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Gene promoter methylation-based biomarkers for early detection and monitoring of oral cancer

Biomarcadores baseados na metilação de promotores de genes para deteção precoce e monitorização de cancro oral

Catarina Martins Diz Lobo Ribeiro

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“Gene promoter methylation-based biomarkers for early detection and monitoring of oral cancer”

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Catarina Martins Diz Lobo Ribeiro

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STUDENT

Catarina Martins Diz Lobo Ribeiro
Student of the 5th year of the Integrated Master in Dentistry at the Faculty of Dental Medicine of the University of Porto

SUPERVISOR

Professor Doutor Rui Manuel Ferreira Henrique
Invited Full Professor at School of Medicine and Biomedical Sciences University of Porto

CO-SUPERVISOR

Doutor Óscar Rapado González
Postdoctoral Researcher (R2A) at Liquid Biopsy Analysis Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago (IDIS), Santiago de Compostela, Spain & Cancer Biology and Epigenetics Group – Research Center of IPO Porto (CI-IPOP) / CI-IPOP@ RISE (Health Research Network) Portuguese Oncology Institute of Porto/ Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), Porto, Portugal.

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“É a Hora!”

Fernando Pessoa

ABSTRACT

Background: According to the WHO's GLOBOCAN network, cancer is a leading cause of death worldwide. The 2022 IARC report notes 389,846 new lip and oral cavity cancer cases and 188,430 deaths globally. Early diagnosis improves treatment outcomes and survival rates. Oral squamous cell carcinomas (OSCC) comprises over 90% of oral malignancies, unfortunately, due to its biological aggressiveness and delayed diagnosis, OSCC has one of the lowest survival rates of all cancers. Epigenetic changes, especially DNA methylation, are crucial in oral carcinogenesis, highlighting the need for effective biomarkers and early detection strategies, including saliva-based liquid biopsies, to reduce mortality and morbidity associated with oral cancer.

Aim: Investigate whether promoter methylation of tumor suppressor genes detected in DNA derived from cells collected by oral lavage samples constitutes an effective panel of biomarkers for the early detection and monitoring of oral cancer.

Data Sources and Methods: Samples of oral rinse with saline solution were collected from three groups: healthy individuals and oral potentially malignant disorders patients from the FMDUP Clinic, and patients with oral cavity or oropharynx carcinoma from IPO Porto. To quantify the methylation of the *RASSF1A* gene promoter, that was previously associated with the presence of oral cancer, DNA was extracted and processed using standard protocols, quantified, and bisulfite-converted for methylation analysis using multiplex quantitative methylation-specific polymerase chain reaction technique (mQMSP).

Results: 39 samples from healthy individuals (control group) and 33 samples from oral cancer patients (cancer group) were analyzed. The A260/A280 ratio and *β-Actin* levels indicate that oral rinse allows for the extraction of a large quantity of DNA with high purity, enabling successful molecular analyses. After DNA extraction and quantification, quantitative assessment of promoter methylation levels of *RASSF1A* was performed using mQMSP. *RASSF1A* showed no amplification compared to the housekeeping gene (*ACTB*) in the control group, indicating that this tumor suppressor gene was not methylated in all samples from healthy individuals. However, when analyzed the methylation

status of *RASSF1A* in the cancer group, we detected its amplification in 3 out of 33 patients (9,09%).

Conclusion: There is a potential of saliva as a non-invasive liquid biopsy for detecting oral cancer through DNA methylation analysis. While the *RASSF1A* promoter showed limited sensitivity but perfect specificity, the search for a salivary gene promoter methylation panel with high sensitivity and specificity remains paramount. Developing such a panel holds promise for significantly improving oral cancer screening, enabling early detection, and ultimately improving patient outcomes. This underscores the importance of ongoing research efforts to use saliva-based biomarkers for the effective management of oral cancer.

KEYWORDS: “oral cancer”, “oral rinse “, gene promoter”, “DNA hypermethylation”, “biomarkers”, “early detection”, “monitoring”.

RESUMO

Introdução: De acordo com a rede GLOBOCAN da OMS, o cancro é uma das principais causas de morte a nível mundial. O relatório da IARC de 2022 refere 389 846 novos casos de cancro do lábio e da cavidade oral e 188 430 mortes a nível mundial. O diagnóstico precoce melhora os resultados do tratamento e as taxas de sobrevivência. Infelizmente, devido à sua agressividade biológica e ao atraso no diagnóstico, o cancro da cavidade oral apresenta taxas de sobrevivência mais baixas. As alterações epigenéticas, especialmente a metilação do ADN, são cruciais na carcinogénese oral, o que realça a necessidade de biomarcadores eficazes e de estratégias de deteção precoce, incluindo biópsias líquidas baseadas na saliva, para reduzir a mortalidade e a morbilidade associadas ao cancro oral.

Objetivo: Investigar se a metilação do promotor de genes supressores tumorais, detetada em DNA derivado de células coletadas através de amostras de lavagem oral, constitui um painel eficaz de biomarcadores para a deteção precoce e monitorização do cancro oral.

Metodologia: Foram recolhidas amostras de lavado oral com soro fisiológico de três grupos: indivíduos saudáveis e doentes com doenças orais potencialmente malignas da Clínica FMDUP, e doentes com carcinoma da cavidade oral ou orofaringe do IPO Porto. Para quantificar a metilação do promotor do gene *RASSF1A*, que foi previamente associado à presença de cancro oral, o ADN foi extraído e processado usando protocolos padrão, quantificado e convertido em bissulfito para análise da metilação usando a técnica mQMSP (multiplex quantitative methylation-specific PCR).

Resultados: Foram analisadas 39 amostras de indivíduos saudáveis (grupo de controlo) e 33 amostras de doentes com cancro oral (grupo de cancro). A relação A260/A280 e os níveis de *β-Actina* indicam que o lavado oral permite a extração de uma grande quantidade de ADN com elevada pureza, possibilitando análises moleculares bem-sucedidas. Após a extração e quantificação do ADN, foi efetuada uma avaliação quantitativa dos níveis de metilação do promotor de *RASSF1A* utilizando mQMSP. O *RASSF1A* não apresentou amplificação em comparação com o gene housekeeping (ACTB) no grupo de controlo, indicando

que este gene supressor de tumor não estava metilado em todas as amostras de indivíduos saudáveis. No entanto, quando analisámos o estado de metilação do *RASSF1A* no grupo de cancro, detetámos a sua amplificação em 3 de 33 doentes (9,09%).

Conclusão: Existe um potencial da saliva como uma biópsia líquida não invasiva para detetar cancro oral através da análise da metilação do ADN. Embora o promotor *RASSF1A* tenha mostrado sensibilidade limitada, mas especificidade perfeita, a procura por um painel de metilação do promotor do gene salivar com alta sensibilidade e especificidade permanece crucial. Desenvolver tal painel promete, assim, melhorar significativamente o rastreio do cancro oral, permitindo a deteção precoce e, em última análise, melhorar os resultados para os pacientes. Isso sublinha a importância das pesquisas contínuas para utilizar biomarcadores baseados em saliva para o controlo eficaz do cancro oral.

PALAVRAS-CHAVE: “cancro oral”, “lavado oral”, “promotor de genes”, “hipermetilação do ADN”, “biomarcadores”, “deteção precoce”, “monitorização”.

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INDEX OF ABBREVIATIONS

ACTB – actin beta;

CpG - cytosine-phosphate-guanine;

DNA - deoxyribonucleic acid;

FMDUP - Faculdade de Medicina Dentária da Universidade do Porto;

HNC – head and neck cancer

HNSCC - head and neck squamous cell carcinoma

HPV- human papilloma virus;

IPO - Instituto Português de Oncologia;

mQMSP – multiplex quantitative methylation-specific PCR;

MSP- methylation-specific PCR;

OC- oral cancer;

OPMD – oral potentially malignant disorders

OSCC- oral squamous cell carcinomas;

PCR - polymerase chain reaction;

qMSP –quantitative methylation-specific PCR;

RASSF1A - RAS association domain family 1 isoform A;

RNA - ribonucleic acid;

rpm – rotation per minute;

TNM - tumor-node-metastasis;

TSG- tumor suppressor gene

UNK – unknown;

Wnt - Wingless-related integration site;

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CHAPTER I

INTRODUCTION AND AIMS

1. INTRODUCTION AND AIMS

According to data from the World Health Organization's GLOBOCAN network, cancer is a leading cause of death worldwide. The International Agency for Research on Cancer 2022 report shows a total of 389,846 new cases of lip and oral cavity cancer and 188,430 deaths globally (1). These cancer types are highly prevalent in South Central Asia, Melanesia and India, with the highest incidence rate in both sexes. Incidence rates are also high in Eastern and Western Europe and Australia/New Zealand, and are linked to various risk factors such as alcohol consumption, betel nut chewing, tobacco smoking and human papilloma virus (HPV) infection (for oral and oropharyngeal cancers), as well as ultraviolet radiation from sunlight exposure (for lip cancer)(2, 3). The condition is, therefore, linked to social and economic status and deprivation, with the highest rates occurring in the most disadvantaged sections of the population.(4)

Oral cancer (OC) rates are currently twice as high in men as in women, and the risk of developing OC increases with age, with more cases occurring in people over the age of 50 (4). There has also been a significant increase in cases in the older population, partly due to increased longevity. It is estimated that there will be a 66.2% increase in new cases over the next 20 years. Between 2020 and 2040, it is estimated that developed countries, including the United States of America, France, the United Kingdom, Japan, Italy, and Australia, will experience an increase in new cases of 24.9% to 50.5%. Developing countries such as China, India, and Brazil, are projected to experience an even greater increase, ranging from 80.1% to 97.8%.

The delayed diagnosis of OC is strongly related to the population's lack of knowledge about its signs and symptoms and the devaluation of self-care, which contributes to the increasing numbers of cases. Moreover, the delayed diagnosis can lead to increased care costs for outpatient or prolonged hospital stays, particularly affecting those who are economically disadvantaged (5).

Early diagnosis of OC leads to the implementation of appropriate treatment in the early stages of disease evolution. This promotes preventive actions, less invasive surgeries, faster convalescence, and better patient survival (5). The treatment of patients with early-stage OC entails a favorable prognosis, with improved rates of survival and quality of life. However, early-stage cancers are often asymptomatic and mimic benign conditions, reducing the likelihood of patients seeking care. Therefore, research efforts should be addressed to improve early detection of OC (4).

OSCC account for over 90% of oral malignancies (4, 6). Unfortunately, due to its biological aggressiveness and delayed diagnosis, OSCC has one of the lowest survival rates of all cancers. In Europe, the five-year survival rate is approximately 50%, while in India, it is below 35%. Importantly, early detection of OC can increase the five-year overall survival rate up to 80%, but this falls to less than 20% when diagnosed at advanced stages with the involvement of regional lymph nodes and distant metastasis (6) (7).

Improved public education and the involvement of physicians and dentists are necessary to detect and promptly refer any suspected malignancies.

Early lesions can be effectively treated with minimal adverse effects, whereas treatments for advanced cancer result in serious sequelae, such as disturbances of facial appearance and oral functions, leading to poor quality of life, and, ultimately, death (7).

The treatment of OC presents clinicians with challenging and complex clinical problems that require a multidisciplinary approach, the solutions to which have a significant impact on patients' survival and quality of life.

The head and neck are responsible for critical functions such as breathing, speech, deglutition, sight, smell, taste, mastication, and jaw function. These functions can be impaired, either temporarily or permanently, by the tumor or its treatment. Furthermore, this may severely affect our facial and dental aesthetics, as well as our self-esteem and self-confidence, while impacting how we are perceived by others. Therefore, due to the functional and aesthetic implications

of treating tumors in this region, it is essential to involve a Multidisciplinary Head and Neck Oncology Team in OC management (7).

Long-term follow-up is necessary for every patient with OC. Clinical and radiologic surveillance is important for detecting tumor recurrence and second primary tumors. Moreover, OC treatment involves a significant morbidity that requires further rehabilitation and treatment, including, but not limited to, speech and swallow rehabilitation, the preservation of the remaining dentition and the restoration of missing dentition, as well as the management of xerostomia. Importantly, the psychologic and social morbidity of the cancer diagnosis and treatment must also not be overlooked and should be addressed (8).

Early detection is crucial in reducing mortality and morbidity associated with this disease and the lack of effective screening protocols was highlighted as a major barrier for early detection. Identifying which oral potentially malignant disorders will develop into malignancy remains a challenge, as the malignant transformation is not consistent. Oral health care professionals could drastically improve the quality of life for patients with potentially malignant oral lesions by using a non-invasive test to detect cancer (9). Then, there is an urgent need to identify new and effective biomarkers for OC screening, diagnosis, and prognosis (6).

Biomarker is a “characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention”, and in this line, various molecules including DNA, RNA, proteins, and metabolites have been identified as potential biomarkers in OSCC (6).

From a molecular standpoint, oral carcinogenesis is a multistep process characterized by an accumulation of multiple genetic and epigenetic alterations in key regulatory genes leading to the transformation of normal oral epithelial cells into OSCC (10). One of the most investigated epigenetic alterations is the DNA methylation which is involved in regulating many cellular processes such as embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, and chromosome stability (11). DNA methylation is a process which involves the covalent addition of a methyl group from the

cofactor S-adenosylmethionine to the carbon-5 position of the pyrimidine ring of cytosine present in cytosine-phosphate-guanine (CpG) dinucleotides (so-called CpG islands) to form 5-methylcytosine. In a great majority of human genes, about 60% of CpG islands are in promoter gene region and normally they are (remaining) unmethylated in transcriptionally active genes and methylated in silenced genes (12, 13). Therefore, DNA methylation in the promoter region is recognized as a common epigenetic mechanism of transcriptional regulation, and its alteration has been involved with development of numerous malignancies. It is widely known that cancer genomes are characterized by widespread aberrations in DNA methylation patterns including site-specific hypermethylation of tumor suppressor genes (TSG) and global DNA hypomethylation of mostly repetitive sequences that induces genomic instability and activates silenced oncogenes (13, 14) (*Figure 1*).

Importantly, DNA methylation alterations are considered an early epigenetic event in cancer playing an important role in tumor development and progression. Thus, CpG island hypermethylation within gene promoter region has been identified in oral carcinogenesis as an important epigenetic mechanism of transcriptional silencing of several tumor-suppressor genes involved in a broad range of cellular processes including cell cycle control, apoptosis, Wingless-related integration site (Wnt) signaling, cell-cell adhesion, and DNA-repair (15). Moreover, these tumor-specific DNA methylation alterations can be detected in body fluids, representing a potential tumor biomarker for diagnosis, prognosis, and treatment response prediction by a liquid biopsy-based approach (9).

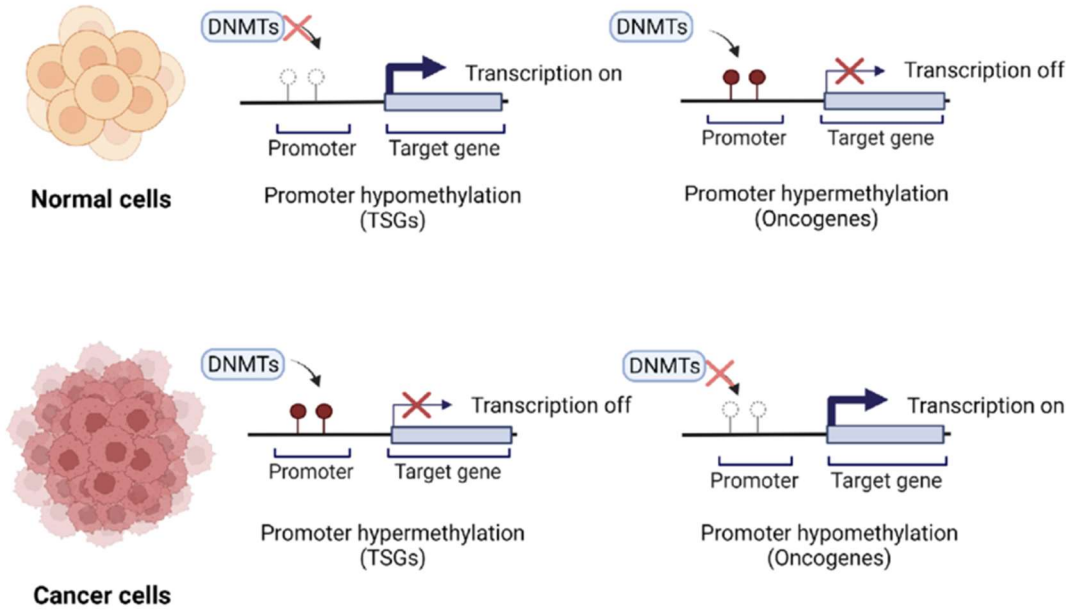

Figure 1. *DNA methylation pattern in different cell types. - In typical cells, the promoter regions of tumor suppressor genes (TSGs), which regulate the cell cycle, are hypomethylated. On the other hand, in oncogenes, acting as transcription factors, these regions are often hypermethylated by DNA methyltransferases (DNMTs). Cancer cells exhibit an inverse pattern, with TSGs displaying hypermethylation and oncogenes featuring hypomethylated promoter regions. Image obtained from "Borges, Barbara. (2022). Epigenetic alterations in canine mammary cancer. Genetics and Molecular Biology. 45. 10.1590/1678-4685-gmb-2022-0131".*

Then, gene promoter hypermethylation is a critical step in OC development and has great potential as a biomarker for early diagnosis, tumor molecular subtyping, prognosis, monitoring, and therapy (10).

Nowadays, effective screening and surveillance approaches consider the collection of genomic material through minimally invasive methods. Since aberrant DNA methylation can be identified in biological fluids such as blood, saliva, urine, sputum, bronchial lavage fluid, or ductal fluids, the study of DNA methylation using liquid biopsies has emerged as a potential and minimally invasive strategy for cancer screening, diagnosis, prognosis, and treatment

monitoring. In the last decade, saliva-based liquid biopsy has been recognized as an attractive approach for cancer detection due to its collection being fast, reliable, cost-effective, and non-invasive, allowing to analyze different molecular markers like genomic and epigenomic alterations involved in the onset and course of the disease (16).

Importantly, since saliva is in direct contact with the oral mucosa and precancerous lesions, the identification of novel salivary biomarkers with diagnostic potential has aroused a great interest for improving OC detection. In this line, since DNA methylation is an early epigenetic event in oral carcinogenesis, the detection of tumor-specific DNA methylation alterations in saliva has emerged as a potential tool for OC management (*Figure 2*).

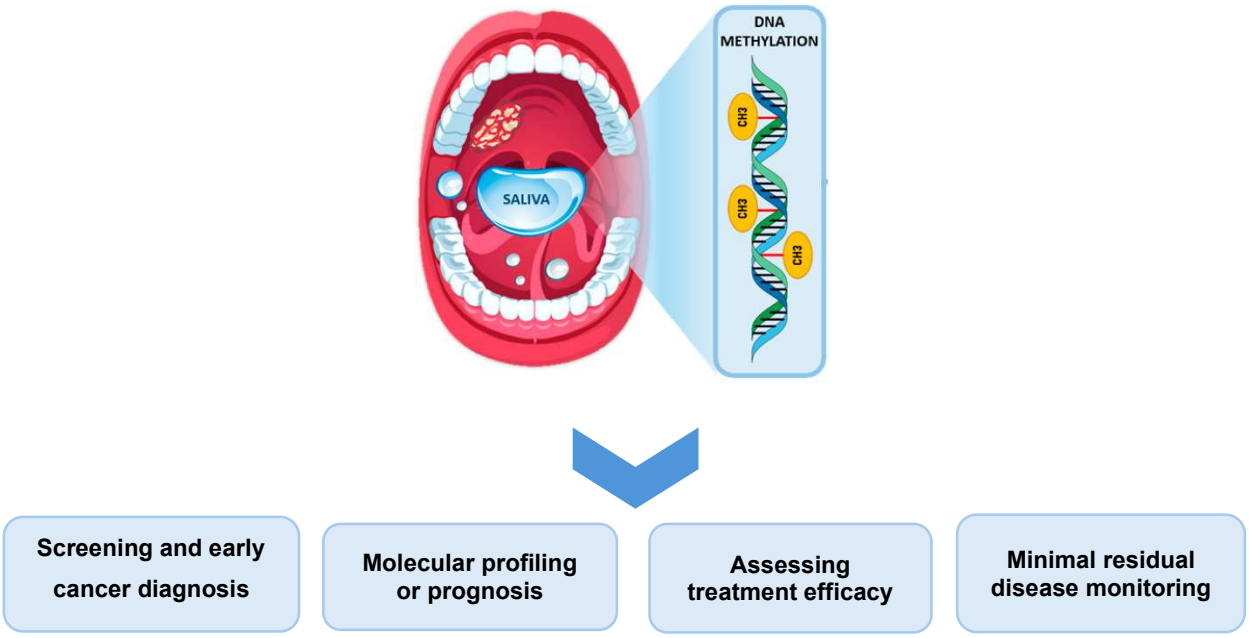

Figure 2. *Potential clinical application of saliva-based DNA methylation biomarkers in oral cancer. Adapted with permission from "Rapado-González Ó, López-Cedrún JL, López-López R, Rodríguez-Ces AM, Suárez-Cunqueiro MM. Saliva Gene Promoter Hypermethylation as a Biomarker in Oral Cancer. J Clin Med. 2021;10(9)".*

In this context, the main goal of this research project is to investigate whether promoter methylation of TSG detected in DNA derived from cells collected by oral lavage samples constitutes an effective panel of biomarkers for the early detection of OC.

CHAPTER II

DATA SOURCES AND METHODS

2. DATA SOURCES AND METHODS

2.1 ETHICAL CONSIDERATIONS

The current investigation received approval from the Health Ethics Committee of the Faculty of Dental Medicine of the University of Porto (Annex 1), the Data Protection Officer of the University of Porto (Annex 2), the responsible for access to clinical records information (Annex 3) and from the Health Ethics Committee and Data Protection Officer of IPO Porto (Annex 4).

The study was conducted on samples collected voluntarily from two populations: a control population from the healthy and oral potentially malignant disorders (OPMD) patients at the Faculdade de Medicina Dentária da Universidade do Porto (FMDUP) Clinic, and a case population from the Head and Neck Clinic at IPO Porto, consisting of patients diagnosed with oral or oropharyngeal cancer.

During the sample collection process, participants, aged 18 years and above, completed a brief questionnaire including data on age, gender, smoking habits (quantified), alcohol consumption (quantified), as well as relevant details to the statistical analysis of biomarkers' performance, such as other potential risk factors, history of cancer, including oral cavity cancer (if so, for how long), current pathologies, usual medication and clinical condition that led to treatment at the FMDUP Clinic.

The patients did not incur in any embarrassment or additional examination to carry out this investigation, and there are no risks, discomforts and/or costs for the participants. It was a free and voluntary participation, whose decision to participate or not to participate did not imply any change in the diagnostic and therapeutic plan. Participants contributed in a voluntary, free, and informed way to scientific research in the field of Oncology, allowing to test new strategies for early detection of oral cavity cancer, with potential impact on improving therapeutic efficacy and survival.

For the purposes of anonymity, the samples and the questionnaire were collected confidentially and numbered (pseudo-anonymization), rather than

identified by name. The patient's data were stored in a protected, encrypted and password-protected database, which was analyzed solely and exclusively for research purposes, guaranteeing the anonymity of the participants with the codification of the records, making it impossible for the researchers to identify them, as only the main researcher has access to the coding key for the records, which will be destroyed 5 years after the end of the project. The general results may also be disseminated to the community of health professionals and the scientific community, through the publication of scientific articles and participation in congresses.

Thus, this study guaranteed the privacy, confidentiality, and protection of personal data, in accordance with current legislation.

2.2 SAMPLE SELECTION

Samples were gathered from a total of 3 groups: healthy individuals from the FMDUP Clinic (control population – n=39), OPMD patients also observed at the FMDUP Clinic (control population – n=3)), and patients diagnosed with carcinoma of the oral cavity at the Head and Neck Clinic at IPO Porto (case population – n=33).

For the control population, oral lavage and oral exfoliative cytology samples were collected from patients at the FMDUP clinic who met the following criteria: age over 18 years old and no prior history of oral cavity or oropharyngeal carcinoma.

Similarly, for the case population, saliva and oral exfoliative cytology samples were collected from patients diagnosed with carcinoma of the oral cavity or oropharynx, at the Head and Neck Clinic at IPO Porto. These patients were also older than 18 years, had a histopathological diagnosis of primary carcinoma of the oral cavity or oropharynx, no prior surgery, radiotherapy or chemotherapy treatment and no previous history of cancer in the last 5-years.

2.3 SAMPLES COLLECTION PROTOCOL

For the oral rinse samples, the patient was asked to rinse his/her mouth with saline solution (10ml – 0,9% NaCl), vigorously during approximately 60 seconds, and discard it into the sterilized sample cup. The samples were then stored in a refrigerator at a temperature between 2-4ºC, to preserve the sample until it was processed, within 24 hours, at the Biobank of IPO Porto (Laboratory of the Epigenetics and Cancer Biology Group of the IPO Porto Research Center), to prevent pathogens proliferation that could alter the sample.

For the oral exfoliative cytology samples, the entire surface of the lesion (premalignant or malignant) – for the OPMD and OC patients and the surfaces of both mucous membranes jugals, dorsum, lateral edge, posterior, and ventral aspect of the tongue – for the healthy control patients were vigorously scrubbed with the scraping brush, rotating the scaling brush repeatedly between 6-8 times. After scraping, we inserted the scraping brush with the collected sample into the "ThinPrep ® PreservCyt®" solution vial, shook it vigorously and rotated it several times counterclockwise so that the oral exfoliated cells were suspended in the liquid medium. The scraping brush was thrown away in the biological waste and the ThinPrep® PreservCyt® solution vial was stored at room temperature for further analysis.

2.4 SAMPLE PROCESSING

2.4.1 DNA EXTRACTION AND QUANTIFICATION

Saliva samples underwent centrifugation at 2600xg for 15 minutes to isolate cell pellets, which were subsequently washed in 500 µL of Phosphate-Buffered Saline (PBS) and preserved at -80 ºC for further analysis (*Figure 3*).

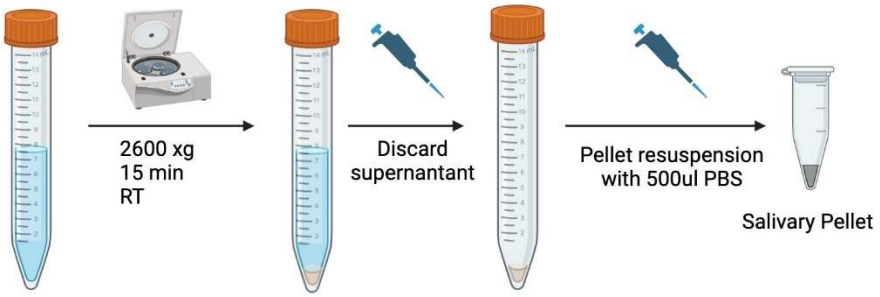

Figure 3. *Schematic representation of saliva sample processing. Created by BioRender (2024).*

For DNA extraction, the cell pellets were treated with SE buffer, 10% SDS, and proteinase K (NZYTech, Portugal). DNA was then extracted from all samples using a standard protocol involving phenol-chloroform, ethanol, and ammonium chloride. Following digestion, samples were transferred to Phase Lock Light 2mL tubes and mixed with 500 µL of phenol-chloroform pH=8 (PC8; Sigma-Aldrich, USA), followed by centrifugation at 13000 rpm for 15 minutes at 4ºC. The aqueous phase was transferred to a new 2 mL tube and the process was repeated. Subsequently, the aqueous phase was transferred to a Safe-Lock 2 mL tube, and DNA precipitation was achieved by mixing with cold absolute ethanol (2x the volume of DNA) (Merck Millipore, Germany) and 7.5M ammonia acetate (1/3 the volume of DNA), then incubating overnight at -20ºC. Following centrifugation at 13,000 rpm for 20 minutes, the supernatant was discarded. The pellets were washed twice with 70% cold ethanol and air-dried. Finally, the pellets were eluted in 30 µL of sterile bi-distilled water. All DNA elutions were stored at -20°C until further use.

The quantification of DNA from the cell pellets was conducted using a NanoDrop Lite Spectrophotometer (Nanodrop Technologies, USA). For each sample employed in this analysis, 1 µL of DNA was quantified.

2.4.2 SODIUM-BISULFITE MODIFICATION

Amongst the methodologies around quantitative evaluation and analysis

of DNA methylation, that can be divided into bisulfite conversion-based and non-bisulfite conversion methods, qPCR, also known as quantitative methylation specific PCR (qMSP), proves to be highly suitable. Similar to traditional qualitative MSP, the qMSP involves DNA treatment with sodium-bisulfite modification (17) , a gold standard procedure in epigenetics studies and DNA methylation analysis. Bisulfite-conversion based methods are only susceptible to unmethylated cytosines and they allow the differentiation in methylation-dependent sequences at CpG dinucleotides, by converting unmethylated cytosine to uracil by deamination, which is subsequently converted to thymine during PCR amplification, while methylated cytosines remain unchanged (5-methylcytosines)(10) (*Figure 4*).

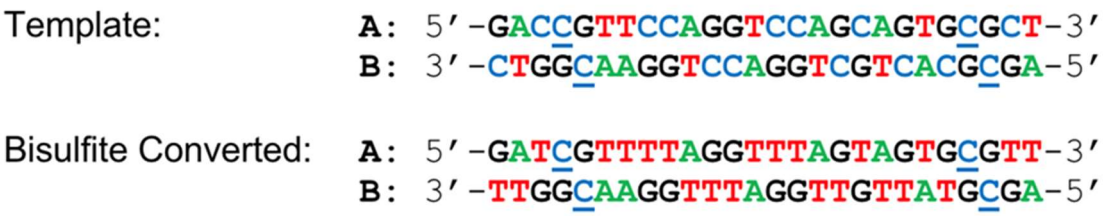

Figure 4. *Bisulfite conversion of double stranded DNA templates. Methylated "C" is underlined in the examples. https://files.zymoresearch.com/protocols/_d5005_d5006_ez_dna_methylation-goldga_o_kit.pdf*

This chemical reaction is divided in 3 steps:

1. **Sulphonation**: addition of sodium bisulfite to the double bond of cytosine to form a cytosine-bisulfite derivative. This reaction is reversible, favored by high temperature and low pH;
2. **Deamination**: irreversible hydrolytic deamination of the cytosine-bisulfite derivative resulting in an uracil-bisulfite derivative, also favored by low pH;
3. **Desulphonation**: removal of the sulphonate group in the uracil-bisulfite to originate an uracil residue, under high pH conditions (18).

For the purposes of the study, all DNA samples were converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) (*Figure 5*), following the manufacturer's instructions.

Initially, 130 µL of CT conversion reagent solution was combined by pipetting an equivalent volume to 20 µL of the previously extracted and quantified DNA samples plus water.

Subsequently, these samples underwent centrifugation at 4000 rpm for 30 seconds, following a program of incubation at 98ºC for 10 minutes to facilitate DNA denaturation, followed by 64°C for 180 minutes, divided in 3 cycles of 60 minutes, to achieve bisulfite conversion, and finally by 4ºC for up to 18h. These steps were carried out in the Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA).

Each sample was then added into a Zymo-Spin™ IC columns, along with 600 µL of M-Binding Buffer, allowing it to incubate for 10 minutes (binding of converted single-stranded DNA to the membrane). The columns were centrifuged at 10,000 rpm for 30 seconds, discarding the *follow-through*. After, 100 µL of M-Wash Buffer were added, followed by another round of centrifugation, with no need of discarding the *follow-through*.

A further addition of 200 µL of M-Desulphonation Buffer was made to the columns, which allowed for the removal of DNA desulfonation residues, followed by a 15 to 20 minutes incubation at room temperature and centrifugation period. These columns were washed twice with 200 µL of M-Wash Buffer interspersed by a series of centrifugation cycles.

Finally, the columns were transferred to 1.5 mL microcentrifuge and safe-lock tubes and 10 µL (for the DNA from the control population) or 30 µL (for the DNA from the case population) of sterile bi-distilled water was added to elute the modified DNA. After 5 minutes of incubation, the columns were centrifuged at 12,000 rpm for 30 seconds, and this process was repeated twice. Quantify the DNA in the *Nanodrop*, selecting the single-chain DNA option (ssDNA). The final yield was 60 µL of bisulfite-converted DNA. Additionally, 4 µL of Human Methylated & Non-methylated DNA (Zymo Research, USA) were also subjected to modification using the previously mentioned protocol and eluted in 20 µL of sterile bi-distilled water (19)

Figure 5. *EZ DNA Methylation-Gold™ Kit (Zymo Research, USA)- https://files.zymoresearch.com/protocols/_d5005_d5006_ez_dna_methylation-goldga_o_kit.pdf*

2.4.3 MULTIPLEX QUANTITATIVE METHYLATION-SPECIFIC PCR (mQMSP)

Quantitative evaluation of promoter methylation levels of *RASSF1A* was conducted using multiplex quantitative methylation-specific PCR (mQMSP). Primers and probes sequences of *RASSF1A* and *β-Actin* are listed in *Table 1.* The internal reference gene, *β-Actin* (*ACTB*), was used as housekeeping gene for assay normalization. The reactions were carried out in 384-well plates employing a QuantStudioTM 12K Flex Real-Time PCR System (Applied Biosystems, USA). Each well contained 1 µL of bisulfite-modified DNA extracted from samples, 5 µL of Xpert Fast Probe MasterMix (GRiSP, Porto, Portugal) with ROX, an optimized volume of primers (F+R)/probe at 10 µM, and sterile bi-distilled water to achieve a final volume of 10 µL (Figure 6).

The PCR running conditions consisted of an initial cycle at 95ºC for 3 minutes to activate the polymerase, followed by 45 cycles at 95ºC for 5 seconds to denature the DNA and 60ºC for 30 seconds to anneal and extend the DNA. All samples were processed in triplicate. Each plate included three non-template controls and two negative controls (Human HCT116 DKO Non-Methylated DNA

from Zymo Research, USA) to ensure the absence of contamination and specificity for methylated DNA templates. Furthermore, serial dilutions (five, in duplicate) of a positive control (Human HCT116 DKO Methylated DNA from Zymo Research, USA) were included on each plate to establish a standard curve and evaluate run efficiency. Run efficiency was considered valid when the values were between 90% and 100%. The results were plotted as relative methylation levels calculated as the ratio between target gene/housekeeping.

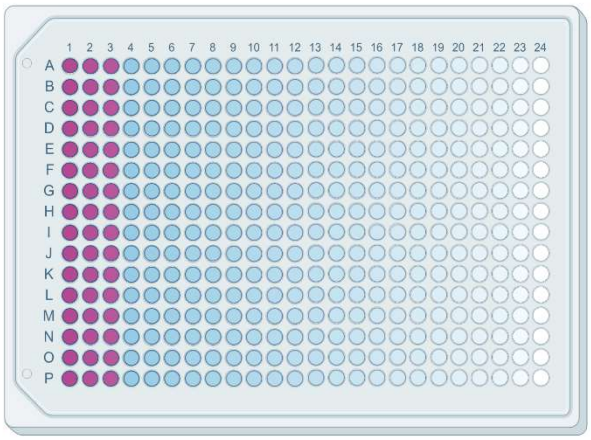

Figure 6. *Plates for analysis of extracted DNA.*

Table 1. *Primers and probes sequences of RASSF1A and β-Actin.*

Genes	**Sequence**	
ACTB	Primers	F – 5´ TGGTGATGGAGGAGGTTTAGTAAGT 3´
		R – 5´ ACCAATAAAACCTACTCCTCCCTTAA 3´
	Probe	5’ Cy5 – ACCACCACCCAACACACAATAACAAACACA – QSY 3’
RASSF1A	Primers	F – 5´ GGGTTTTGCGAGAGCGC 3´
		R – 5´ CGAAACCACAAAACGAACCC 3´
	Probe	5’ Fam – TCGTTTAGTTTGGATTTTGGGGGAGGC – BHQ 3’

CHAPTER III

RESULTS

3. RESULTS

3.1 STUDY COHORT

A total of 39 control and 33 OC patients were included in in our study cohort (*Table 2*).

For the control group, the mean±SD and median (IQR) age of the healthy controls was 63.33±10.52 years and 64 (57.00-71.00) years, respectively, with a range from 41 to 83 years. Concerning gender, 18 were female (46.15%) and 21 were male (53.85%).

Based on risk factors, 6 controls reported tobacco smoking, 14 were ex-smokers, and 24 reported alcohol consumption almost daily. The clinical conditions that led to treatment at the FMDUP Clinic were the following: dental prosthesis consultations (n=11), caries and restorative treatments (n=8), dental scale appointments (n=4), temporomandibular joint, bruxism, or orofacial pain appointments (n=3), tooth extractions (n=3), external whitening (n=1), appointments to remove sutures (n=1) and appointments for oral check-ups (n=8).

For the cancer group, the mean±SD and median (IQR) age of this case population was 63.24±12.07 years and 61 (55.00-69.50) years, respectively, with a range from 42 to 93 years. Regarding gender, 14 were female (42.42%) and 19 were male (57.58%). Based on the risk factors. 17 reported tobacco smoking, 6 were ex-smokers, and 18 reported alcohol consumption almost daily. Regarding to tumor anatomic location, 13 patients had a primary tumor in the tongue, 9 in the floor of mouth, 4 in the gingiva, 3 in the palate, 2 in the buccal mucosa, and 2 in the retromolar trigone. The mean of maximum tumor size was 31,75 mm and in terms of macroscopic clinical appearance 17 patients had endophytic ulcers, 9 had exophytic ulcers, 3 had exophytic ,3 had leucoplakia and 1 had leukoerythroplakia. According to the tumor-node-metastasis (TNM) staging, 6 patients were at Stages I and II, 9 at Stages III - IV whereas for 18 patients this data was not available, and in histopathological grading 5 patients had well, 7 patients had moderately differentiated carcinomas, 3 had poorly, whereas in 18

patients this data was not available either.

Table 2. *Demographic and clinical characteristics of enrolled participants.*

	Oral Cancer (n=33)	Healthy individuals (n=39)
Age (mean ±SD)	63.24 ± 12.07	63.33±10.52
Gender, n (%)		
Male	19 (57.58)	21 (53.85)
Female	14 (42.42)	18 (46.15)
Smoking habits, n (%)		
Smoker	17 (51.52)	6 (15.38)
Non-smokers	10 (30.30)	19 (48.72)
Ex-smokers	6 (18.18)	14 (35.90)
Drinking habits, n (%)		
Drinker	18 (54.55)	24 (61.54)
Non-drinkers	15 (45.45)	15 (38.46)
OPMD, n (%)		
Yes	6 (18.18)	0
No	27 (81.82)	39 (100)
Tumor site, n (%)		
Tongue	13 (39.39)	-
Floor of mouth	9 (27.27)	-
Buccal mucosa	2 (6.06)	-
Retromolar trigone	2 (6.06)	-
Gingiva	4 (12.12)	-
Palate mole	3 (9.09)	-
Maximum tumor size (mean ±SD)	31.75 ± 9.89	-
Macroscopic clinical appearance, n (%)		
Endophytic ulcer	17 (51.52)	-
Exophytic ulcer	9 (27.27)	-
Exophytic	3 (9.09)	-
Leucoplakia	3 (9.09)	-
Leukoerythroplakia	1 (3.03)	-
Clinical TNM staging, n (%)		
I-II	6 (18.18)	-
III-IV	9 (24.24)	-
Unknown	18 (54.55)	-
Histopathological grading, n (%)		
Well	5 (15.15)	-
Moderately	7 (21.21)	-
Poorly	3 (9.09)	-
Unknown	18 (54.55)	-

3.2 DNA QUANTIFICATION AND QUALITY

A total of 72 salivary DNA samples (33 controls and 39 cases), were successfully extracted and quantified.

The mean salivary DNA concentration for the control group was 753.02 ng/µL± 223.98, with a range between 115.9 and 1127 ng/µL, as showed in *Table 3*. Meanwhile, the mean salivary DNA concentration for the case group was 773.29 ng/µL± 213.97 with a range between 72.2 and 1159.6 ng/µL, as depicted in *Table 4*.

The ratio of absorbance wavelengths at 260 nm and 280 nm was used to assess the purity of isolated DNA samples. A ratio of 1.7–2.0 is considered to be pure for DNA, while lower absorbance ratios may indicate the presence of protein, phenol or other contaminants (20). As we shown in Tables 2 and 3, the salivary DNA samples showed high A260/280 ratios, indicating the high purity of isolated DNA for further analysis.

Table 3. *Nanodrop quantification and quality analysis of healthy control (HC) salivary DNA samples (N=39).*

CODE SAMPLE	DNA CONCENTRATION (ng/uL)	A260/280
HC-08	493	1,83
*HC-68**	115,9	1,61
*HC-100**	766,7	1,85
HC-07	727	1,83
HC-32	930,5	1,85
HC-29	1109,2	1,88
HC-33	1076,3	1,88
HC-04	441,5	1,81
HC-42	906,4	1,81
HC-54	368,9	1,78
HC-52	667	1,82
HC-61	846,3	1,85
HC-97	775,9	1,88
HC-37	918,7	2
HC-56	882,9	1,9

HC-47	643,5	1,92
HC-86	624,5	1,89
HC-09	1227	1,89
HC-64	801,8	1,86
HC-99	915,1	1,94
HC-72	1173,1	1,88
HC-22	939	1,85
HC-21	924,8	1,84
HC-80	450,1	1,94
HC-98	819,4	1,91
HC-92	612,8	1,84
HC-82	668,8	1,91
HC-74	782,4	1,91
HC-67	539,9	1,9
HC-96	583,2	1,88
HC-43	859,4	1,81
HC-34	774,1	1,91
HC-25	684,2	1,88
HC-15	769,6	1,85
HC-11	665,5	1,89
HC-24	755,5	1,92
HC-59	754,4	1,93
HC-65	425,4	1,88
HC-91	947,9	1,85

Table 4. *NanoDrop quantification and quality analysis of oral cancer (OC) salivary DNA samples (N=33).*

CODE SAMPLE	DNA CONCENTRATION (ng/uL)	A260/280
OC-18	847,8	1,88
OC-06	466,6	1,83
OC-08	799,2	1,94
OC-01	459,6	1,81
OC-04	1159,6	1,78
OC-15	895,7	1,86
OC-14	841,2	1,83
OC-13	72,2	1,61
OC-11	688,6	1,9
OC-09	880,6	1,82
OC-21	641,9	1,83

OC-20	1014,7	1,87
OC-39	752,6	1,98
OC-25	940,9	1,93
OC-27	836	1,88
OC-22	809,5	1,94
OC-24	886,4	1,84
OC-30	768,8	1,86
OC-40	1005,8	2,02
OC-41	759,4	1,94
OC-34	765	1,92
OC-35	834,7	1,87
OC-26	884,7	1,84
OC-38	262,6	1,83
OC-36	919,8	1,84
OC-19	821,9	1,94
OC-42	970,5	1,95
OC-43	620,2	1,87
OC-44	758,6	1,87
OC-45	585,9	1,9
OC-46	724,5	1,91
OC-47	787,7	1,88
OC-48	1055,4	1,91

3.3 *RASSF1A* PROMOTER METHYLATION STATUS

The methylation status of *RASFF1A* gene promoter was evaluated by mQMSP in the saliva samples of 33 OC patients and 39 healthy controls.

The *ACTB* gene was selected as an endogenous control. This protein lacks CpG sites, ensuring that the PCR-amplified sequence remains unaffected by methylation status, thereby facilitating control over variables in DNA samples. Moreover, the *ACTB* sequence is exclusive to humans, and as the primers designed at IPO Porto target the human actin gene sequence, it enables the detection of human DNA only.

PCR standard curves were used to quantify the quantity of target nucleic

acid present in samples, by plotting the threshold cycle (CT) values against the logarithm of known input amounts of a standard material. Then, standard curves were generated for *ACTB* and *RASSF1A* genes through serial dilutions of a positive control whose initial amount is known (100000 copies) with an efficiency within 90-100% (Figure 7 *and* *Table 5*, *Figure 8* *and* *Table 6*).

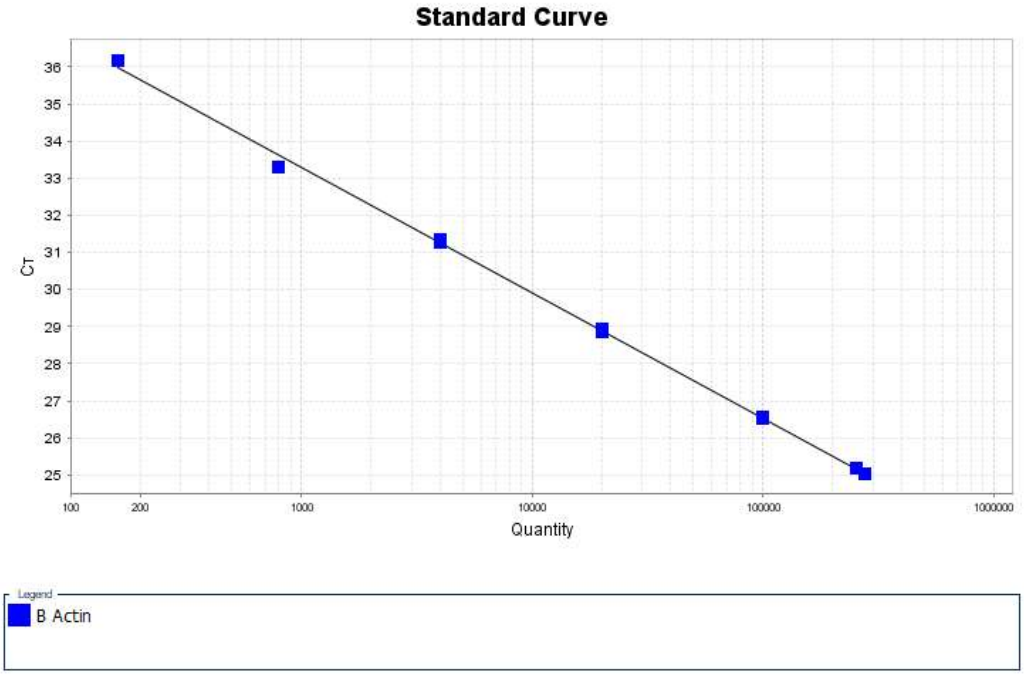

Figure 7. *Standard curve for B-Actin values.*

Table 5. Standard Line Table and Equation for B-Actin.

Standard Line Table and Equation		
Slope	-3.386	y= -3.386x + 44.455
Y- inter	43.455	
R^2	0.998	Ct= -3.386 quantity + 43.455
Eff%	97.398	

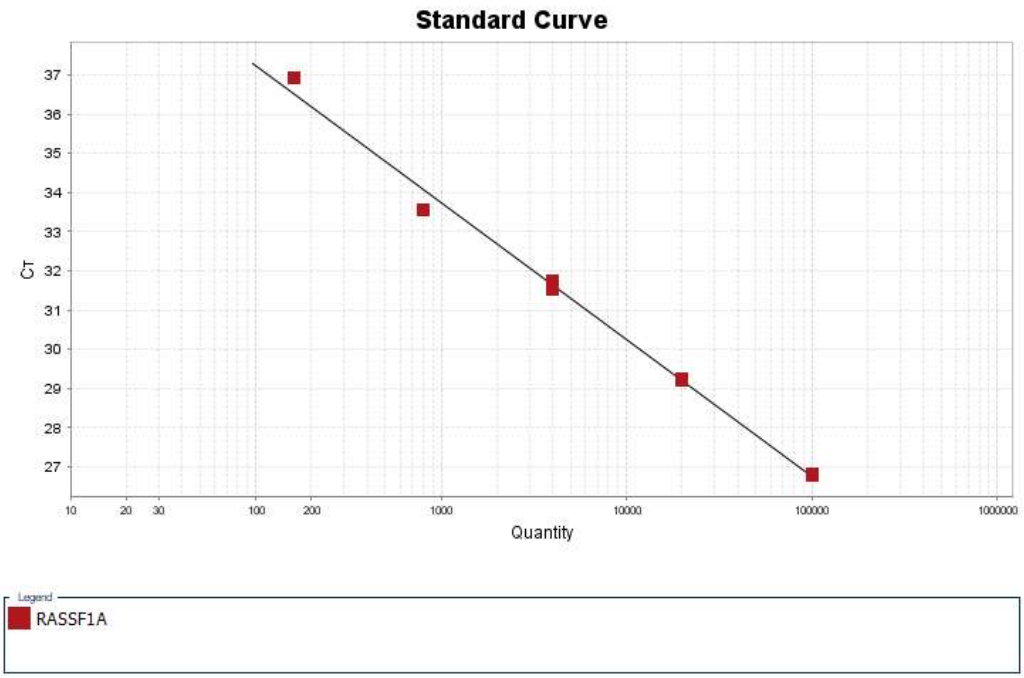

Figure 8. *Standard curve for RASSF1A values.*

Table 6*. Standard Line Table and Equation of RASSF1A.*

Standard Line Table and Equation		
Slope	-3.501	y= -3.501x + 44.257
Y- inter	44.257	
R^2	0.994	Ct= -3.501 quantity + 44.257
Eff%	93.037	

Regarding the amplifications plots of control group *RASSF1A* gene did not exhibit amplification compared to housekeeping gene (*ACTB)*, indicating that this TSG was not methylated in all samples from healthy individuals (Figure 9). However, when analyzed the methylation status of *RASSF1A* in the cancer group, we detected its amplification in 3 of 33 patients (9,09%), as displayed in *Figure 10*.

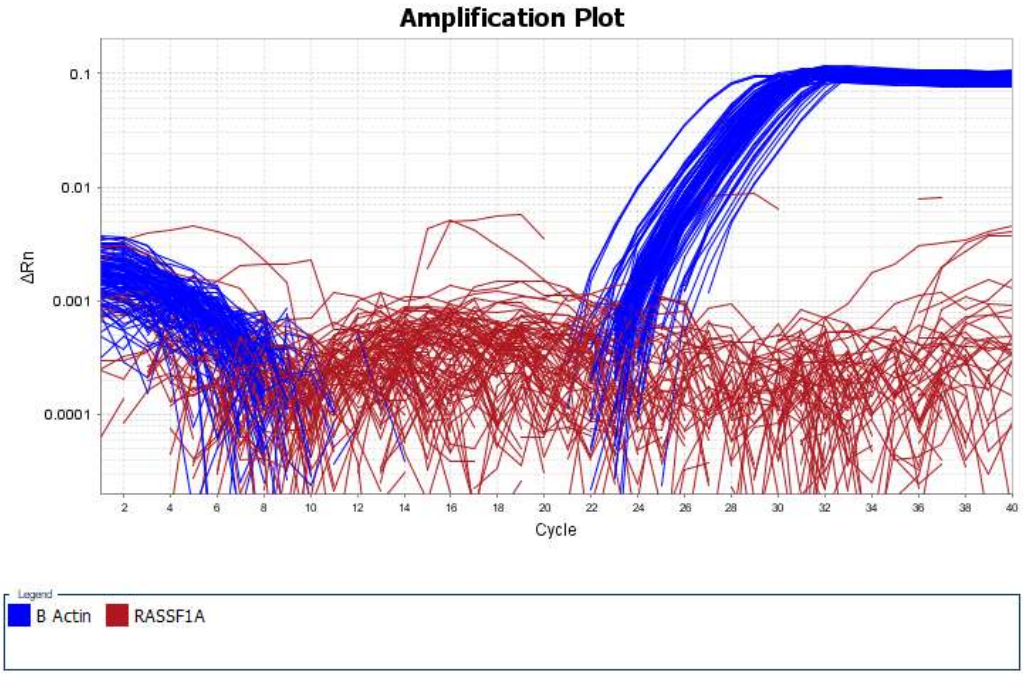

Figure 9. *B-Actin and RASSF1A amplification plot for control group.*

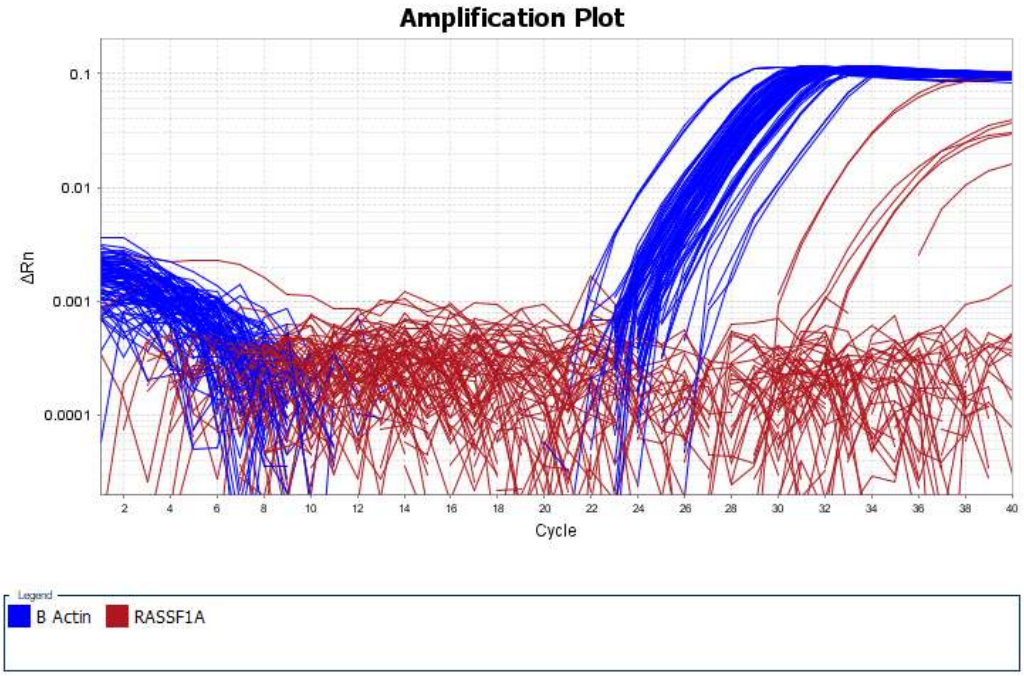

Figure 10. *B-Actin and RASSF1A amplification plot for cancer group.*

CHAPTER IV

DISCUSSION

4. DISCUSSION

Head and neck squamous cell carcinomas (HNSCCs) include tumors found in the oral cavity, pharynx, larynx, paranasal sinuses, nasal cavity, and salivary glands, representing some of the most aggressive cancers, affecting each year ~780,000 new patients worldwide (21).

Currently, early detection of OC remains challenging due to the lack of effective early diagnostic tools, resulting in delayed diagnosis, which leads to poor prognosis and low survival rates with a significant negative impact on the healthcare system (22). Although the oral cavity is easily accessible for visual examination, around 60% of OC patients are often diagnosed at an advanced stage associated with a high risk of locoregional recurrence and distant metastasis (23). Therefore, there is an urgent need for identifying highly sensitive and specific tumor non-invasive markers for OC detection.

Since epigenetic alterations such as DNA methylation represent an early event in carcinogenesis and may be detected in biological fluids, the analysis of DNA methylation in liquid biopsies has emerged as an attractive and minimally invasive strategy for developing biomarkers with potential clinical applications. In this line, scientific evidence has uncovered the potential clinical application of salivary DNA methylation for HNC diagnosis (24).

Numerous authors have reported gene-promoter methylation of widely known TSGs such as *p16, MGMT, DAPK* or *ECAD* in salivary DNA from HNC patients, highlighting its potential as tumor biomarkers (25) (26) (27). Epigenetic silencing of TSGs is widely recognized as an early and driving event in oncogenic process because of the transcriptional repression of genes which regulate key cellular functions, such as cell cycle, apoptosis, differentiation, and DNA repair (28). Moreover, the presence of promoter hypermethylation of TSGs in saliva has been associated with an increased risk of HNC, suggesting the important role of these epigenetic alterations as early markers of carcinogenesis in HNC (27). Then, the analysis of gene-promoter methylation through saliva has emerged as potential non-invasive strategy for the early detection and monitoring of head and

neck tumors. This highlights the potential of salivary DNA methylation analysis as a valuable tool in HNC research and clinical practice, offering insights into tumorigenesis and providing avenues for improved diagnosis and management strategies (21, 24, 26, 27, 29, 30).

Taking into account this context, in the present research project, we have evaluated the gene promoter methylation status of *RASSF1A* as a potential tumor epigenetic alteration for OC detection using saliva. The *RASSF1A* gene plays a pivotal role in suppressing tumorigenesis through several diverse cellular mechanisms, including cell cycle regulation, migration inhibition, microtubular stabilization, and apoptosis induction *(*27*).* Importantly, the silencing of *RASSF1A* by promoter hypermethylation has been reported in various types of cancer (27), evidencing its role in the tumor initiation and development and its utility as diagnostic, prognostic, and therapeutic biomarker (31).

Focusing on our results, *RASSF1A* gene promoter was methylated in 9,09% of salivary rinses from OC patients whereas no methylation was observed in the healthy control group, indicating a high specificity as a tumor biomarker. In the same line, other researchers have also investigated the hypermethylation of *RASSF1A* gene in saliva samples from HNC patients (21, 26, 29, 30).

Righini et al. analyzed the promoter methylation of *RASSF1A* in salivary rinses from 60 HNC patients and 30 healthy individuals by MSP. They found *RASSF1A* methylation in 16.6% of OC patients while none of the healthy individuals showed amplification for that gene (30), which is in line with our results. In another study, Lim et al. evaluated in saliva the methylation status of *RASSF1A* in 88 OC patients and 122 healthy individuals, detecting methylation in 40.9% of cases and in 12.2% of controls using conventional MSP technique (21). Similarly, González-Pérez et al., also investigated by MSP technique the promoter methylation of *RASSF1A* in saliva samples from 43 OSCC patients and 40 healthy individuals identifying *RASSF1A* hypermethylation in 23.3% and 2%, respectively (29). More recently, Liyanage et al. investigated the promoter hypermethylation of a panel of 4 TSG (*p16*, *RASSF1A*, *TIMP3*, and *PCQAP/MED15*) in saliva from oral and oropharynx cancer patients

identifying by MSP salivary *RASSF1A* methylation in a 68.5% and 64.7% of oral and oropharynx cancer patients, respectively (26). Overall, a higher salivary promoter methylation of *RASSF1A* has been reported in OC patients compared to our study cohort. This discrepancy may be attributed to the fact that conventional MSP has been the most used technique for the detection of aberrant *RASSF1A* methylation in saliva. The observed differences in the values of *RASSF1A* methylation between the two methods may be explained by the overestimation of methylation while using the MSP, which is known to be a qualitative having lower specificity compared to the qMSP method that we used. Indeed, qMSP is known to be a quantitative method that shows better sensitivity and specificity, reason why it was used in this research study.

Interestingly, *RASSF1A* promoter methylation has been also reported in oral tumor tissues with an estimated frequency of 40% (32-34) suggesting an important role of this TSG in oral carcinogenesis.

In the last decade, saliva has attracted considerable attention within the scientific community as a non-invasive liquid biopsy medium for genomic and epigenomic analysis, with a particular focus on cancer diagnosis, particularly in the context of head and neck carcinomas. As a diagnostic strategy, this approach is fast, reliable, cost-effective, and non-invasive. Additionally, due to saliva's direct contact with the oral mucosa the early detection of premalignant and malignant oral lesions using salivary biomarkers has aroused a great interest as screening approach. Several salivary biomarkers have been discovered through omics technology with potential for the diagnosis of HNC as well tumors more distant to the oral cavity. Particularly, the identification of tumor-specific DNA methylation markers in saliva has emerged as promising approach for early OC detection. In this line, to advance in salivary diagnostics, , the correct selection and implementation of saliva collection methodologies are paramount to ensure the presence of significant biomarkers levels in the samples, particularly given that the duration of saliva collection has a substantial impact on the reliability of the collected samples, where both excessively short and long collection periods may result in inconsistent quantities (21, 24, 26, 29, 35).

Additionally, ongoing advances in technology represent an opportunity to identify novel epigenetic markers in cancer research. In this line, sequencing and microarray technologies have emerged as a powerful strategy to discover new cancer-related genes that are specific and sensitive for early cancer detection, particularly in the context of OC. Specifically, methylation arrays has emerged as a highly sensitive, unexpensive, and time-efficient method for the characterization of tumor methylome and identification of novel methylated markers in saliva (17, 24, 26).

Several constraints were identified throughout this investigation. Firstly, the sample size of study cohort was limited to 33 OC patients and 39 controls. A larger case-control study could provide more evidence regarding the clinical value of salivary promoter *RASSF1A* methylation in OC and precancer detection.

Secondly, oral precancer controls were not included for testing salivary promoter *RASSF1A* methylation. Regarding this point, as we previously mentioned in the data sources and methods section, saliva samples from OPMD patients were also collected at FMDUP Clinic, however, the number of samples was not representative for analysis at this moment. After completion of the recruitment of OPMD cohort, promoter salivary *RASSF1A* methylation will be also tested.

Thirdly, regarding the clinicopathological characteristics of the patients diagnosed with oral carcinoma at the Head and Neck Clinic at IPO Porto, it should be noted that the clinical TNM staging, and the histopathological grading of the tumors remained unknown in some patients, as most of them are still waiting for surgery, after which that information will be available, following histopathological assessment. Consequently, it was not possible to statistically analyze the clinicopathological characteristics, as they were only available for some patients.

Fourthly, the promoter *RASSF1A* methylation was not evaluated in tissue tumor samples. Although the objective of this research is focused on saliva; due to the limited number of patients with positive *RASSF1A* methylation, it could be interesting to evaluate the methylation status in the tumor tissues to confirm the presence or absence of methylation in each case and correlate the results.

Finally, due to time constraints, sampling limitations and the fact that it was the only optimized gene at the Cancer Biology and Epigenetics Group – IPO Porto, it was only possible to evaluate the *RASSF1A* gene. This significantly restricts our capacity to analyze and interpret the results particularly in relation to the potential occurrence of hypermethylation in other TSGs. Future projects should explore the methylation of various genes to identify a gene methylation signature with high sensitivity and specificity for early OC detection.

CHAPTER V

CONCLUSIONS

5. CONCLUSIONS

-Saliva is as a potential liquid biopsy for detecting tumor DNA methylation alterations.

-RASSF1A promoter was methylated in 9.09% of OC patients and unmethylated in 100% of healthy individuals, disclosing low sensitivity but perfect specificity.

-The identification of salivary gene promoter methylation panel with high sensitivity and specificity for detecting OC would allow to improve oral screening in a non-invasive way.

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