. Quality assessment of the included studies according to Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) criteria. (A) Proportion of studies with low, high, or unclear risk of bias. (B) Proportion of studies with low, high, or unclear concerns regarding applicability.

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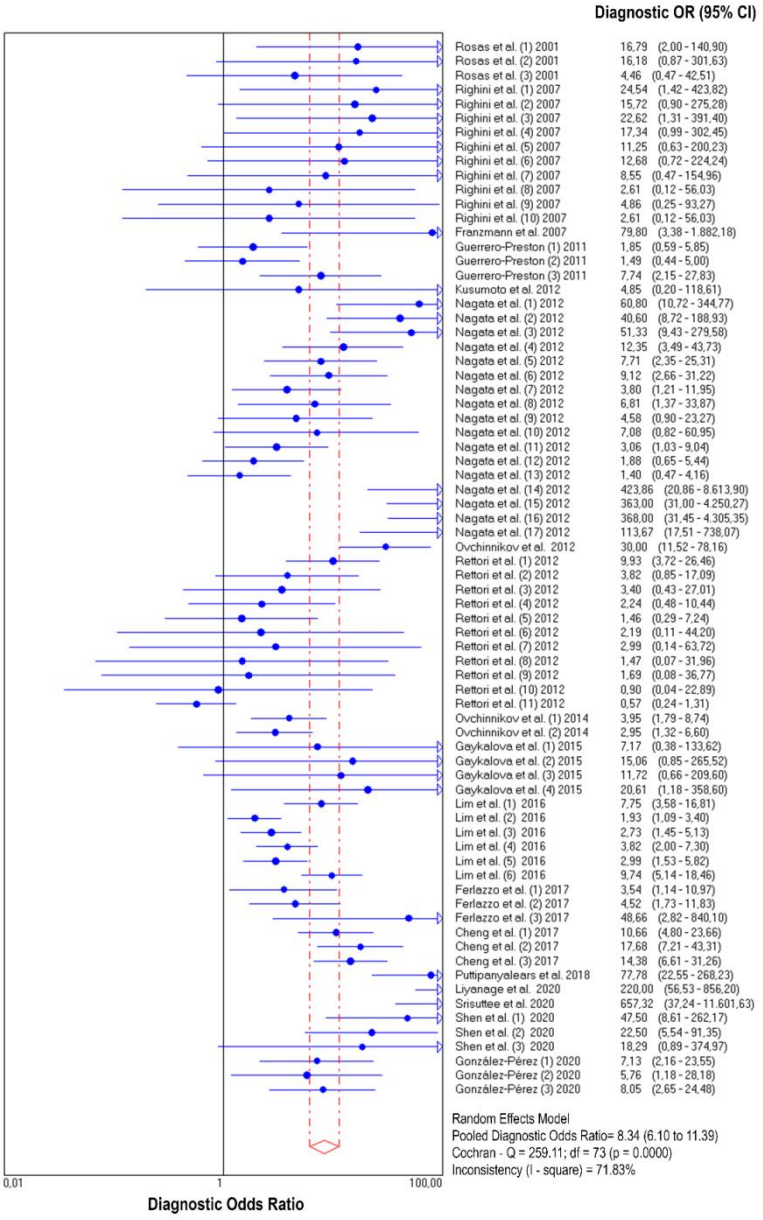

Figure S2. Forest plot of pooled dOR salivary DNA methylation for the diagnosis of HNC. *Abbreviations*: dOR, diagnostic odds ratio; HNC, head and neck cancer.

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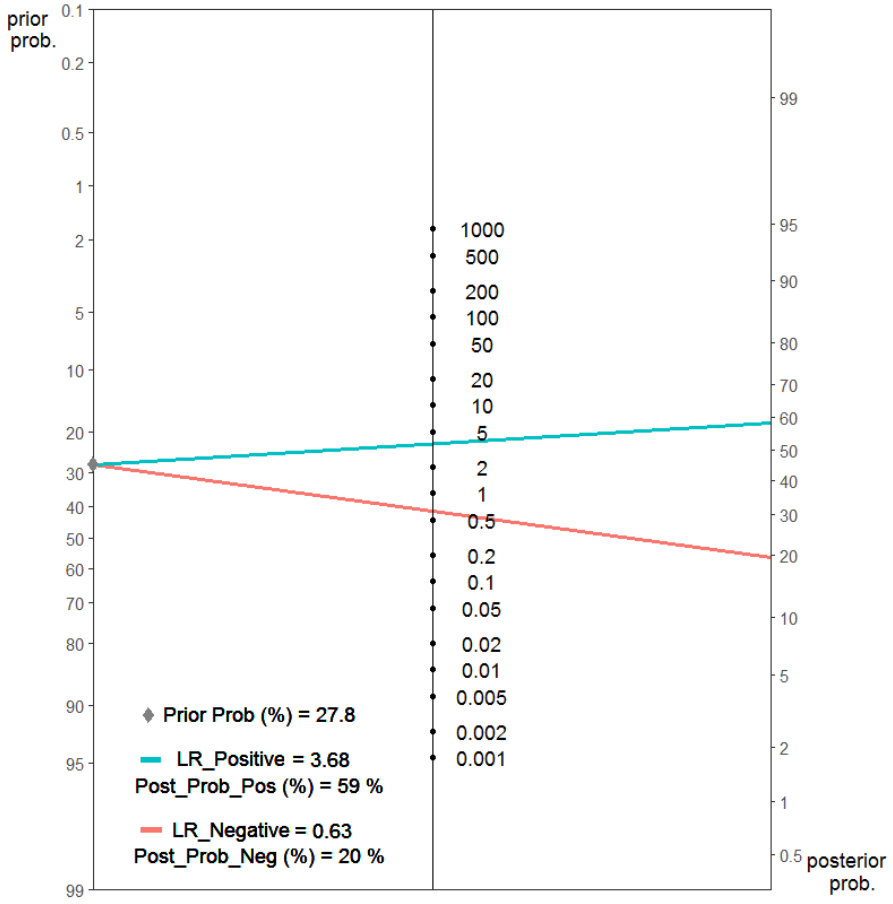

Figure S3. Fagan´s monogram evaluating the clinical utility of salivary DNA methylation for differentiating HNC patients. *Abbreviations*: HNC, head and neck cancer.

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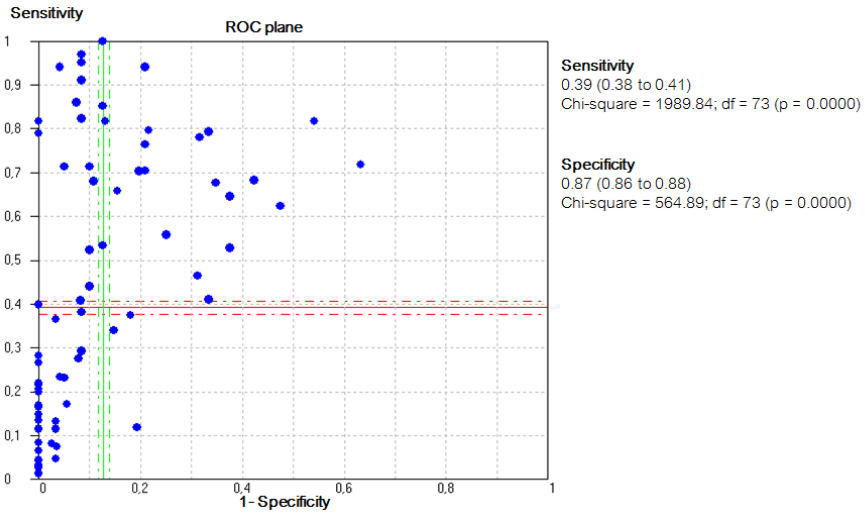

Figure S4. Representation of sensitivity against (1-specificity) in ROC space for each study of salivary methylation in the diagnosis of HNC. *Abbreviations*: ROC, summary receiver operator characteristic; HNC, head and neck cancer.

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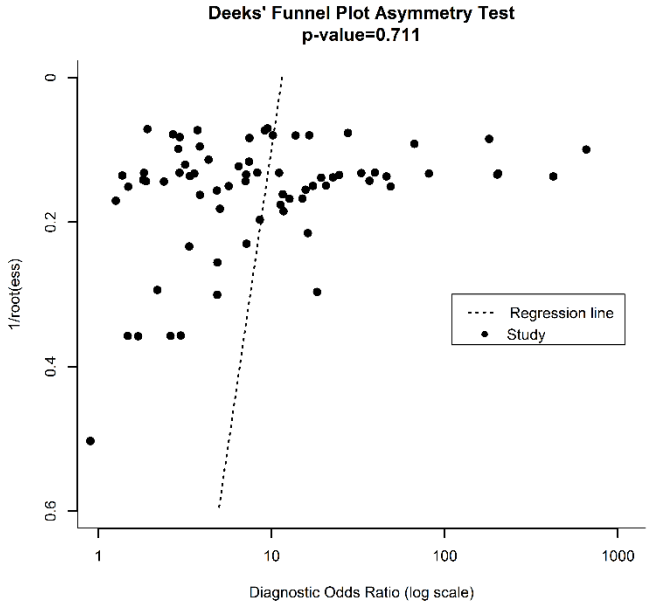

Figure S5. Deeks' funnel plot asymmetry test for the assessment of potential bias of included studies.

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Table S1. Results of meta-regression analysis.

Var	Coeff.	*p*-value	RDOR	[95%CI]
Cte.	-1.549	0.0592	-	-
S	0.023	0.7139	-	-
Sample type	0.739	0.1276	2.09	(0.81;5.44)
Anatomic tumor location	0.631	0.0204	1.88	(1.11;3.19)
Technique	-0.483	0.2752	0.62	(0.26;1.48)
Gene profiling	1.502	0.0000	4.49	(2.34;8.62)
Sample size	0.371	0.3493	1.45	(0.66;3.18)

No. studies = 74
Filter OFF
Add ½ to all cells of the studies with zero

Var	Coeff.	*p*-value	RDOR	[95%CI]
Cte.	-0.864	0.1082	-	-
S	0.021	0.7316	-	-
Anatomic tumor location	0.731	0.0016	2.08	(1.33;3.24)
Gene profiling	1.421	0.0000	4.14	(2.19;7.84)

No. studies = 74
Filter OFF
Add ½ to all cells of the studies with zero

Abbreviations: CI, confidence interval; Coeff., coefficient; Std. Err., standard error; RDOR, relative diagnostic odds ratios.

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Table S2. Subgroup analysis of salivary DNA methylation for HNC detection based on different covariates.

Subgroups	No of study units	Sensitivity (95% CI)	I²(%)	Specificity (95% CI)	I² (%)	PLR (95% CI)	I² (%)	NLR (95% CI)	I² (%)	DOR (95% CI)	I² (%)	AUC (95% CI)
Total	74	0.39 (0.38-0.41)	96.33	0.87 (0.86-0.88)	87.07	3.68 (2.97-4.57)	73.99	0.63 (0.57-0.69)	96.35	8.34 (6.10-11.38)	71.83	0.81 (0.77-0.84)
Sample type												
Saliva	16	0.55 (0.52-0.57)	94.98	0.81 (0.79-0.83)	92.30	3.04 (2.20-4.21)	82.52	0.57 (0.47-0.69)	90.55	6.33 (3.90-10.27)	79.34	0.78 (0.72-0.83)
Oral Rinse	58	0.34 (0.32-0.35)	96.28	0.91 (0.90-0.92)	81.02	4.07 (3.08-5.39)	66.18	0.65 (0.59-0.73)	96.39	9.42 (6.30-14.08)	67.84	0.82 (0.78-0.86)
Samples size												
>100	25	0.43 (0.41-0.45)	97.64	0.84 (0.83-0.86)	91.61	3.24 (2.35-4.46)	84.32	0.56 (0.46-0.67)	97.79	6.88 (4.24-11.19)	84.15	0.79 (0.73-0.84)
<100	49	0.35 (0.33-0.40)	94.89	0.91 (0.94-0.93)	80.63	4.12 (3.06-5.54)	60.01	0.68 (0.61-0.76)	94.46	9.58 (6.44-14.27)	53.11	0.82 (0.78-0.86)
Anatomic Tumor Location												
HNC	43	0.31 (0.29-0.33)	96.77	0.86 (0.85-0.87)	90.43	3.03 (2.27-4.03)	73.63	0.75 (0.96-0.82)	95.39	5.78 (3.86-8.67)	70.11	0.81 (0.75-0.88)
OC	33	0.63 (0.60-0.65)	92.22	0.87 (0.85-0.89)	76.63	4.02 (2.99-5.41)	73.79	0.40 (0.31-0.50)	92.45	13.07 (8.19-20.88)	73.08	0.88 (0.84-0.93)
OPC	8	0.70 (0.62-0.77)	86.26	0.86 (0.81-0.89)	88.41	3.67 (2.88-4.69)	88.67	0.41 (0.19-0.90)	90.75	13.26 (3.17-55.42)	83.12	0.87 (0.72-1.00)
Technique												
MSP	48	0.47 (0.45-0.49)	95.08	0.85 (0.83-0.86)	87.22	3.59 (2.81-4.57)	72.53	0.59 (0.52-0.67)	94.49	9.06 (6.30-13.03)	70.46	0.82 (0.78-0.85)
qMSP	26	0.29 (0.27-0.31)	97.15	0.92 (0.90-0.93)	84.79	3.65 (2.31-5.77)	76.18	0.69 (0.60-0.79)	97.12	6.81 (3.70-12.54)	74.82	0.78 (0.71-0.86)
Gene profiling												
Single gene	62	0.32 (0.31-0.34)	95.45	0.87 (0.86-0.88)	88.12	3.17 (2.53-3.97)	69.76	0.71 (0.66-0.77)	94.50	6.02 (4.45-8.13)	62.54	0.77 (0.73-0.81)
Combination gene	12	0.73 (0.69-0.76)	95.29	0.88 (0.58-0.91)	78.40	5.76 (3.92-8.48)	54.71	0.22 (0.12-0.40)	96.78	36.97 (16.81-81.32)	73.62	0.92 (0.88-0.96)

Abbreviations: AUC, area under the SROC; dOR, diagnostic Odds Ratio; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CI, confidence interval; HNC, head and neck cancer; OC, oral cancer; OPC, oropharyngeal cancer; MSP, methylation-specific polymerase chain reaction; qMSP, quantitative-MSP.

OBJECTIVE 3.4.2

SALIVARY DNA METHYLATION AS AN EPIGENETIC BIOMARKER FOR HEAD AND NECK CANCER. PART II: A CANCER RISK META-ANALYSIS

Figure S1. Funnel plot for studies (of 18 studies) on the association between salivary DNA hypermethylation and HNC

Figure S2. Funnel plot for studies (of 9 studies) on the association between salivary hypermethylation of *p16* gene promoter and HNC

Figure S3. Funnel plot for studies (of 5 studies) on the association between salivary hypermethylation of *MGMT* gene promoter and HNC

Figure S4. Funnel plot for studies (of 4 studies) on the association between salivary hypermethylation of *DAPK* gene promoter and HNC

Figure S5. Funnel plot for studies (of 4 studies) on the association between salivary hypermethylation of *TIMP3* gene promoter and HNC

Figure S6. Funnel plot for studies (of 3 studies) on the association between salivary hypermethylation of *RASSF1A* gene promoter and HNC

Figure S7. Funnel plot for studies (of 3 studies) on the association between salivary hypermethylation of *APC* gene promoter and HNC

Table S1. The Newcastle-Ottawa Scale (NOS) for assessing the quality of included studies

Table S2. Subgroup analysis of salivary DNA methylation for HNC detection based on different covariates.

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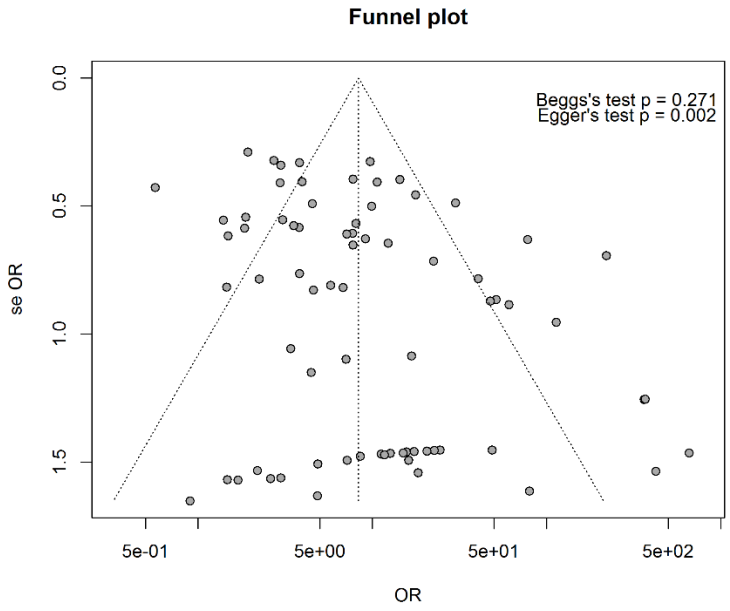

Figure S1. Funnel plot for studies (of 18 studies) on the association between salivary DNA hypermethylation and HNC. *Abbreviations:* HNC, head and neck cancer.

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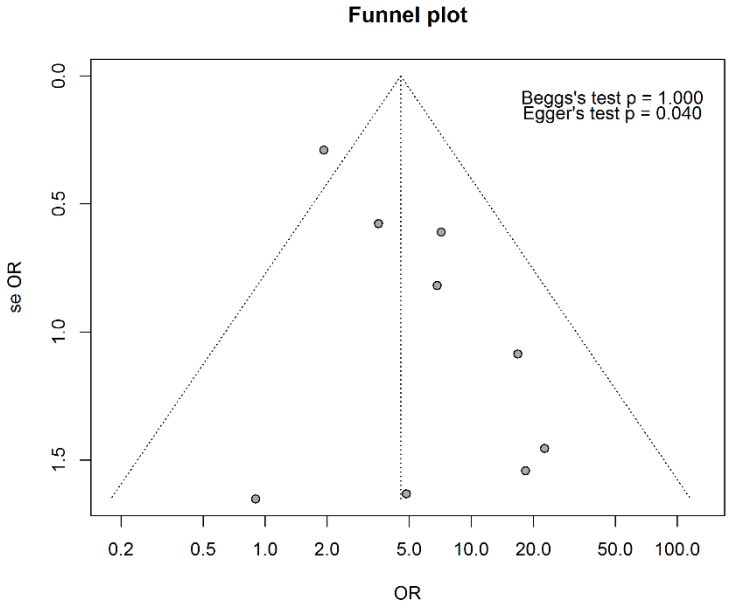

Figure S2. Funnel plot for studies (of 9 studies) on the association between salivary hypermethylation of *p16* gene promoter and HNC. *Abbreviations:* HNC, head and neck cancer.

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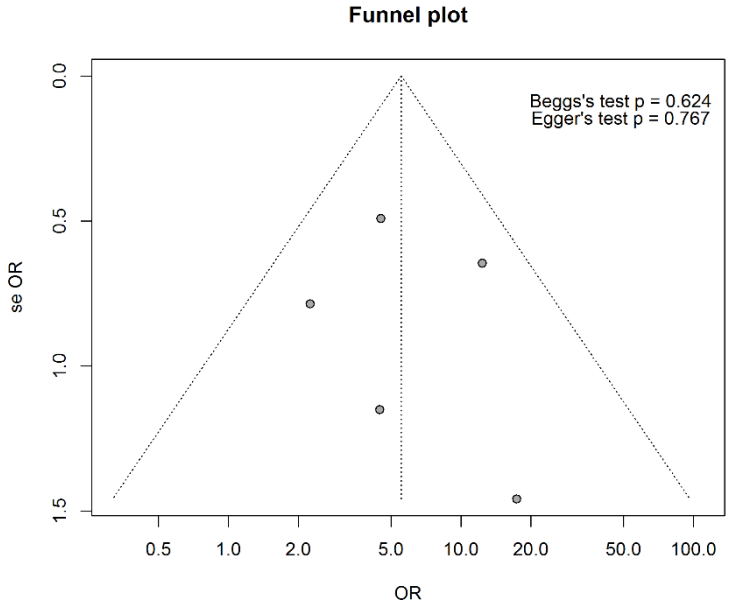

Figure S3. Funnel plot for studies (of 5 studies) on the association between salivary hypermethylation of *MGMT* gene promoter and HNC. *Abbreviations:* HNC, head and neck cancer.

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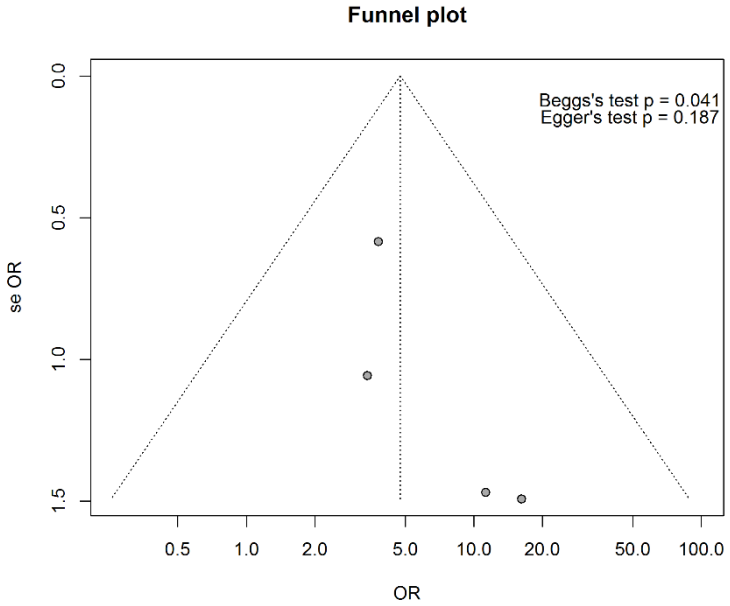

Figure S4. Funnel plot for studies (of 4 studies) on the association between salivary hypermethylation of *DAPK* gene promoter and HNC. *Abbreviations:* HNC, head and neck cancer.

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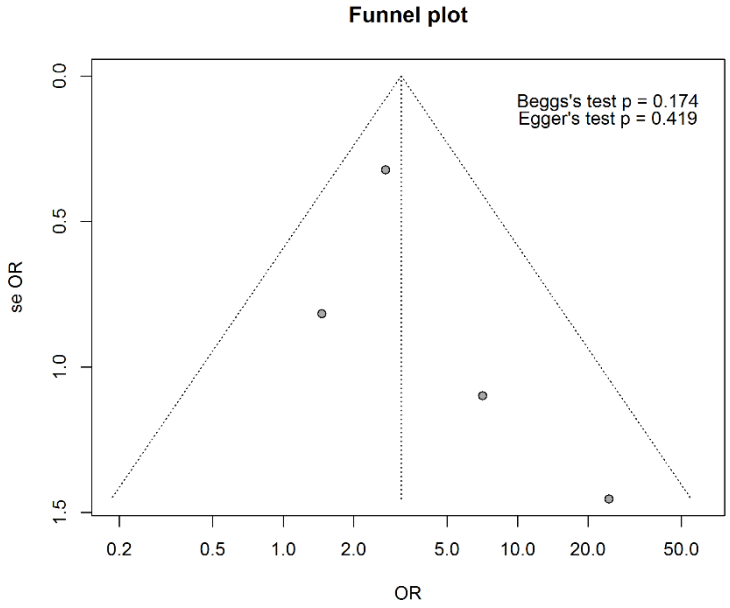

Figure S5. Funnel plot for studies (of 4 studies) on the association between salivary hypermethylation of *TIMP3* gene promoter and HNC. *Abbreviations:* HNC, head and neck cancer.

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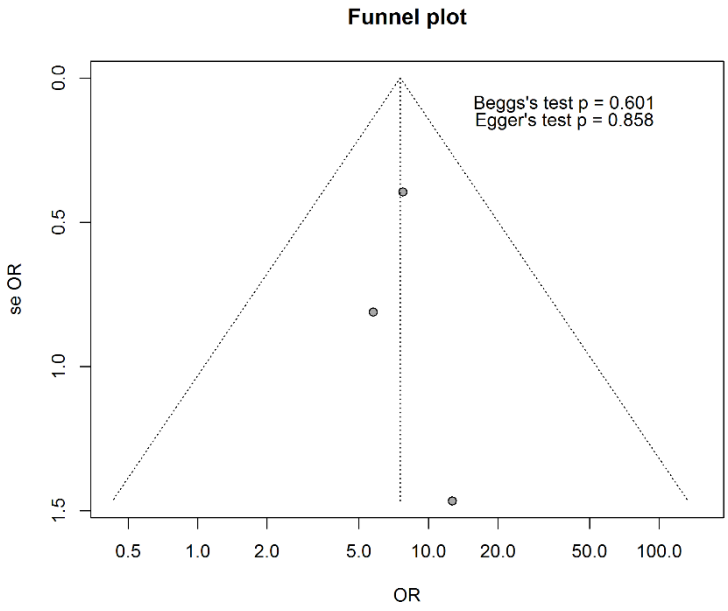

Figure S6. Funnel plot for studies (of 3 studies) on the association between salivary hypermethylation of *RASSF1A* gene promoter and HNC. *Abbreviations:* HNC, head and neck cancer.

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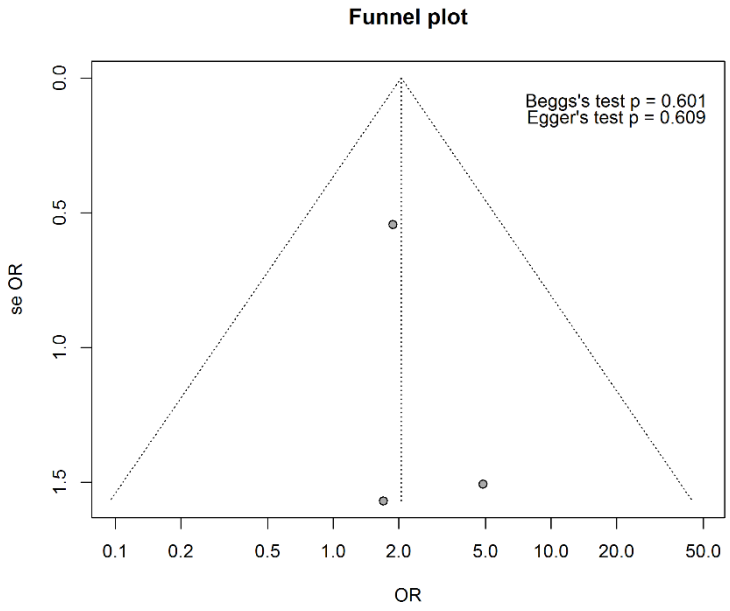

Fig. S7. Funnel plot for studies (of 3 studies) on the association between salivary hypermethylation of *APC* gene promoter and HNC. *Abbreviations:* HNC, head and neck cancer.

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Table S1. The Newcastle-Ottawa Scale (NOS) for assessing the quality of included studies.

Study	Subject Selection (Max. 4★)	Study Comparability (Max. 2★)	Ascertainment of exposure (Max. 3★)	Final quality assessment
Rosas et al.; 2001	★★★★	0	★★★	7
Righini et al.; 2007	★★★★	0	★★★	7
Franzmann et al.; 2007	★★	★★	★★★	7
Guerrero-Preston et al.; 2011	★★★★	0	★★★	7
Nagata et al.; 2012	★★★★	0	★★★	7
Ovchinnikov et al.; 2012	★★★★	0	★★★	7
Ksumoto et al.; 2012	★★★★	0	★★★	7
Rettori et al.; 2012	★★★★	0	★★★	7
Ovchinnikov et al.; 2014	★★★★	0	★★★	7
Gaykalova et al.; 2015	★★★★	0	★★★	7
Lim et al.; 2016	★★★★	0	★★★	7
Ferlazzo et al.; 2017	★★★★	★★	★★★	9
Cheng et al.; 2017	★★★★	0	★★★	7
Puttipanyalears et al.; 2018	★★★★	0	★★★	7
Liyanage et al.; 2020	★★★★	★★	★★★	9
Srisutte et al.; 2020	★★★★	★	★★★	8
Shen et al.; 2020	★★★★	0	★★★	7
González-Pérez et al.; 2020	★★★★	★	★★★	8

Note: "★" means one point

Table S2. Subgroup analysis of salivary DNA methylation for HNC detection based on different covariates.

Subgroups	No of study units	Heterogeneity		Model	Effect size	
		I^2(%)	*P*-Q-test		OR (95% CI)	*P-value*
Sample type						
Saliva	16	79	<0.01	R	6.33 (3.90−10.27)	<0.01
Oral Rinse	58	68	<0.01		9.42 (6.30−14.08)	<0.01
Samples size						
N>100	25	84	<0.01	R	8.34 (6.10−11.39)	<0.01
N<100	49	53	<0.01		9.58 (6.44−14.27)	<0.01
Tumor Location						
HNC	43	70	<0.01	R	5.78 (3.86−8.67)	<0.01
OC	33	73	<0.01		13.07 (8.19−20.88)	<0.01
OPC	8	80	<0.01		13.26 (3.17−5.42)	<0.01
Technique						
MSP	48	70	<0.01	R	9.06 (6.30−13.03)	<0.01
qMSP	26	75	<0.01		6.81 (3.70−12.54)	<0.01
Gene profiling						
Single gene	62	63	<0.01	R	6.02 (4.46−8.13)	<0.01
Combination gene	12	74	<0.01		36.79 (16.81−81.32)	<0.01

Abbreviations: HNC, head and neck cancer; OC, oral cancer; OPC, oropharyngeal cancer; MSP, methylation-specific polymerase chain reaction; qMSP, quantitative-MSP.

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OBJECTIVE 3.4.3

GENOME-WIDE DNA METHYLATION PROFILING OF ORAL TONGUE SQUAMOUS CELL CARCINOMA

Figure S1. DNA methylation level of CpG sites identified by the Infinium HM850K DNA methylation assay. Displayed are averages of normalized beta-values of each CpG in 6 OTSCC patients (y-axis) and 6 adjacent normal tissues (x-axis).

Figure S2. Unsupervised hierarchical heatmap clustering of the random 44 hypermethylated DMCpGs and 956 hypomethylated DMCpGs between tumor and adjacent normal samples analyzed on the Illumina Infinium MethylationEPIC BeadChip (HM850K) platform. DNA methylation values were represented as colors with red representing hypermethylated DMCpGs and green representing hypomethylated DMCpGs. The row represents individual CpGs and the column represents individual samples. *Abbreviations:* DMCpGs, differentially methylated CpGs; T, tumor; N, normal control.

Figure S3. Gene Ontology (GO) enrichment analysis of the hypermethylated and hypomehtylated DMCpGs.

Figure S4. ROC curves of the 11 selected DNA methylation markers for discriminating early-OTSCC patients from healthy controls from TCGA cohort. ROC curve and AUC values were estimated for *A2BP1*, *ACSS3*, *ANK1*, *CA3*, *ALDH1A2*, *GFRA1*, *GABRB3*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B* using R software.

Table S1. Clinicopathological characteristics of OTSCC patients.

Table S2. List of candidates differentially hypermethylated CpGs sites identified in OTSCC in HM850K array and in TCGA database.

Table S3. Independent analysis of sensitivity and specificity of each individual gene in detecting OTSCC based on HM850K array data and TCGA database.

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Table S4. Sensitivity and specificity of each individual gene in saliva for discriminating OTSCC patients from healthy individuals.

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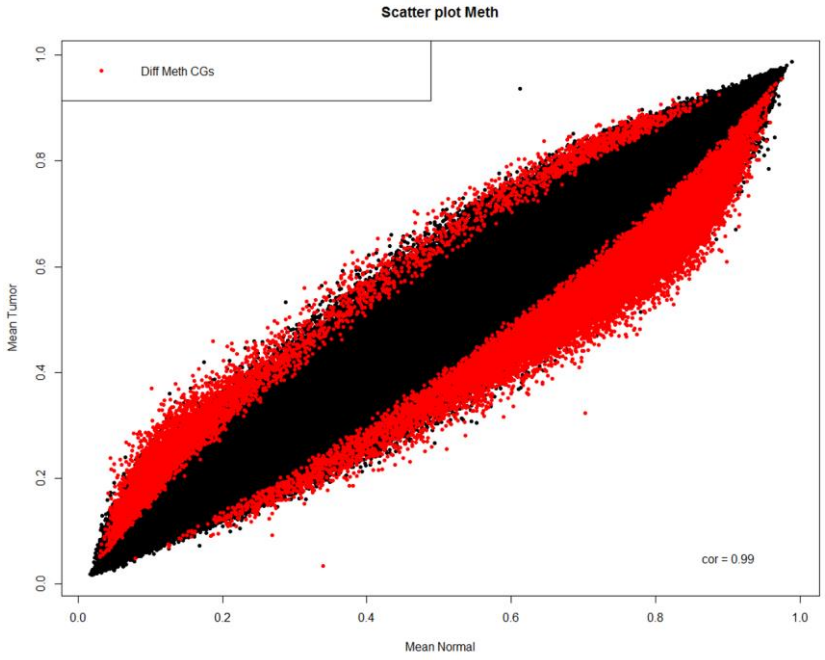

Figure S1. DNA methylation level of CpG sites identified by the Infinium HM850K DNA methylation assay. Displayed are averages of normalized beta-values of each CpG in 6 OTSCC patients (y-axis) and 6 adjacent normal tissues (x-axis). *Abbreviations:* OTSCC, oral tongue squamous cell carcinoma; CpG, cytosine-guanine dinucleotide.

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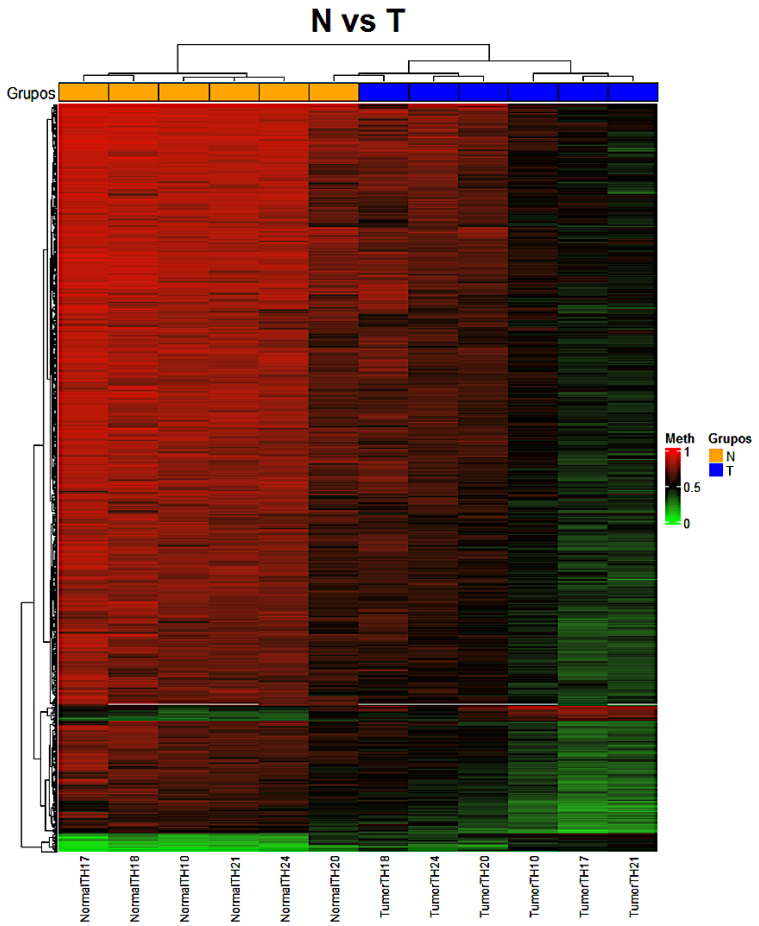

Figure S2. Unsupervised hierarchical heatmap clustering of the random 44 hypermethylated DMCpGs and 956 hypomethylated DMCpGs between tumor and adjacent normal samples analyzed on the Illumina Infinium MethylationEPIC BeadChip (HM850K) platform. DNA methylation values were represented as colors with red representing hypermethylated DMCpGs and green representing hypomethylated DMCpGs. The row represents individual CpGs and the column represents individual samples. *Abbreviations:* DMCpGs, differentially methylated CpGs; T, tumor; N, normal control.

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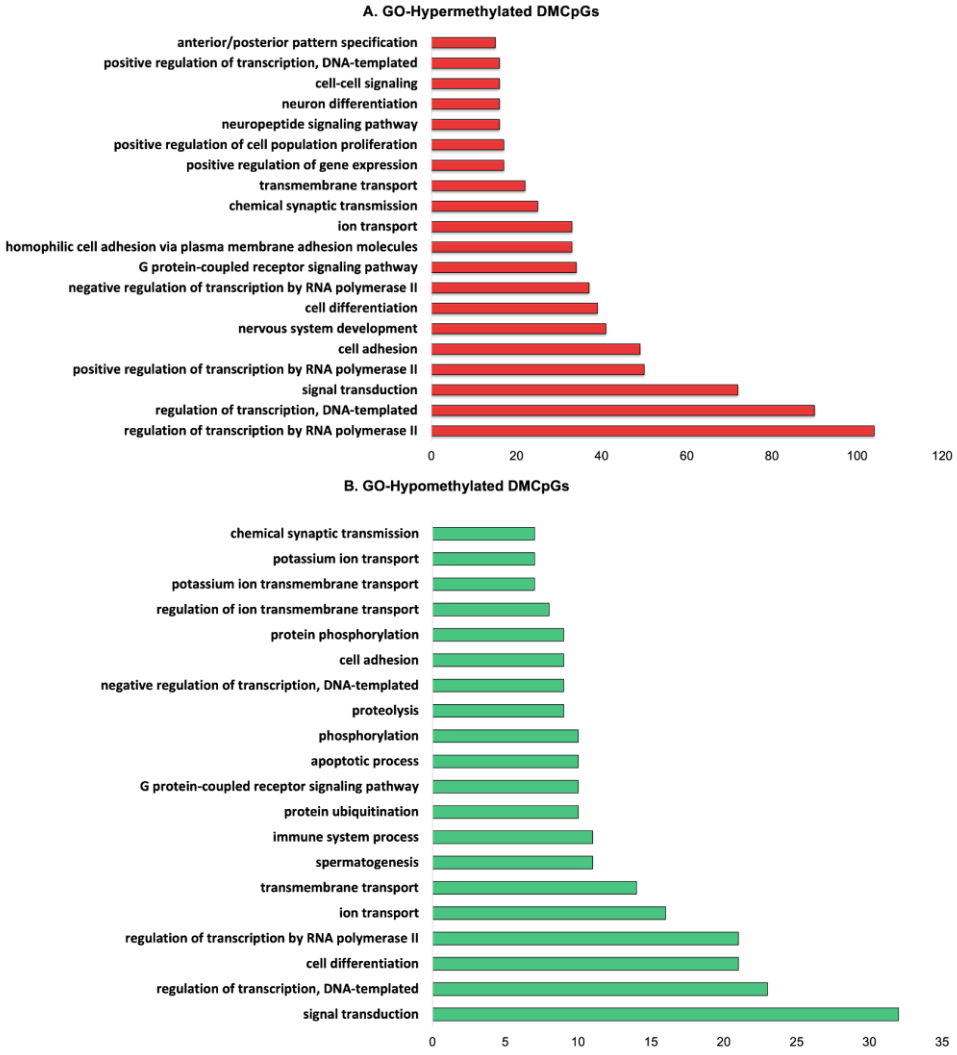

Figure S3. Gene Ontology (GO) enrichment analysis of the hypermethylated and hypomethylated DMCpGs. *Abbreviations:* DMCpGs, differentially methylated CpGs.ñ

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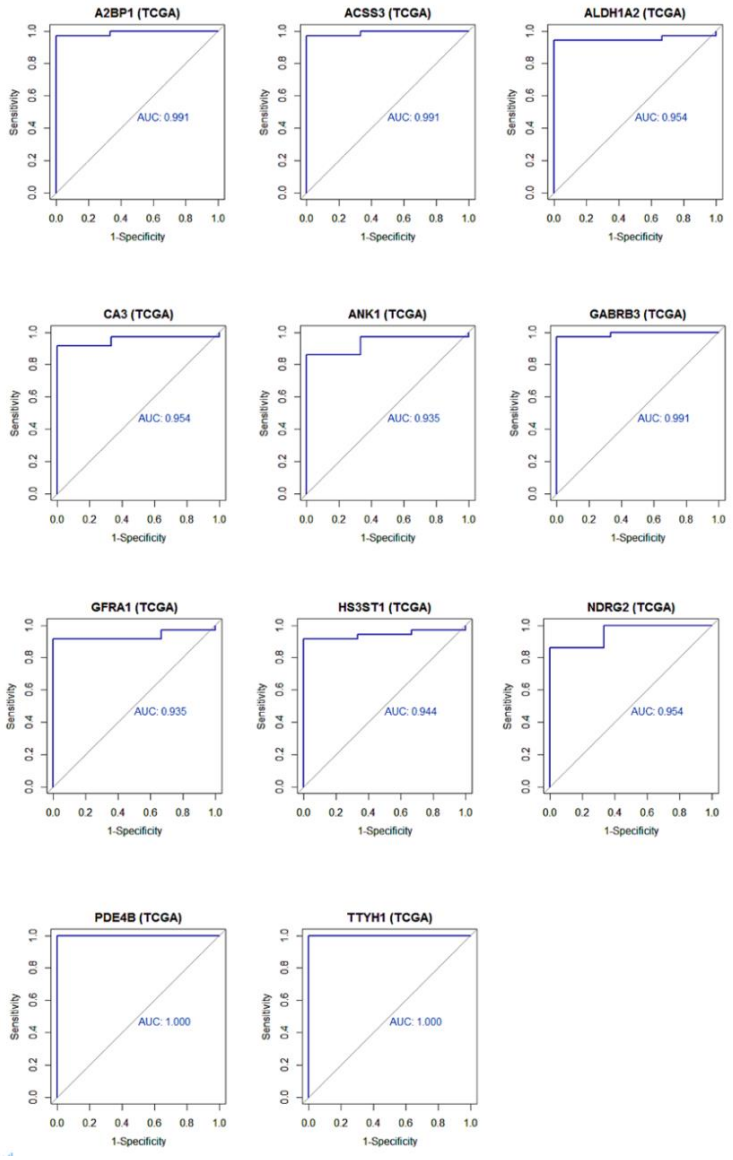

Figure S4. ROC curves of the 11 selected DNA methylation markers for discriminating early-OTSCC patients from healthy controls from TCGA cohort. ROC curve and AUC values were estimated for *A2BP1*, *ACSS3*, *ANK1*, *CA3*, *ALDH1A2*, *GFRA1*, *GABRB3*, *HS3ST1*, *NDRG2*, *TTYH1*, and *PDE4B* using R software. *Abbreviations:* ROC, receiver operating characteristic; TCGA, The Cancer Genome Atlas; OTSCC, oral tongue squamous cell-carcinoma.

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Table S1. Clinicopathological characteristics of OTSCC patients.

ID-sample (Tumor/Normal)	Gender	Age	Lifestyle Habits (Tobacco/Alcohol)	OPMDs	Anatomic tumor location	TNM-Stage	HPV-status	Grade of differentiation
TH-10	Female	72	No/No	No	Tongue (Right-side)	T1N0M0	Negative	Moderate
TH-17	Female	72	No/No	No	Tongue (Dorsal-surface)	T1N0M0	Negative	Moderate
TH-18	Female	76	No/No	Leukoplakia	Tongue (Left-side)	T2N0M0	Negative	Moderate
TH-20	Female	50	Yes /No	No	Tongue (Left-side)	T1N0M0	HPV-11	Moderate
TH-21	Male	83	No/No	No	Tongue (Left-side)	T2N0M0	Negative	Moderate
TH-24	Female	74	No/No	Leukoplakia	Tongue (Left-side)	T1N0M0	Negative	Moderate

Abbreviations: OTSCC, oral tongue squamous cell carcinoma; TNM, Tumor-Node-Metastasis; HPV, Human Papillomavirus; OPMDs, oral potentially malignant disorders.

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Table S2. List of candidates differentially hypermethylated CpGs sites identified in OTSCC in HM850K array and in TCGA database.

		Array		TCGA	
	Target ID	***P*-Value**	**FDR**	***P*-Value**	**FDR**
A2BP1	**cg00853103**	0.00077743	0.04159519	0.00059852	0.00271331
	cg01610605	0.00022216	0.0362395	0.00045861	0.00244421
	cg02230017	0.00029811	0.03651831	0.00020478	0.00139248
	cg03072621	0.00096034	0.04359476	0.0001283	0.00109055
ACSS3	**cg10789281**	0.00111746	0.04512193	0.00053384	0.00259296
	cg11659501	0.00053214	0.03901607	0.00072525	0.00281086
	cg24711649	0.00149096	0.04872774	0.0066387	0.01399227
ALDH1A2	**cg14805347**	0.00036468	0.03718973	0.00125506	0.004064
	cg19890277	0.00053193	0.0390083	0.00715761	0.01431522
ANK1	**cg05166490**	0.00093822	0.04337063	0.00275784	0.00694566
	cg17331296	0.00110689	0.04504166	0.00202171	0.00549906
CA3	**cg05355225**	0.00124821	0.04636033	0.00679037	0.01399227
	cg07560510	0.00081224	0.0419932	0.00514372	0.0116591
	cg17480760	0.00086025	0.0425077	0.00781455	0.01518255
	cg21346043	0.00031741	0.0367245	0.00067557	0.00281086
GABRB3	**cg01690182**	0.00124136	0.04628132	0.00145461	0.00449607
	cg08063317	0.00087238	0.04260831	0.02624924	0.04056701
	cg18026588	6.3691E-05	0.03615241	8.23E-05	0.00108757
GFRA1	**cg12087643**	0.00120058	0.04590193	0.00198032	0.00549906
	cg23524195	0.0007037	0.04079635	0.00898355	0.01607583
	cg25617725	0.00058154	0.03944276	0.00228187	0.00596797
	cg27341926	0.00153771	0.04913425	0.0063133	0.01384852
HS3ST1	**cg01089498**	0.00078184	0.0416351	0.00096606	0.00328459
	cg14820199	0.00129548	0.04682392	0.02108918	0.03361718
NDRG2	**cg20902783**	0.00086407	0.04253266	0.01091586	0.01855697
	cg24112713	8.7088E-05	0.03615241	0.02125793	0.03361718
PDE4B	**cg00046625**	0.00121843	0.04606214	3.2222E-06	0.00017176
	cg22336004	0.00155873	0.04934766	0.00011196	0.00108757
	cg22488256	0.00056832	0.0393133	0.00020171	0.00139248
	cg24637364	0.00110459	0.04501718	7.6735E-05	0.00108757
TTYH1	**cg09474331**	0.00011652	0.03615241	5.0518E-06	0.00017176
	cg15241920	0.00067216	0.04068777	0.0001064	0.00108757
	cg21883754	0.00044288	0.0380507	0.00328889	0.00771187

Abbreviations: FDR, false discovery rate.

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Table S3. Independent analysis of sensitivity and specificity of each individual gene in detecting OTSCC based on HM850K array data and TCGA database.

		Array			TCGA		
	Target ID	**Specificity**	**Sensitivity**	***P*-Value**	**Specificity**	**Sensitivity**	***P*-Value**
A2BP1	**cg00853103**	1	0.83	0.0021645	1	0.97	0.00035666
	cg01610605						
	cg02230017						
	cg03072621						
ACSS3	**cg10789281**	0.83	1	0.004329	1	0.97	0.00021884
	cg11659501						
	cg24711649						
ALDH1A2	**cg14805347**	0.83	1	0.00757576	1	0.94	0.00518189
	cg19890277						
ANK1	**cg05166490**	0.83	1	0.00757576	1	0.86	0.00339206
	cg17331296						
CA3	**cg05355225**	0.83	1	0.004329	1	0.97	0.00076595
	cg07560510						
	cg17480760						
	cg21346043						
GABRB3	**cg01690182**	0.83	1	0.004329	1	0.97	0.00095776
	cg08063317						
	cg18026588						
GFRA1	**cg12087643**	0.83	1	0.00757576	1	0.91	0.00339206
	cg23524195						
	cg25617725						
	cg27341926						
HS3ST1	**cg01089498**	0.83	1	0.01298701	1	0.88	0.00339206
	cg14820199						
NDRG2	**cg20902783**	0.83	1	0.00757576	1	0.83	0.00339206
	cg24112713						
PDE4B	**cg00046625**	0.83	1	0.00757576	1	1	4.27E-08
	cg22336004						
	cg22488256						
	cg24637364						
TTYH1	**cg09474331**	0.83	1	0.00757576	1	1	4.29E-08
	cg15241920						
	cg21883754						

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Table S4. Sensitivity and specificity of each individual gene in saliva for discriminating OTSCC patients from healthy individuals.

	Target ID	Specificity	Sensitivity	*P*-Value
A2BP1	**cg00853103** **cg02230017** **cg03072621**	1	1	0.00518936
ALDH1A2	**cg14805347** **cg19890277**	1	0.75	0.03333333
ANK1	**cg05166490** **cg17331296**	1	0.75	0.03333333
GFRA1	**cg23524195**	0.83	0.75	0.03333333
PDE4B	**cg00046625** **cg24637364**	0.83	1	0.0123705
TTYH1	**cg09474331** **cg15241920** **cg21883754**	0.83	1	0.00952381

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GLOSSARY OF ABBREVIATIONS

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GLOSSARY OF ABBREVIATIONS

A:	adenomas
A2BP1:	RNA binding fox-1 homolog 1
ACSS3:	acyl-CoA synthetase short chain family member 3
AGO:	argonaute proteins family
AJCC:	American Joint Commission on Cancer
ALDH1A2:	aldehyde dehydrogenase 1 family member A2
ALU:	Arthrobacter luteus
ANK:	ankyrin 1
AUC:	area under the receiver operating characteristic curve
BC:	benign controls
BcL-XL:	B-cell lymphoma extra-large
BEAMing:	beads, emulsions, amplification, and magnetics
BMI:	body mass index
bp:	base pairs
CA3:	carbonic anhydrase 3
CAPP-seq:	Cancer Personalized Profiling by deep sequencing
ccfDNA:	circulating cell-free DNA
ccfRNA:	circulating cell-free RNA
CDH1:	E-cadherin
CDKN2A:	cyclin dependent kinase inhibitor 2A
CEA:	carcinoembryonic antigen
cfDNA:	cell-free DNA
cfMeDIP-seq:	cell-free Methylated DNA immunoPrecipitation and high-throughput sequencing

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cfRNA:	cell-free RNA
CI:	confidence interval
circRNAs:	circular RNAs
CNAs:	copy number aberrations
CNV:	copy number variations
Coeff.:	coefficient
COSMIC:	Catalogue of Somatic Mutations in Cancer
CpG:	cytosine-guanine dinucleotide
CpGIs:	CpG islands
CRC:	colorectal cancer
CT:	computed tomography
CTCs:	circulating tumor cells
ctDNA:	circulating tumor DNA
ctRNA:	circulating tumor RNA
CV:	coefficient of variation
DAPK:	death associated protein kinase
ddPCR:	droplet digital PCR
DDR1:	discoidin domain receptor tyrosine kinase 1
DFS:	disease-free survival
DIANA:	DNA Intelligent Analysis
DMCpGs:	differentially methylated CpGs
dOR:	diagnostic odds ratio
dsRNA:	double-strand RNA
ECM:	extracellular matrix
EGFR:	epidermal growth factor receptor
EMT:	epithelial to mesenchymal transition
EVs:	extracellular vesicles
ExonBnd:	exon boundary
EXP5:	exportin-5 protein
FAM134-B:	family with sequence similarity 134 member B

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FAmut:	fractional abundance of mutant copies
FDR:	false discovery rate
FFPE:	formalin fixation and paraffin-embedding
FIT:	fecal immunochemical testing
FN:	false negative
FP:	false positive
FRAP:	ferric reducing antioxidant power
GABRB3:	gamma-aminobutyric acid type A receptor subunit beta3
GC:	gastric cancer
GEO:	gene expression omnibus
gFOBT:	guaiac fecal occult blood testing
GFRA1:	GDNF family receptor alpha 1
GO:	gene ontology
GTP:	guanosine triphosphate
HC:	healthy controls
HNC:	head and neck cancer
HNSCC:	head and neck squamous cell carcinoma
HOMD:	Human Oral Microbiome Database
HPV:	human papillomavirus
HPV-ve:	HPV-negative
HPV+ve:	HPV-positive
HR:	hazard ratio
HS3ST1:	heparan sulfate-glucosamine 3-sulfotransferase 1
I^2:	I-square
Ig:	Immunoglobulin
InDels:	insertion/deletion variants
IQR:	interquartile range
KEGG:	Kyoto Encyclopedia of Genes and Genomes
LC:	lung cancer

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lncRNAs:	long non-coding RNAs
MAF:	mutant allele frequency
MGMT:	O-6-methylguanine-DNA methyltransferase
microRNAs:	miRNAs/miRs
miRbase:	miRNA data base
miRISC:	miRNA-induced silencing complex
miRNA-seq:	miRNA-sequencing
miRNome:	miRNA profile
mRNA:	messenger RNA
m*SEPT9*:	methylated *Septin9*
MSP:	methylation-specific PCR
MT-DNA:	mutant DNA
MTHFR:	methylenetetrahydrofolate reductase enzyme
N:	normal control
NA:	not available
NCCs:	non-cancer controls
NcRNAs:	non-coding RNAs
NDRG2:	NDRG family member 2
NGF:	nerve growth factor
NGS:	Next-Generation Sequencing
NLR:	negative likelihood ratio
NOS:	Newcastle-Ottawa Scale
NP:	not performed
nt:	nucleotides
OC:	oral cancer
OCSCC:	oral cavity squamous cell carcinoma
OEC:	oesophageal cancer
OFNASET:	Oral Fluid NanoSensor Test
OPC:	oropharyngeal cancer
OPMD:	oral potentially malignant disorders

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OPSCC:	oropharyngeal squamous cell carcinoma
OR:	odds ratio
OS:	overall survival
OSCC:	oral squamous cell carcinoma
OTSCC:	oral tongue squamous cell carcinoma
PC:	pancreatic cancer
PCR:	polymerase chain reaction
PDE4B:	phosphodiesterase 4B
PFS:	progression-free survival
PI3K/AKT:	phosphoinositide 3-kinase/protein kinase B
piRNAs:	piwi-interacting RNAs
PLR:	positive likelihood ratio
Pre-miRNA:	precursor miRNA
Pri-miRNA:	primary miRNA
PTEN:	Phosphatase and tensin homolog
PRISMA:	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSA:	prostate-specific antigen
qMSP:	quantitative-MSP
qPCR:	quantitative real-time PCR
QUADAS-2:	Quality Assessment of Diagnostic Accuracy Studies-2 tool
RAN-GTP:	GTP-binding nuclear protein RAN
RBP1:	retinol binding protein 1
RDOR:	relative diagnostic odds ratios
RISC:	RNA-induced silencing complex
RNAi:	RNA interference
RNA-seq:	RNA-sequencing
ROC:	receiver operating characteristic
RQ:	relative quantification

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RT-qPCR:	reverse transcription-qPCR
scfDNA:	salivary cell-free DNA
SD:	standard deviation
Se:	sensivity
SFAAs:	salivary free-amino acids
SKB:	Salivaomics Knowledge Base
SMIRs:	small molecule inhibitors of miRNAs
snoRNAs:	small nucleolar RNA
snRNAs:	small nuclear RNAs
SNVs:	single nucleotide variants
Sp:	specifity
SPARC:	secreted protein acidic and rich cysteine
SPK:	Saliva Proteome Knowledge
SPKB:	Saliva Proteome Knowledge Base
SOX6:	SRY-box transcription factor 6
SPW:	Salivary Proteome Wiki
SROC:	summary receiver operating characteristic
Std. Err.:	standard error
stDNA:	salivary tumor DNA
T:	tumor
TCGA:	The Cancer Genome Atlas
TLDA:	TaqMan Low-Density Human Array
TN:	true negative
TNM:	Tumor-Node-Metastasis
TP:	true positive
TRBP:	TAR RNA-binding protein
TSS:	transcription start sites
TST170:	TrueSight 170 panel
TTYH1:	tweety family member 1
UICC:	Union for International Cancer Control

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USFDA:	United States Food and Drug Administration
UTR:	untranslated regions
VAF:	variant allele frequency
VCE:	video capsule endoscopy
VEP:	Ensembl Variant Effect Predictor
WT-DNA:	wild-type DNA

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ANNEX 1

Ethical Considerations

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DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonómico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 29/10/2013 el estudio:

Título: Detección de biomarcadores tumorales en saliva. Biomarksa project
Promotor: María Mercedes Suárez Cunqueiro
Código de Registro CEIC de Galicia: 2013/371

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y el la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

Emite un **INFORME FAVORABLE** para la realización del estudio en los centros y con los investigadores siguientes

Centros	Investigadores Principales
USC (Facultad de Mediciana e Odontoloxía)	María Mercedes Suárez Cunqueiro
C.H. Universitario de Santiago	Rafael López López

En Santiago de Compostela, a 05 de noviembre de 2013
La Secretaria

Paula M. López Vázquez

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DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE SANTIAGO-LUGO

Cristina Márquez Riveras, Secretaria del Comité de Ética de la Investigación de Santiago-Lugo,

CERTIFICA:

Que este Comité evaluó en su reunión del día 21/06/18 el estudio:

Título: Detección de biomarcadores en saliva y plasma de pacientes con cáncer de cabeza y cuello
Versión:
Promotor/a:IDIS, CHUS, CHUAC, INIBIC
Investigador/a: Rafael López López; M.ª Mercedes Suárez Cunqueiro; José Luis López-Cedrún
Código de Registro:2018/003

Y que este Comité, tomando en consideración la pertinencia del estudio, el conocimiento disponible, los requisitos legales aplicables y los Procedimientos Normalizados de Trabajo del Comité, emite un dictamen **FAVORABLE** para la realización del citado estudio.

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Y HACE CONSTAR QUE:

1.- El Comité Territorial de Ética de la Investigación de Santiago-Lugo cumple tanto en su composición como en sus PNTs los requisitos legales vigentes.

2.- La composición actual del Comité Territorial de Ética de la Investigación de Santiago-Lugo es:

- **Juan Manuel Vázquez Lago (Presidente).** Médico especialista en Medicina Preventiva y Salud Pública. Área de Gestión Integrada de Santiago.
- **Pilar Rodríguez Ledo (Vicepresidenta).** Médico especialista en Medicina Familiar y Comunitaria. Área de Gestión Integrada de Lugo.
- **Cristina Márquez Riveras (Secretaria).** Enfermera. Dirección Xeral de Saúde Pública. Consellería de Sanidade.
- **Lorenzo Armenteros del Olmo (Secretario Suplente).** Médico especialista en Medicina Familiar y Comunitaria. Área de Gestión Integrada de Lugo.
- **Francisco Campos Pérez.** Biólogo. Fundación Instituto de Investigación Sanitaria de Santiago de Compostela.
- **Rosana Castelo Domínguez.** Farmacéutica de Atención Primaria. Área de Gestión Integrada de Santiago.
- **Ricardo García Martínez.** Licenciado en Derecho. Área de Gestión Integrada de Lugo.
- **Jaime Gulín Dávila.** Farmacéutico especialista en Farmacia Hospitalaria. Área de Gestión Integrada de Lugo.
- **Víctor Herrán Carreira.** Paciente. ADIL-Asociación de Diabéticos Lucense.
- **María Jesús Lamas Díaz.** Farmacéutica especialista en Farmacia Hospitalaria. Área de Gestión Integrada de Santiago.
- **Guillermo José Prada Ramallal** Médico especialista en Farmacología Clínica. Área de Gestión Integrada de Santiago. Fundación Instituto de Investigación Sanitaria de Santiago de Compostela.
- **Carlos Rodríguez Moreno.** Médico especialista en Farmacología Clínica. Área de Gestión Integrada de Santiago.
- **Sandra Vidal Martínez.** Enfermera. Área de Gestión Integrada de Santiago
- **Rafael Carlos Vidal Pérez.** Médico especialista en Cardiología. Área de Gestión Integrada de Lugo.

Para que conste donde proceda, y a petición de quien proceda, en Santiago de Compostela,

La Secretaria del Comité Territorial de Ética de la Investigación de Santiago Lugo,

Cristina Márquez Riveras

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ANNEX 2

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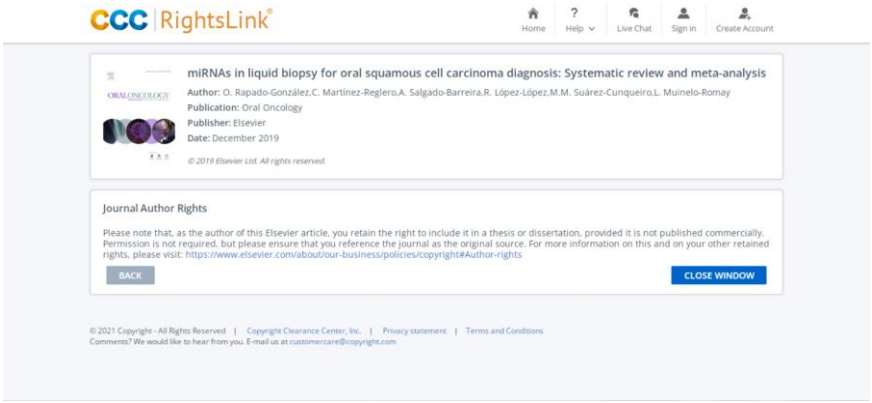

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miRNAs in liquid biopsy for oral squamous cell carcinoma diagnosis: Systematic review and meta-analysis

Author: O. Rapado-González,C. Martínez-Reglero,A. Salgado-Barreira,R. López-López,M.M. Suárez-Cunqueiro,L. Muinelo-Romay
Publication: Oral Oncology
Publisher: Elsevier
Date: December 2019

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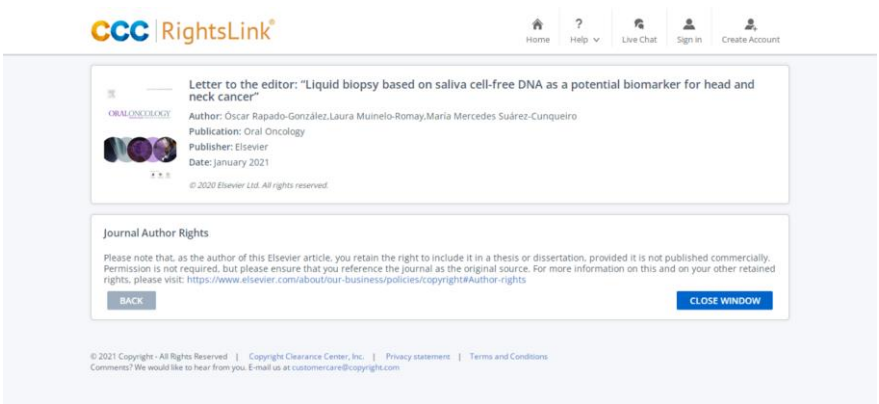

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Letter to the editor: "Liquid biopsy based on saliva cell-free DNA as a potential biomarker for head and neck cancer"

Author: Óscar Rapado-González,Laura Muinelo-Romay,María Mercedes Suárez-Cunqueiro
Publication: Oral Oncology
Publisher: Elsevier
Date: January 2021

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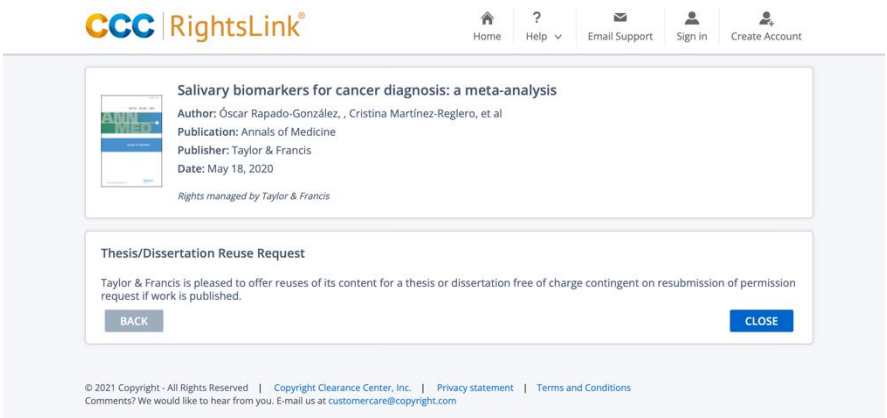

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Salivary biomarkers for cancer diagnosis: a meta-analysis

Author: Óscar Rapado-González, , Cristina Martínez-Reglero, et al
Publication: Annals of Medicine
Publisher: Taylor & Francis
Date: May 18, 2020

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ANNEX 3

Publications derived from this Thesis

(In chronological order)

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Cancer Salivary Biomarkers for Tumours Distant to the Oral Cavity

https://www.mdpi.com/1422-0067/17/9/1531/htm

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Human Salivary microRNAs in Cancer

https://www.jcancer.org/v09p0638.htm

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Liquid Biopsy in Oral Cancer

https://www.mdpi.com/1422-0067/19/6/1704

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Circulating microRNAs as promising biomarkers in Colorectal Cancer

https://www.mdpi.com/2072-6694/11/7/898

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Cell-free microRNAs as Potential Oral Cancer Biomarkers: Forms Diagnosis to Therapy.

https://www.mdpi.com/2073-4409/8/12/1653

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MiRNAs in Liquid Biopsy for Oral Squamous Cell Carcinoma Diagnosis: Systematic Review and Meta-analysis

https://www.sciencedirect.com/science/article/abs/pii/S1368837519303768?via%3Dihub

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A Novel Saliva-Based miRNA Signature for Colorectal Cancer Diagnosis

https://pubmed.ncbi.nlm.nih.gov/31757017/

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Salivary Biomarkers for Cancer Diagnosis: A Meta-analysis

https://www.tandfonline.com/doi/full/10.1080/07853890.2020.1730431

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Liquid Biopsy based on Saliva Cell-Free DNA as a Potential Biomarker for Head and Neck Cancer

https://www.sciencedirect.com/science/article/abs/pii/S1368837520304528?via%3Dihub

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Saliva Gene Promoter Hypermethylation as a Biomarker in Oral Cancer

https://www.mdpi.com/2077-0383/10/9/1931

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Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part I: A Diagnostic Accuracy Meta-Analysis

https://www.mdpi.com/2075-4426/11/6/568

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Salivary DNA Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part II: A Cancer Risk Meta-Analysis

https://www.mdpi.com/2075-4426/11/7/606

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Liquid biopsies have emerged as an innovative tool in the precision oncology representing a valuable complement to traditional tumor biopsy for detecting, characterizing, and monitoring cancer. The objective of this PhD Thesis was to identify biomarkers with clinical utility in liquid biopsies of saliva and blood from colorectal and head and neck cancer patients. In colorectal cancer, we performed a pioneer characterization of salivary microRNAs and identified potential diagnostic biomarkers. In head and neck cancer, different molecular approaches were carried out to improve its noninvasive characterization, including characterization of salivary cell-free DNA, assessment of somatic mutations in blood and saliva, and profiling of salivary DNA methylation.