UNIVERSITÀ DEGLI STUDI DI PALERMO

Dipartimento di Medicina di Precisione in Area Medica, Chirurgica e Critica (Me.Pre.C.C.)

Dottorato di ricerca in Oncologia e Chirurgia Sperimentali

IDENTIFICATION OF EPIGENETIC BIOMARKERS IN HPV-RELATED CARCINOMAS

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A.A. 2023/2024 – Cycle XXXVII

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Abstract

Head and neck squamous cell carcinomas (HNSCC), especially those localised in the oropharynx and oral cavity, have increased significantly in recent decades, with persistent Human papillomavirus (HPV) infection identified as the main aetiological cause. Conventional clinical and pathological evaluation is often insufficient to accurately assess the risk of malignant transformation in precancerous lesions. This limitation often delays the diagnosis of oral cancer, contributing to unfavourable outcomes for many patients. Therefore, the need for correct patient risk stratification and appropriate patient triage has led to the search for new diagnostic and prognostic molecular biomarkers, specifically among the epigenetic modification of DNA methylation. In this context, the PreCursor-M+ kit (Fujirebio, Tokyo, Japan), designed to analyse hypermethylation of the two onco-suppressor genes FAM19A4 and miR124-2 in cervical samples from high-risk HPV-positive women, was used to assess the abnormal methylation level of 111 oral rinse samples. The samples were distinguished according to the histologically diagnosed lesion: oral squamous cell carcinoma (OSCC), potentially malignant lesions (OPMD) and benign lesions (BL). To these, a group of healthy individuals was added (NL). In each group, samples were selected to have approximately equal numbers of HPV-positive and HPV-negative samples. HPV detection was performed using INNO-LiPA® HPV Genotyping Extra II diagnostic kit (Fujirebio, Tokyo, Japan), which allows the identification of thirty-two distinct genotypes, distinguished into twelve low-risk HPVs and twenty high-risk HPVs. Analysis of the results showed how hypermethylation correlated with the severity of the diagnosis. A positive hypermethylation result, as indicated by the kit, was more common in OSCC ($p<0.0001$), while a negative result was more common in NL and BL ($p= 0.0023$ and $p= 0.0162$). HPV positivity was positively correlated with hypermethylation only in OSCCs (32.4%, $p= 0.0006$), although statistical significance was also maintained in HPV-negative patients ($p= 0.0007$). Among HPV-positive OSCC, those infected with HPV16 showed a higher methylation level. Furthermore, the methylation levels of the two targets taken individually increased from the NL group to the BL and OPMD groups and finally to OSCCs. This study has shown how assessing hypermethylation of two targets can help diagnose OSCC and identify those patients affected by OPMDs who could be at risk of developing cancer. The relatively small number of patients did not allow for the detection of statistically significant differences in the effect of HPV infection on target methylation levels. It is therefore imperative to increase the sample size to confirm these preliminary results and to outline new significant information.

Summary

In recent years, oncology research has focused on identifying rapidly detectable molecular biomarkers, such as DNA mutations or epigenetic aberrations, which could potentially allow appropriate and accurate diagnosis of cancer and risk stratification of patients with premalignant lesions.

Human papillomavirus is directly involved in deregulating methylation patterns that coincide with the progression towards the cancer phenotype. Research has focused mainly on cervical cancer due to its entirely HPV-induced etiopathogenesis, allowing the commercialisation of the PreCursor-M+ diagnostic kit (Fujirebio, Tokyo, Japan), which assesses the hypermethylation level of the two targets FAM14A4 and miR124-2.

Literature on head and neck cancers is relatively scarce and mainly focuses on neoplastic lesions. To fill the gaps in the literature, this study used the aforementioned diagnostic approach to analyse four different groups of samples, i.e. healthy patients, benign lesions, potentially malignant disorders and oral cancers, which can be partially considered as a step-by-step evolution from healthy mucosa to cancerous epithelium.

The study's results depict a scenario in which the higher the target methylation, the more severe the diagnosis, which is consistent with what is observed in premalignant and malignant cervical lesions.

Although HPV positivity did not appear to be the sole cause of aberrant hypermethylation in oral cancers, the detection of HPV16 was associated with higher levels of methylation.

Future confirmation of these preliminary results in larger sample cohorts would allow the inclusion of this new molecular approach in the diagnosis of oral cancer and the early identification of patients at risk of developing cancerous lesions.

CHAPTER 1

Background Rationale and Objectives

1.1 Introduction

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common malignancy in the world. They are associated with high morbidity and mortality, with 650,000 new cases and more than 350,000 deaths annually [1,2]. The term HNSCC refers to a highly heterogeneous group of malignant neoplasms distributed in various anatomical sites of the upper aerodigestive tract, including the oral cavity, lips, sinuses, oropharynx, hypopharynx, nasopharynx, and larynx, of which oral and laryngeal cancers are the most widespread [3,4]. Traditionally, the etiopathogenesis of HNSCCs has been associated with two risk factors, namely tobacco use and alcohol abuse, to which ~75% of new cases are related. Recently, however, a novel new risk factor for HNSCC has emerged: persistent Human papillomavirus (HPV) infection, which appears to be responsible for the significant increase, recorded since the 1990s, in oral squamous cell carcinoma (OSCC) in the narrow sense (i.e. up to the anterior palatal pillars) and oropharyngeal squamous cell carcinoma (OPSCC) [5–9]. Although the exact route of transmission and natural history of oral HPV infection are poorly studied and still not fully understood, orogenital contact has been reported as the primary route of oral transmission [10]. Recent statistics show that more than 80% of men and women engage in oral sex, making it the second most common sexual practice after vaginal sex [11–14]. Probably due to a lack of awareness of risks and protection methods, condom or dental dam use during oral sex is minimal, making oral transmission of sexually transmitted infections inevitable [11,15–17].

According to the World Health Organization (WHO), HPV is the most common sexually transmitted infection, with about 80% of sexually active women and men infected at least once in their lifetime [18,19]. The virus infects basal keratinocytes of mucosal and cutaneous epithelia, proliferating asymptomatically before being eradicated within months. However, if the infection persists for 12-24 months, the patient risks developing benign and malignant hyperproliferative lesions [20]. In particular, the International Agency for Research on Cancer (IARC) distinguished mucosa-infecting genotypes in high-risk (hrHPV) and low-risk genotypes (lrHPV) according to strong epidemiological, biological and/or biochemical evidence of association with the development of malignant lesions. Specifically, the hrHPV group includes twelve carcinogenic types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), classified as Group I, one (HPV68) classified as probably carcinogenic in Group 2A, and twelve HPV types (HPV26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, 97) classified as possibly carcinogenic in Group 2B [21].

The genome is a circular double-stranded DNA of about 8.0 kb, encoding a regulatory sequence (LCR), four early regulatory genes (E2, E4, E5, E6, E7) and two late structural genes encoding capsid proteins (L1 and L2) [22,23].

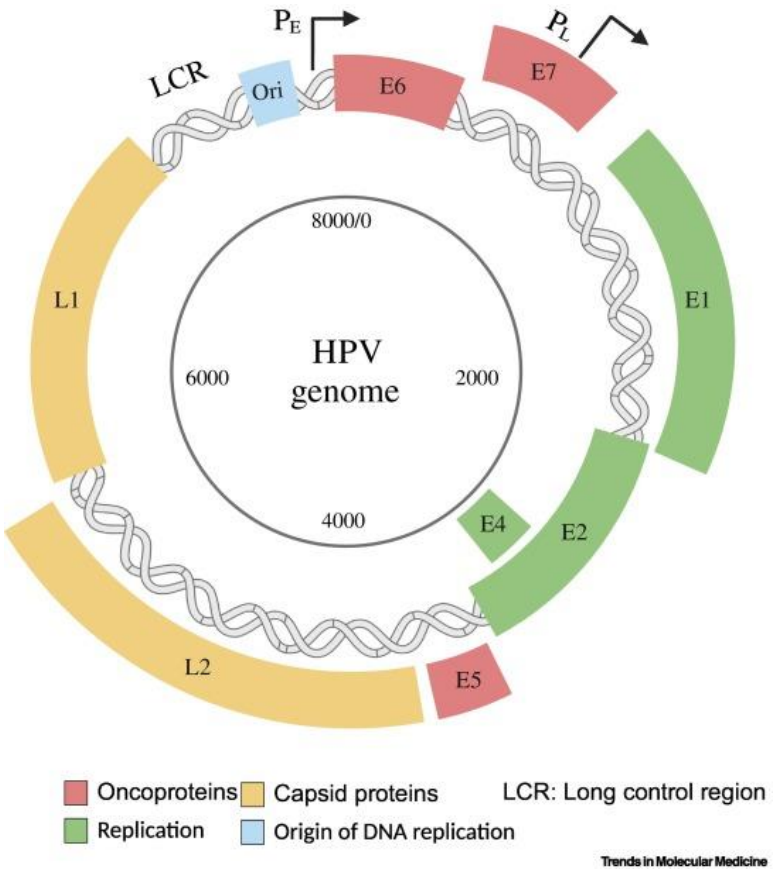


Figure 1. HPV genome, 8000 bp divided into: a regulatory sequence (LCR), four early genes and (E2, E4, E5, E6, E7) two structural genes (L1 and L2) [24].

The E6 and E7 proteins play a key role in the oncogenic process. They deregulate the cell cycle by inducing proteasome-mediated degradation of the tumour suppressor proteins p53 and pRB (retinoblastoma protein) [23].

Analyzing the similarities and differences within the genome sequence enables the taxonomic classification of HPV [22]. Indeed, the two-hundreds or more different genotypes identified till now are distributed in five different species, namely: Alphapapillomaviruses, Betapapillomaviruses, Gammapapillomaviruses, Mupapillomaviruses, and Nupapillomaviruses. Skin infections predominantly involve beta and gamma species, while the alpha genus comprises the clinically relevant mucosal types [25].

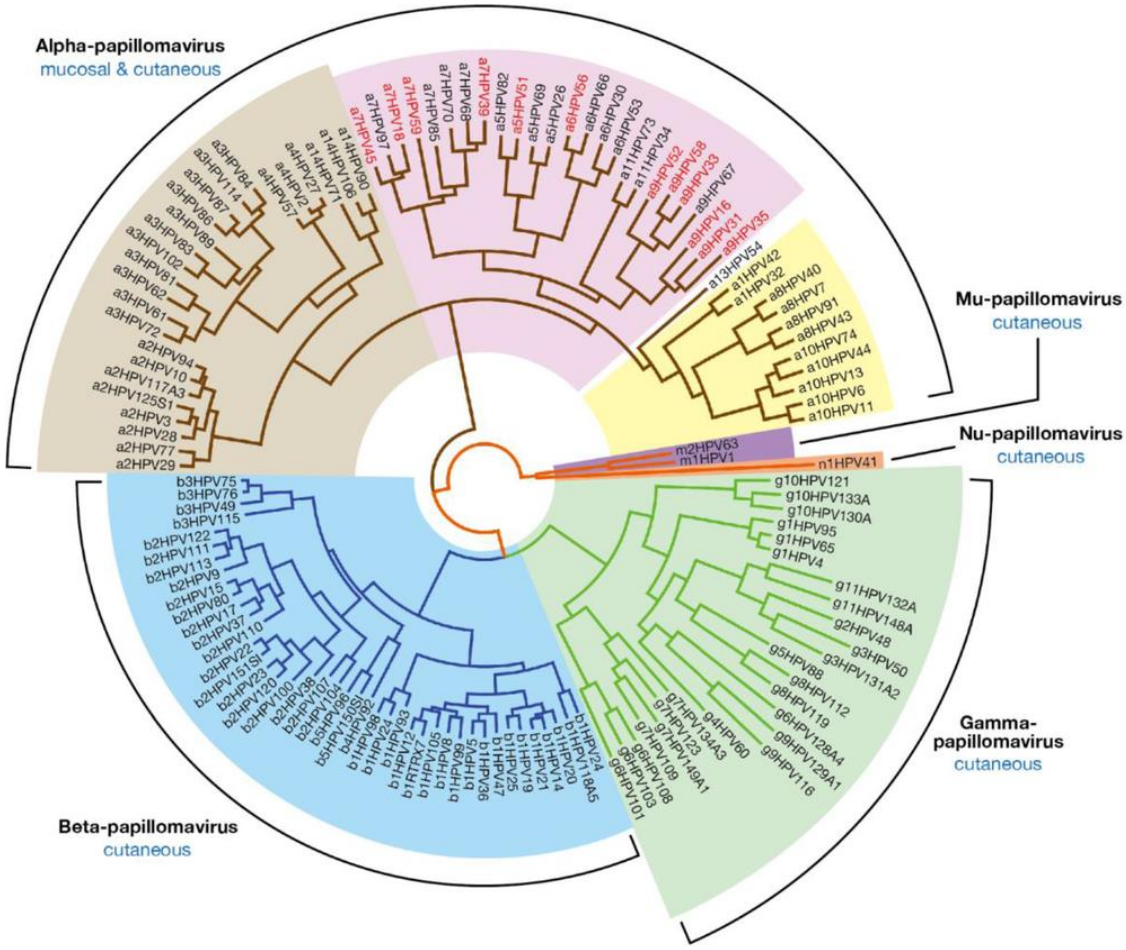


Figure 2. Evolutionary tree of Human papillomaviruses. The five genera are pictured in different colours: Alpha (low-risk cutaneous in light brown; low-risk mucosal in yellow; high-risk in pink), Beta (blue), Gamma (green), Mu (purple), and Nu (orange) [26].

Data from the US collected between 2017 and 2021 identified 47,984 new cases of HPV-associated cancers each year, making it one of the most common viruses associated with human carcinogenesis [27]. Almost all cases (99.7%) of cervical cancer have an HPV-induced aetiopathogenesis [28], while other anogenital cancers have significant yet lower correlation rates with persistent HPV infection: 88% of anal cancers [29], 43% of vulvar cancer [30], 70% of vaginal cancers [31] and 50% of penile cancers [18,32,33]. Data about HPV-induced HNSCC are less defined, with prevalence rates ranging from 20 to 80%, with HPV16 being the most implicated genotype [34,35]. This heterogeneity is caused by multiple factors, such as a frequently ambiguous description of tumour localisation, geographical differences, diverse demographic profiles of patients, and the HPV detection method employed [36]. Nevertheless, HPV has been described as a well-defined risk factor for OPSCC, with 20-70% of cases being HPV-related, while lower and more uncertain incidence rates have been described for other subsites, namely oral cavity (0-37%) and larynx (23-33%) [37]. According to recent estimates, in a short time, HPV-OPSCC will surpass HPV-related cervical cancer in incidence rates, and it is assumed that by 2030 it will be the predominant form of head and neck cancer [38].

The correct and careful assessment of HPV involvement in HNSCC carcinogenesis is imperative, as HPV-positive and negative HNSCC, mainly OPSCC, show crucial differences that impact patient management. The distinction between the two subgroups begins with socio-demographics, where individuals diagnosed with HPV-negative malignancies are characterised by lower socioeconomic status, tobacco and alcohol dependence, older average age, and poor oral hygiene [39]. In addition, HPV-negative tumours have a significantly different molecular profile, with frequent mutations in p53 and cell cycle regulators, which is reflected in a poorer response to chemotherapy and radiotherapy, a higher incidence of metastases and a negative prognosis, with a survival rate of 31% compared to 79% for the HPV-positive subgroup [6–9].

As concerning oral cancer, its onset of oral carcinomas may be preceded by the development of oral potentially malignant disorders (OMPD), a heterogeneous group of oral conditions and diseases which include oral leukoplakia, its rare verrucous-proliferative variant, oral lichenoid lesions, oral submucosal fibrosis, oral lichen planus, and erythroplakia [40]. The rate of risk of malignant transformation of these mucosal abnormalities is variable but

appears to be strongly influenced by the presence of dysplasia, which has been associated with a 12.3% percentage of tumoral progression over a period between 5 months and sixteen years [41,42]. HPV involvement in OPMD development is highly debated, with a recent meta-analysis depicting a 22.5% rate of pooled HPV DNA prevalence [43]. Other oral manifestations, defined as benign lesions, are oral condylomas and oral squamous cell papillomas, which are difficult to differentiate both clinically and histologically. Traditionally associated with HPV, specifically low-risk genotypes like HPV6 or HPV11, such alterations are scarcely studied in large cohorts, with studies to 1998 reporting a positivity rate of 50% for papillomas and 75% for condylomas [36,44].

Clinical and pathological examinations are not always effective in delineating the precise risk stratification of malignant progression of pre-cancerous lesions, resulting in frequent late diagnosis of oral cancer, and leading to a poor prognosis for a large number of patients.

In this scenario, identifying new molecular biomarkers with diagnostic and/or prognostic value could aid in improving early diagnosis, thus leading to an adequate diagnosis, improved treatment, and better patient survival and quality of life [42]. Specifically, in recent decades, the search for new ways to identify and characterise the progression towards the cancer phenotype has focused on epigenetics. In short, this term refers to all the heritable and non-heritable mechanisms involved in gene expression that add to the information contained in DNA sequences [45].

Among these, the most widely characterised are DNA methylation, post-translational modifications of histone proteins and non-coding RNAs (ncRNAs). These epigenetic regulators do not act separately but are dynamically linked to each other in the regulation of gene expression. Disruption of this complex epigenetic control mechanism can affect the structure and integrity of the genome and alter the expression of genes critically involved in tumorigenesis and chronic and degenerative diseases [46].

In detail, DNA methylation consists of the enzymatic addition of a methyl group (CH_3) to the 5-carbon position of cytosines (C) next to a guanine (G), forming the so-called CpG dinucleotides. These are particularly abundant in CpG or CGI islands, which are approximately 1kb long segments located at regulatory sequences (i.e. promoters) of many genes [47].

This process is catalysed by methyltransferase enzymes, classified into DNMT1, DNMT3A and DNMT3B. It participates in many physiological events

such as cell differentiation and maintenance of cellular identity, embryonic development, repression of repetitive elements, X-chromosome inactivation and genomic imprinting [48]. More specifically, DNMT1 works only on hemimethylated DNA, and thus it is described as a maintenance methyltransferase; conversely, DNMT3a and DNMT3b are defined as de novo methyltransferases because of their ability of modifying both hemimethylated and unmethylated sequences [49].

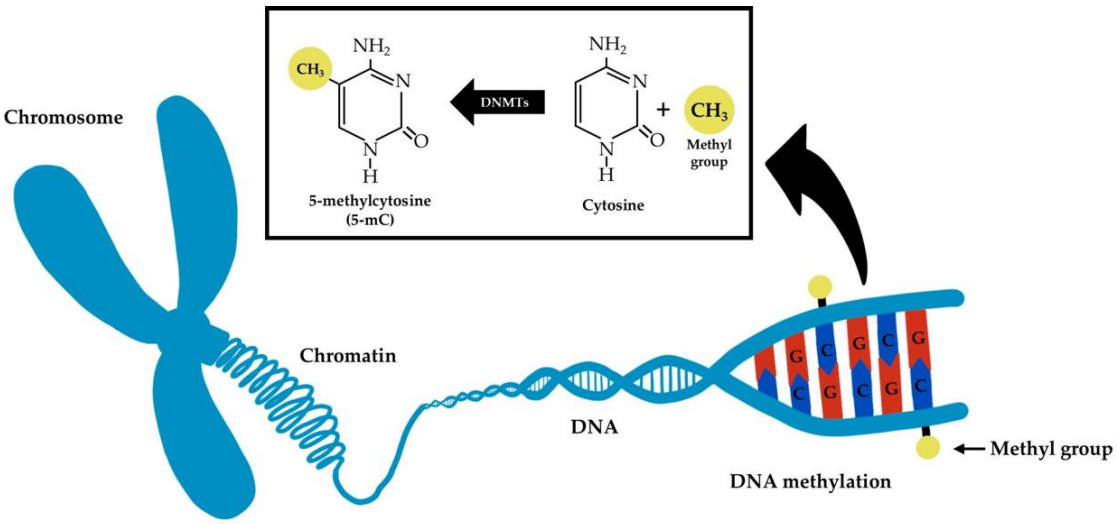


Figure 3. Simplified graphical representation of the DNA methylation process. DNMTs enzymes add a methyl group (CH_3) to the 5-carbon position of cytosines (C) in CpG dinucleotides [50].

Promoter hypermethylation is generally associated with transient or permanent transcriptional repression and gene silencing, whereas hypomethylation favours transcriptional events. This mechanism involves methyl-CpG binding domain (MBD) proteins, which recognise methylated CpG and recruit other epigenetic factors and chromatin remodelling factors that create condensed DNA, making it less accessible to proteins and inducing transcriptional repression. The maximum level of methylation is what can be seen in heterochromatin and inert chromosomes (i.e., X chromosome in females) [51]. In the slow progression towards cancerous phenotype, alongside the genetics and cytogenetics modification is the abnormal epigenetic regulation, which comprises also DNA methylation levels impairment. Briefly, it appears that cancer cells are characterized by a general hypomethylated state, accompanied by the hypermethylation and silencing of onco-suppressors genes.

DNA methylation is thus a reversible mechanism that could be a useful diagnostic and prognostic tool to identify the neoplastic lesion itself and predict prognosis and response to therapy [51]. The abnormal increase or decrease in methylation could help predict the rate and likelihood of malignant transformation or disease reversal, or the occurrence of relapse well in advance.

Moreover, the concept that methylation changes are more easily reversible than DNA mutations has led to the recent development of epigenetic drugs or epi-drugs, which reverse the altered methylation pattern of cancers and reactivate tumour-suppressor genes [52,53].

Beyond aberrant methylation generally associated with tumourigenesis, methylation dysregulation strictly associated with virus-induced carcinogenesis can be described.

It is well-known that HPV can hamper the cellular methylation machinery, inducing a state of hypermethylation [54]. This effect is carried out through the direct and indirect influence of E6 and E7 oncoproteins over methyltransferase enzymes. In particular, both proteins indirectly induce the overexpression of DNMT1 via the degradation of p53 and pRb. DNMT1 is also stimulated by a direct interaction with the E7 protein, which simultaneously induces the expression of DNMT3A and DNMT3B [55].

The quest for methylation biomarkers in HPV-positive cancers has mainly focused on preneoplastic and neoplastic lesions of the cervix. Indeed, numerous studies have led to the identification of several specific genes (i.e. *miR-124, EPB41L3, CADM1, MAL, PAX1, FAM19A4, SOX1*) whose abnormal hypermethylation is associated with high sensitivity to cell premalignant and malignant phenotype and which could then serve as prognostic and diagnostic biomarkers [45,56–60].

Among the promising targets identified in the literature, two, namely FAM19A4 and miR124-2, stand out from the rest and hence have already been included in diagnostic assays that can be used in HPV-based cervical cancer screening. This approach uses the bisulphite conversion method to highlight the hypermethylation of the two targets: bisulphite treatment deaminates unmethylated cytosines to uracils, leaving methylated cytosines unaffected. The former will correspond to thymines after amplification by polymerase chain reaction (PCR). Primers designed to pair only with the originally methylated DNA of the two targets allow their methylation to be detected.

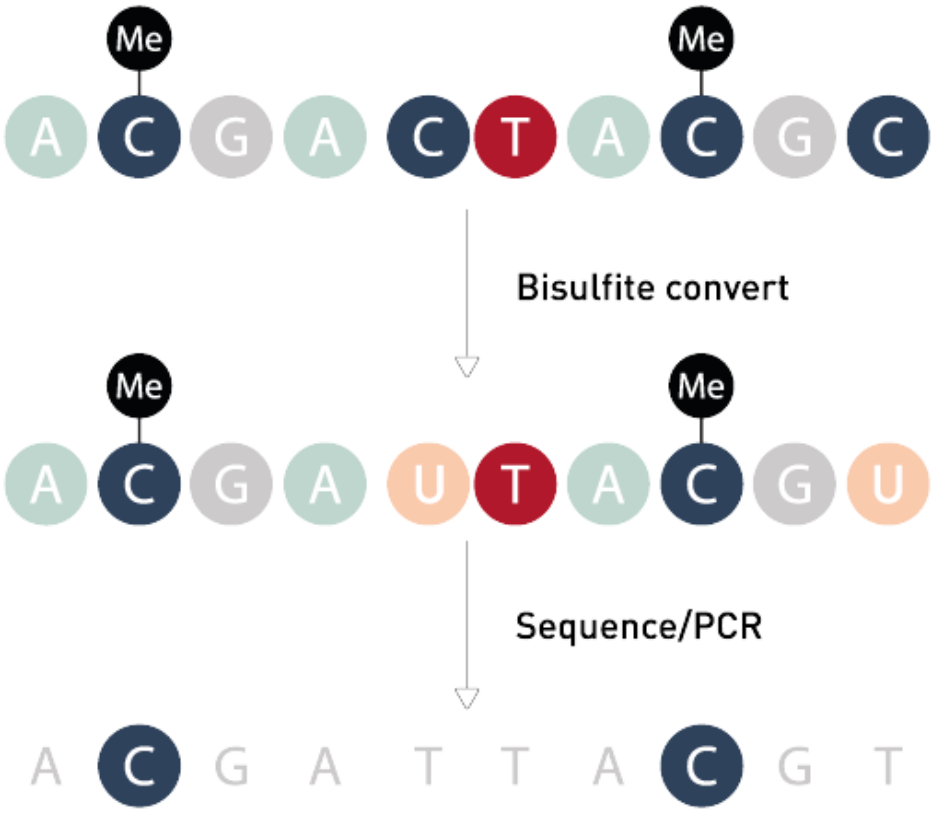


Figure 4. Graphical simplification of the bisulphite conversion process [61].

Multiple studies have already shown the robustness of an approach evaluating the methylation levels of the two targets simultaneously, which has been shown as equally or more sensitive than cytology and colposcopy triage [58,62].

In particular, detectable methylation has been strictly associated with high sensitivity to cervical cancerous lesions: 98.3% of cervical carcinomas are detected, regardless of histotype, HPV genotype, geographical area and sample type [63,64]. Moreover, the result of the assay is predictive of the prognosis of high-grade cervical preneoplastic lesions (CIN2/3): high methylation level is indicative of progression to the cancer phenotype, while low methylation is indicative of lesion regression [63,65,66].

Talking more specifically about the two targets, the FAM19A4 gene, now known by the HGNC (*HUGO Gene Nomenclature Committee*) symbol TAFA4 or TAFA chemokine-like family member 4, is a gene located in chromosome three, a member of the TAFA family. The latter includes five genes encoding small, secreted proteins sharing sequence similarities with the CC chemokine family [67]. The highest expression levels have been found in the brain tissue where they are postulated to function as brain-specific chemokines, however, it has been found in several other sites, such as intestines, spleen and liver, breast, ovary and uterus, prostate and testis. Since its first

identification in 2003, it has been linked with several functions: phagocytosis, signal transduction, regulation of signalling receptor activity and membrane potential, and superoxide anion generation [68].

Whole Genome Bisulphite Sequencing (WGBS) analyses performed on HPV-negative and HPV-positive oral cancer specimens have shown statistically significant hypermethylation of its promoter in neoplastic lesions compared to normal adjacent tissue [69–73].

MiR124-2 (or hsa-miR124-2) is one of three precursors' genes (i.e., miR124-1, miR124-2, and miR124-3) which are processed together forming miR124 [74]. Its encoding gene is located on chromosome eight, and like any other miRNA, it acts as a post-transcriptional regulator, inducing transcriptional silencing. Its expression has been registered in tissues like the colon, breast and brain, and it has been linked with events such as body mass index regulation and response to antidepressants, regulation of alternative splicing and differentiation of neuronal cells [75]. Its role as a tumour-suppressor manifests through the inhibition of proliferation, aggressiveness, apoptosis, and invasion of tumoral cells, as observed in osteosarcoma, pancreatic adenocarcinoma, breast and lung cancers, hepatocellular carcinoma cell lines and oral squamous cell cancers [74,75].

In general, it has been shown that microRNA genes are several times more often methylated than protein-coding genes, and regarding miR124-2, its methylation-dependent silencing has been registered in a plethora of cancers, from lung squamous cell carcinoma [76] to ovarian cancer [77], from breast cancer [78] to cervical cancer [79,80] and colorectal carcinoma [81], where its silencing is associated with poor clinical prognosis [82].

Concerning OSCC, Hunt et al. [83] demonstrated in vitro how miR124-2 can downregulate the expression of integrin beta-1 (ITGB1), involved in adherence processes and motility of OSCC cells, hence reducing invasive and migrating genotypes.

Understanding and measuring the methylation patterns of cancers could lead to discovering diagnostic and prognostic biomarkers proposing an easy and efficient tool for overcoming diagnostic limitations, identifying oral cancer precociously and eventually providing the basis for epigenetic drug discovery.

1.2 Objectives

To proceed with the rapid identification of potential epigenetic biomarkers, we opted for the use of the PreCursor-M+ kit (Fujirebio, Tokyo, Japan) on oral rinse samples. This validated CE-IVD method is designed to detect the level of hypermethylation of promoters associated with the two onco-suppressor genes FAM19A4 and miR124-2. This approach allows better risk stratification in hrHPV-positive women characterised by cytological/histological abnormalities, distinguishing between patients who might develop cervical carcinoma and those whose lesions might regress. Specifically, high methylation levels are associated with a high risk of preneoplastic lesions evolving into cervical cancer in the short term, in contrast to lesions related to low, or undetectable, methylation levels.

The use of the same method in oral HPV-positive patients with potentially malignant and/or neoplastic lesions would make it possible to assess the correlation between hypermethylation of the two onco-suppressors and the development of oral cavity neoplasia, to use them as potential prognostic markers.

CHAPTER 2

Materials and Methods

2.1 Patient recruitment and Sample collection

During the three-year duration of the PhD project, 215 stored samples were initially selected. The samples originally belonged to male and female patients admitted to the Operative Unit of Oral Medicine and Dentistry for Frail Patients of the University Hospital Policlinic, P. Giaccone, Palermo. All subjects underwent a dental examination that revealed the presence of a suspected HPV-related lesion. To assess the presence of the virus, each patient independently performed an oral rinse with 10 ml of Original Mint Scope® mouthwash (Procter & Gamble), carefully reaching all parts of the oral cavity and avoiding gargling, which was then sent to the Microbiology and Virology Unit of the University Hospital Policlinic, P. Giaccone, Palermo. Each patient was instructed not to eat, drink or use oral hygiene products for at least one hour before sampling.

At the same time, a biopsy was taken and sent to the Anatomical Pathology Unit of the same hospital, where the HE-stained histological examination made it possible to classify the previously identified lesions as oral squamous cell carcinoma (OSCC), potentially malignant disorders (OPMD) or benign lesions (BL).

Sample selection was based on two different criteria: the availability of a histological diagnosis of squamous cell carcinoma, potentially malignant lesions (erythroplakia, leukoplakia, lichen planus) and benign lesions (papillomas), and the need to have an equal number of HPV-positive and negative patients in each group. To these, was added a control reference group of healthy individuals, also consisting of approximately the same proportion of positive and negative individuals.

The University Hospital Policlinic, P. Giaccone, Palermo ethical committee board approved the study and, as the data were pseudonymized for analysis, the need for consent was waived.

2.2 Sample processing

Before the virological examination, each sample needed an initial processing to isolate the cellular component. The procedure consisted of first centrifugation at 1,600 rpm for 10 minutes, followed by the pellet resuspension in 1-3 mL of phosphate-buffered saline (PBS), according to cellular yield. The samples dispensed into 1.5 mL Eppendorf tubes (1 mL each), were subjected to a second centrifugation at 13,000 rpm for 5 min. After discarding the supernatant, each pellet was stored at -20°C or processed immediately.

2.3 DNA extraction, HPV detection and genotyping

The first step required for virological diagnosis was DNA extraction, which was carried out using the Elitech Ingenius automated system (Elitech Group, Turin, Italy). The DNA concentration was quantified using a Qubit fluorometer (Thermofisher Scientific, Waltham, Massachusetts, USA) and each sample was then subjected to viral genome detection and genotyping. The latter involved the use of the validated CE-IVD INNO-LiPA® HPV Genotyping Extra II diagnostic kit (Fujirebio, Tokyo, Japan), which allows the identification of thirty-two distinct genotypes, distinguished in twelve lrHPV (HPV6, HPV11, HPV40, HPV42, HPV43, HPV44, HPV54, HPV61, HPV62, HPV81, HPV83, HPV89) and twenty types classified as hrHPV (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV67, HPV68, HPV26, HPV53, HPV66, HPV70, HPV73, HPV82).

The technique involves a PCR amplification using biotinylated primers followed by a reverse hybridization assay. The PCR reaction simultaneously amplifies two targets: a 65 bp region of the HPV L1 gene using SPF10-based primers, and HLA-DBP1 as an endogenous control to assess sample adequacy. In the reverse hybridization step, the PCR products bind to DNA probes immobilized on a membrane strip: the endogenous control probe, 32 genotype-specific probes, and two additional generic “HPV control” probes. Since SPF10-based primers can amplify more than 54 HPV genotypes, some samples may be positive only for the HPV control probes. In this case, genotyping required a

nested PCR with PGMY09/PGMY11 and GP05 + /GP06 + primers as described elsewhere [84], followed by indirect Sanger sequencing. Specifically, the workflow consisted of purification of PGMY09/PGMY11 or GP05+/GP06+ amplification products using Microcon®Centrifugal Filters (Merck KGaA, Darmstadt, Germany), followed by amplification using the BigDyeTM Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA). Purified products from Centri-SepTM Spin Columns (Thermo Fisher Scientific, Waltham, MA) were then sequenced on an ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). Genotypes were then obtained by BLAST sequence alignment.

2.3 Methylation analysis

Following the manufacturer's protocol, the methylation analysis required an initial treatment with sodium bisulphite, performed with the EZ DNA Methylation™ kit (Zymo Research, Irvine, USA). During this procedure, the bisulphite acts on the unmethylated cytosines by deaminating them into uracils, while leaving the methylated cytosines intact. Following amplification by PCR, the former will correspond to thymines.

For the conversion to occur correctly, a precise amount of DNA, 200ng, was necessary, the corresponding volume of which was appropriately calculated.

200 ng of DNA was used for conversion for all samples that produced enough DNA. In contrast, for samples with concentrations below the minimum acceptable, the maximum allowable volume of extracted DNA (45 µl) was used to add the maximum amount of ng for conversion, as per the kit's protocol.

Following the conversion process, the converted DNA samples were subjected to real-time multiplex qPCR of the PreCursor-M+ kit (Fujirebio, Tokyo, Japan). The method involves using primers designed to pair only with the originally methylated DNA of the two target regions corresponding to the promoter sequences of the FAM19A4 and miR124-2 genes. In addition, a third pair of primers allows the amplification of β-actin (β-Act), which verifies the quality and recovery of the DNA after bisulphite treatment and allows the relative quantification of the methylation level.

Gene amplification is detected with fluorescent hydrolysis “TaqMan” probes.

Each run required the testing of a calibrator containing a standard concentration of DNA fragments containing the three targets (FAM19A4, miR124-2 and β-actin).

2.3 Real-time PCR results analysis

The quantitative evaluation required the initial normalisation of the two target genes compared to the endogenous control (β-Act) by calculating the ΔCt, i.e., the difference between the target's Ct and the control's Ct. The value of ΔCt was then used to evaluate the methylation level using the ΔΔCt method, which is based on the application of the following formula:

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$$

Livak's method, using the $2^{-\Delta\Delta C}$ calculation formula, was used to express the ΔΔCt figure as '% of methylated cells in the sample'. This percentage is distributed on a scale ranging from 0 (unmethylated) to values above 0 (partially methylated or methylated samples depending on the value). This formula indicates how often the gene is methylated in the test compared to the calibrator, which normalised the data to the housekeeping gene.

2.4 Statistical analysis

For categorical variables, data are presented as numbers and percentages, and continuous data are expressed as mean ± standard deviation [SD] unless otherwise specified.

A binomial test was performed to compare two mutually exclusive proportions or percentages in groups.

The multicomparison chi-square test was used to define significant differences among groups. If the chi-square test was positive [p-value <0.05], residual analysis with continuity correction for Z-test was performed to localize the highest or lowest significant presence. In addition to the analyses between three or more modalities of a variable, the chi-square goodness of fit was used. Test for normal distribution was performed by Shapiro-Wilk test.

One-way ANOVA test was used to test the difference between the means of several subgroups of a variable. If the ANOVA test was positive (p<0.05) then the post hoc test was used by Scheffé's method for pairwise comparison of subgroups. Before the ANOVA test, Levene's Test for Equality of Variances was performed. If the data was no not normally distributed the Kruskal-Wallis test was performed in multi-comparison among three or more unpaired samples. If the Kruskal-Wallis test was significant (p-value <0.05), the Conover post hoc test was used for pairwise comparison of subgroups.

The relationship between Infections and other parameters was calculated using the chi-square test or Fisher's exact test (dichotomous vs dichotomous), or the Mann-Whitney test (dichotomous vs no normal continuous data). For this step, we define the following variables:

- diagnoses: no lesions= 0, benign lesions= 1, potentially malignant lesions = 2, cancer lesions= 3 (the scale was defined according to the severity of the disease).
- age.
- sex: male= 1, female= 0.
- genotype risk: hr or hr/lr= 1, lr= 0; HPV infected: yes= 1, no= 0.
- hypermethylation: negative= 0, positive= 1.

Multiple linear regression was used to find the best-fitting model to describe the relationship between the dichotomous characteristic of interest (the dependent variable: diagnoses) and a set of independent variables such as hypermethylation, age, sex, genotype risk, HPV infection, and the number of genotypes.

All tests with $p<0.05$ were considered significant. All data were analysed using the Matlab statistical toolbox version 2008 (MathWorks, Natick, MA, USA) for 32-bit Windows

CHAPTER 3

Results

The reduced availability of research funds combined with the high cost of materials led to a reduction in the number of samples analysed from 215 to 123. The cohort therefore consisted of 123 patients. From this initial cohort, 11 samples were additionally excluded due to low DNA input, leaving a total of 111 samples. Of this final group, 61.5% (67/111) were men and 43.6% (44/111) were women, with an overall mean age of 55.7 (±16.2).

The percentage of HPV-positive samples was 48.6% (54/111), of which 81.5% (44/54) were single infections, and 66.7% (36/54) displayed at least one oncogenic HPV type (Table 1).

The cohort was subdivided into four groups depending on the diagnosis.

Each group was composed as follows (Table 1):

- NL group composed of 20 patients with no lesions, with 45% (9) males and 55% (11) females, with ages in the range 19-51, mean 39.2 y.o., and standard deviation (SD) of 9.8 y.o.
- BL group, composed of 37 patients with benign lesions, with 59.5% (22) males and 40.5% (15) females, with ages in the range 16-82, mean 51.1 y.o., and standard deviation (SD) of 15.6 y.o.
- OPMD group composed of 17 patients with potentially malignant lesions, with 52.9% (9) males and 47.1% (8) females, with ages in range 47-82, mean 66.4 y.o. and standard deviation (SD) of 10.6 y.o.
- OSCC group composed of 37 patients with carcinoma, with 73% (27) males and 27% (10) females, with ages in the range 40-92, mean 64.5 y.o., and standard deviation (SD) of 12.3 y.o.

Table 1 also shows the negative or positive samples for hypermethylation according to the cut-offs for cervical lesions.

A total of 29 genotypes were identified; the prevalent types were HPV16, with a rate of 25.9% (14/54), HPV6, with 14.8% (8/54), and HPV66, with 12.9% (7/54).

In Table 2 we reported the clinical information among patients with no lesions (NL), benign lesions (BL), potentially malignant lesions (OPMD) and cancerous lesions (OSCC). Patients with no lesions were significantly younger than patients with benign lesions (39.2 vs 51.1, $p<0.05$), potentially malignant lesions (39.2 vs 66.4, $p<0.05$) and cancerous lesions (39.2 vs 64.5, $p<0.05$). A similar ratio was found for patients affected by benign lesions compared to patients with potentially malignant lesions (51.1 vs 66.4, $p<0.05$) and cancerous lesions (51.1 vs 64.5, $p<0.05$).

Genotype risk and diagnosis were significantly correlated ($p= 0.0033$), with the OSCC group characterized by a substantially higher rate of high-risk genotypes, alone or together with low-risk genotypes in multiple infections ($p= 0.0279$). Conversely, lrHPVs were significantly more frequent in the BL group ($p= 0.0048$).

Hypermethylation analysis was performed considering the positivity cut-offs of the kit developed for cervical samples.

Hence, a positive result, i.e. a ΔΔCt value above the cut-off for cervical lesions for at least one of the two targets, was significantly more frequent in cancerous lesions ($p<0.0001$). A negative result, i.e. a ΔΔCt value below the cut-off designed for cervical lesions for both targets, was significantly more frequent in patients without lesions and diagnosed with benign lesions ($p=0.0023$ and $p=0.0162$, respectively). The graphical representation of these results is depicted in Figure 5, which clearly shows the progressive decrease in the number of hypermethylation-positive samples from OSCC (64.9%), to OPMD (35.3%), BL (18.9%) and NL (5%), as well as the progressive increase of hypermethylation-negative samples from OSCC (34.2%), to OPMD (64.7%), BL (81.1%) and NL (95%).

Looking specifically at the HPV-positive patients, the OSCC group was the only group showing a significant positive correlation between HPV-positivity and hypermethylation (32.4%, $p=0.0006$). However, HPV-negative patients in the same group also had a significant positive hypermethylation result (32.4%, $p=0.0007$).

Similarly, HPV-negative patients had a significant negative hypermethylation result in the NL group (50%, $p=0.0105$).

Table 3 shows the association analysis between hypermethylation and age, sex, diagnoses, HPV infection, number of genotypes, and genotype risk. A significant correlation was found between hypermethylation and age ($p<0.0001$), and hypermethylation and symptoms ($p<0.0001$). About the latter, the post hoc Z-test showed that the NL and BL lesions were more frequent among the negative hypermethylated (26%, $p=0.0023$; 41.1%, $p=0.0162$, respectively), while the OSCCs were more frequent in the group positive for hypermethylation (63.2%, $p<0.0001$) (Table 3).

The significant variables defined in Table 2 were then analysed with the disease variable by multiple linear regression analysis, as shown in Table 4. From this analysis, it was found that among age, genotype risk, and hypermethylation, age and total hypermethylation were positive predictors of diagnoses (R-partial= 0.37, p= 0.0077; R-partial= 0.29, p= 0.0381, respectively).

Specifically considering HPV-positive samples, an analysis was performed to identify a possible correlation between the identified genotype, diagnosis, and hypermethylation. In multiple infections, each single genotype was analysed individually (Table 6).

A correlation was found for genotypes 53, 61, 62, 81, and 82 with hypermethylation of samples negative for lesions, genotypes 6, 16, 42, and 61 with hypermethylation of benign lesions, 54 and 62 with hypermethylation of potentially malignant lesions, and for genotypes 16 (n=6), 31, 44, 56, 58, 62, 66 (n=3), 67, 68 (n=2), 81, 83, 120 (n=2) with hypermethylation of cancerous lesions. In particular, in the hypermethylated OSCC samples, some genotypes were more frequent than others, namely HPV16, HPV68, and HPV120, but unfortunately, the data were not statistically significant.

To overcome the limitation imposed by the above cut-offs, the ΔΔCt ratio ($2^{-\Delta\Delta Ct}$) calculation was used to perform a relative quantification of methylation levels, expressed as a percentage.

This evaluation, performed individually for both targets, showed that the methylation levels correlated with the severity of the oral lesion (Figures 6 and 7). This observation was confirmed by the statistical analysis performed on mean and median ΔΔCt scores of the two targets (Table 6). In particular, $2^{-\Delta\Delta Ct}$ FAM19A4 scores were significantly correlated with the diagnosis ($p<0.0001$), with the post hoc test showing how the score for OSCC was greater than NL (median: 0.28 vs 0.0, $p<0.05$), BL (median: 0.28 vs 0.0, $p<0.05$), and OPMD (median: 0.28 vs 0.03, $p<0.05$).

A similar result was found for mir124-2 $2^{-\Delta\Delta Ct}$ values (9.5 vs 7.4, $p<0.05$), for which the OSCC group score was greater than NL (median: 048 vs 0.14, $p<0.05$), and BL (median: 048 vs 0.09, $p<0.05$).

The same ΔΔCt ratio scores were used to evaluate the correlation between hrHPVs or lrHPVs detected and the diagnosis, as shown in Table 7. From this analysis emerged how FAM19A4 ΔΔCt ratio scores varied significantly among lrHPV positive samples, with the OSCC score significantly higher than the BL one (mean: 0.743 vs 0.083, $p<0.05$).

Then the OSCC samples positive for HPV16 were specifically selected to evaluate whether their methylation levels were higher compared to the other HPV-positive samples. Figures 8 and 9 show that HPV16 was significantly associated with hypermethylation of both targets.

CHAPTER 4

Discussion

Virus-induced carcinogenesis represents a significant burden in the global public health scenario, with over 10% of tumours caused by viral infections [85]. Harald Zur Hausen first defined the role of HPV as an oncovirus, and since then its involvement in the initiation and development of tumours has been extended from cervical carcinoma to a wide range of other malignancies [18].

The last challenge in this regard is represented by HPV-related HNSCCs, particularly those in the oropharynx and oral cavity, which appear to have met a significant increase since the nineties, especially in high-income countries like the US and the Scandinavian geographic area [86]. However, the epidemiology of oral HPV infection and the natural history of the virus in the oral cavity are still poorly understood, leading to under-diagnosis or delayed diagnosis of the disease [87]. Adding to the complexity of the diagnosis are the potentially malignant lesions, which are not per se clear precursors of oral neoplastic lesions, and whose association with HPV infection is far from being defined [40].

Untangling the complex skein of cancer development has led to the identification of molecular biomarkers, which help define the diagnosis, prognosis and treatment of malignancies. These include DNA mutations identified in liquid biopsies or epigenetic modifications such as small non-coding RNAs or DNA methylation. Among these, silencing of tumour suppressor genes through DNA methylation of promoter regions, is an event closely correlated with HPV-induced tumorigenesis, as the deregulation of methyltransferases has been identified among the functions carried out by E6 and E7 proteins [55].

In this regard, the research on cervical carcinoma has produced interesting and

promising results, leading to the placing on the market of diagnostic kits designed to identify the hypermethylation of two targets, miR124-2 and FAM19A4.

To begin with, a study performed on HPVE6 E7-immortalised keratinocyte cell lines infected with seven hrHPV genotypes (16, 18, 31, 33, 45, 66, 70) showed a significant increase in promoter methylation levels of a fourteen genes panel during the different stages of immortalisation, with a gene-specific timing and independently from the genotype. Looking specifically at the two targets of interest, considerable hypermethylation of miR-124-2 was observed from the earliest stage of pre-immortalization, while the increase of FAM19A4 methylation level was registered only in the last stage of immortalisation [88] .

Following in vitro experiments, the involvement of their aberrant methylation in cervical tumorigenesis has been demonstrated in several studies involving large cohorts of patients, effectively demonstrating how measuring their methylation levels has strong diagnostic and prognostic performances in cervical cancer and precancerous lesions. Specifically, this approach can correctly detect 98.3% of cervical cancers and improves triage of hrHPV-positive patients. Hypermethylation-positive patients are at risk of developing the cervical cancer phenotype, while hypermethylation-negative have a low cancer risk that also extends to fourteen years [63].

The credibility of these targets in the context of HPV-related oral cancer is based on several lines of evidence, including preliminary results from WGBS analyses showing cancer-associated methylation deregulation in oral cancer tissue.

To our knowledge, this is the first study to analyse miR124-2 and FAM19A methylation levels in oral samples. In particular, we used oral rinse samples, a well-proven method for diagnosing oral HPV infection, let alone much less invasive and perhaps just as informative as tissue samples.

To begin with, our cohort showed general characteristics coherent with data known from the literature. For instance, the mean age significantly increased from the NL group to the BL, OPMD and OSCC groups. This finding displays, on the one hand, the higher exposure of young individuals to develop a frequently asymptomatic oral infection due to their involvement in risky sexual behaviour, and, on the other hand, the age-related progression towards carcinomas [16].

Regarding HPV positivity, the distribution of hrHPV and lrHPV in the four groups

showed, again in accordance with the literature, that high-risk genotypes were significantly more prevalent in cancerous lesions than in the other groups, whereas low-risk types were significantly more prevalent in benign lesions [21,89].

Hypermethylation results were observed using two different approaches: an analysis performed under the lens of the hypermethylation positivity cut-off developed for cervical samples, in parallel with a ΔΔCt ratio-dependent analysis. Both ways painted a picture highly suggesting the involvement of hypermethylation in the progression towards oral cancer.

In the first case, a positive hypermethylation result in the total sample, i.e. one of the two target ΔΔCts above the cervix-defined cut-offs, was significantly more frequent in cancerous lesions ($p < 0.0001$), whereas a negative hypermethylation result was significantly more frequent in patients without lesions and with benign lesions ($p = 0.0023$ and $p = 0.0162$, respectively).

As for ΔΔCt Ratio analysis, it showed, again, how the two targets, taken together or separately, are highly methylated in oral cancer samples and, in addition, the level of their hypermethylation increases progressively from patients with no diagnosed lesions to those with BL, OPMDs and OSCCs. In other words, the analysis of hypermethylation percentages alone allows a clear differentiation between the four groups and their diagnosis.

These findings suggest a possible prognostic role of FAM19A4 and miR124-2 methylation assessment: patients with benign lesions and, especially, OPMDs showing a positive hypermethylation result of one or both targets should receive close follow-up.

As cervical cancer has a 99.7% correlation rate with HPV infection, the assessment of the two target methylation levels in an HPV-negative scenario has not been evaluated in the literature. The same cannot be said for oral cancer, whose association with persistent HPV infection amounts to much lower rates: from the 10.6% figure reported by the National Cancer Database (NCDB) [90] to the broad range of 0-37% described in the recent meta-analysis by Katirachi et al [34].

The cohort analysed, where the sample selection was specifically designed to have approximately equal numbers of HPV-positive and HPV-negative samples in each group, allowed a separate hypermethylation analysis in HPV-positive and HPV-negative samples.

In particular, the significance of the correlation between hypermethylation and

OSCC diagnosis was maintained in both cases: HPV-positive samples were significantly correlated with hypermethylation (p-value= 0.0006), similar to HPV-negative ones, which showed a correlation p-value of 0.0007 with hypermethylation.

These data were complemented by the finding that when in the no lesions group the virus could not be found, the same was true for hypermethylation (p= 0.0105).

These pieces of evidence contributed to the assumptions that the methylation of the two targets is not strictly correlated with HPV infection but may be one of the epigenetic alterations involved in the progression to oral cancer.

Literature findings describe how aberrant methylation of miR124-2 and FAM19A4 promoters is a phenomenon that could characterise the neoplastic evolution per se, without the intervention of a persistent viral infection. Indeed, FAM19A4 was one in a multitude of genes analysed through WGBS whose corresponding datasets can be found on the *Gene Expression Omnibus* (GEO) platform under the following accession numbers GSE38532 [72,73], GSE41114 [72], GSE46802 [70,71]. These studies showed a FAM19A4 promoter's statistically significant hypermethylation in oral cancer tissue biopsies compared to adjacent non-cancerous tissue.

There is talking specifically about Sheu J.J. et al [72], and Lee C.H. et al [73], which analysed tumour and non-tumour pair-wise samples of a cohort of OSCC patients exposed to risk factors such as alcohol, areca nut and smoke, with the WGBS (Whole Genome Bisulphite Sequencing) platform Illumina HumanMethylation27 BeadChip (HumanMethylation27_270596_v.1.2). Among the tested targets, the CpG island located upstream from FAM19A4 gene showed a significant difference in methylation between cancerous tissue and healthy tissue with adjusted p-values of $1.70e^{-27}$, $1.04e^{-26}$.

Using the same WGBS platform, Towle R. et al [70,71] tested thirty biopsies corresponding to dysplastic, in situ carcinoma (CIS), OSCC, and adjacent normal tissues, obtained from ten patients undergoing surgical removal of OSCC or CIS. Different methylation patterns were found between the normal and the CIS/OSCC tissues, with FAM19A4 counted among the genes showing a significant alteration in the methylation level (p-value= $1.01e^{-04}$, $4.34e^{-04}$).

Similarly, Pickering C.R. et al [69] showed through Illumina HumanMethylation450 BeadChip (HumanMethylation450_15017482_v.1.2) the differences in the methylation framework of forty-two OSCC, two normal

oral epithelial tissues, and two normal blood samples. Also in this case, FAM19A4 was differently methylated in non-CpG island regions, located within the gene's sixth exon and first intron (p-values of 0.0025 0.0036 and 0.004, respectively).

MiR124-2, on the other hand, does already have a clearly defined role as a tumour suppressor; hence, its methylation-dependent silencing has already been analysed and defined in several histotypes that go beyond HPV-related cervical cancer, i.e., ovarian, breast, and colorectal cancers [76,78,81].

Studies on HPV have often highlighted the different aberrant dynamics induced by the distinct HPV genotypes. These differences lie in the peculiar molecular characteristics identified between cutaneous and mucosal types, lr and hrHPVs, and between individual high-risk types. The main differences concern the two oncoproteins E6 and E7 [91] which influence carcinogenic potential and can interfere with methyltransferase activity.

Specifically, hrHPV E6 targets the cellular E3 ubiquitin ligase E6AP, thereby mediating the transfer of ubiquitin peptides to p53, which is then targeted to the proteasome for degradation. Low-risk and cutaneous genotypes, on the contrary, are unable to activate this pathway [55].

Since the two oncoproteins are responsible for the impaired methylation scenario, it can be assumed that the aberration in methylation is induced differently depending on the HPV risk classification.

The ΔΔCt Ratio approach was used to assess whether the hypermethylation was indicative of the correlation between hr or lrHPV genotypes and the diagnosis, but only close-to-significance p-value results were found.

In the analysed cohort, correlations were found between hypermethylation, defined again as above-cut-off values for at least one target, and some genotypes. This was the case for HPV16, which was found to be associated with hypermethylation both in cancerous lesions and, more surprisingly, in benign lesions. However, due to the relatively small number of HPV-positive samples, it was not possible to outline statistically significant associations.

Speaking specifically about positive OSCCs, the separate analysis of FAM19A4 and miR124-2 methylation percentages in HPV16 positive OSCC samples compared to those positive for other genotypes revealed a visually striking difference. Indeed, hypermethylation was significantly higher when HPV16 was detected.

HPV16 is undoubtedly the genotype whose involvement in tumorigenesis has

been better characterised, being the most frequently identified genotype in cervical, anal, penile, oropharyngeal and oral cancers [92]. Even if some authors still deny the causal relationship between infection and oral neoplasia, the evidence of HPV16 association with higher methylation levels indicates a possible peculiar methylation and molecular landscape, and thus a further suggestion of the actual involvement of HPV, at least HPV16, in oral carcinogenesis.

The statistical analysis performed allowed us to highlight another notable finding: higher hypermethylation in older patients. As shown in the multiple linear regression analysis in Table 2, both age and total methylation were defined as positive predictors of the diagnosis. In other words, a positive hypermethylation result tells us with high confidence that the patient does indeed, have an oral lesion.

This association, also described in a cohort of HSIL patients by Kaliff et al. [93], correlates with the severity of the diagnosis and allows for further insight. In fact, during the ageing process, the methylation pattern of the genome undergoes some major changes, with most studies suggesting a change towards hypermethylation at specific CpG sites, so significant that they allow the delineation of the so-called "epigenetic clocks", such as the Horvath [94], Hannum [95], Weidner [96] or PhenoAge [97] clocks.

Since also tumorigenesis is associated with epigenetic aberrations, and cancer itself is an age-related condition, multiple study groups have focused on its correlation with epigenetic age acceleration. It follows that Levine et al. [98], Lu et al. [99], Yu et al. [100], highlighted how cancer risk is associated with methylation age. However, more research is needed to understand the epigenetic connection between senescence and malignant transformation [101].

In conclusion, this study has shown for the first time how assessment of hypermethylation of two targets, the FAM19A4 and mir124-2 promoters, can aid in the diagnosis of OSCC and, more importantly, identify those patients affected by OPMDs at risk of cancer progression. To prove the exact prognostic value of hypermethylation assessment in the OPMD context will require a close follow-up of patients, as well as the design of future longitudinal studies.

The relatively low number of patients, especially HPV positives, did not enable bringing out the statistically significant differences in the effect of HPV infection on targets' methylation level.

Therefore, increasing the sample size to confirm these preliminary results and outline new significant information is imperative.

The future of this topic would certainly include studies of methylation-related changes in transcriptomic and proteomic pathways. This would allow an understanding of the biomolecular processes affected by aberrant methylation of FAM19A4 and mir124-2 and thus directly involved in the progression to the cancer phenotype [101].

Furthermore, a deeper understanding of these dynamics would provide the basis for the future development of epi-drugs specifically designed to treat oral diseases.

CHAPTER 5

Tables and Figures

Table 1. Clinical information: age, sex, diagnoses, and infection type, in the total sample group.

Parameters	**Sample data**
Patients	111
Age	55.7 ± 16.2
Sex	
Male	60.4% (67)
Female	39.6% (44)
Diagnoses	
NL	18.0% (20)
BL	33.3% (37)
OPMD	15.3% (17)
OSCC	33.3% (37)
HPV infected	48.6% (54)
Number genotypes	
One	81.5% (44)
Two	7.4% (4)
Three	3.7% (2)
More than three	7.4% (4)
Genotype risk	
hr/hr-lr	64.8% (35)
lr	35.2% (19)
Hypermethylation	
Negative	65.8% (73)
Positive	34.2% (38)

NL = No Lesions; BL = Benign lesions; OPMD = potentially malignant lesions; OSCC = Cancer lesions; lr= low risk; hr= high risk.

Table 2. Characteristics and comparison among patients belonging to the four groups.

Parameters	NL n=20	BL n=37	OPMD n=17	OSCC n=37	Analysis among groups p-value (test)
Age					p<0.001* (A)
mean±SD	39.2 ± 9.8	51.1 ± 15.6	66.4 ± 10.6	64.5 ± 12.3	NL<BL, p<0.05* (Sh) NL<OPMD, p<0.05* (Sh) NL<OSCC, p<0.05* (Sh) BL<OPMD, p<0.05* (Sh) BL<OSCC, p<0.05* (Sh)
median (IQR)	41 (31.5, 48)	53 (40, 60.8)	68 (57.5, 74)	66 (54.8, 73.3)	
Sex					
Male	45% (9)	59.5% (22)	52.9% (9)	73% (27)	p=0.18 (C)
Female	55% (11)	40.5% (15)	47.1% (8)	27% (10)	
HPV infected	50% (10)	48.6% (18)	41.2% (7)	51.2% (19)	0.92 (C)
Number of genotypes					
One	30% (6)	40.5% (15)	100% (7)	43.2% (16)	p=0.45 (F)
Two	5% (1)	5.4% (2)	0.0% (0)	2.7% (1)	
Three	5% (1)	2.7% (1)	0.0% (0)	0.0% (0)	
More than three	10% (2)	0.0% (0)	0.0% (0)	5.4% (2)	
Genotype risk					p=0.0033 * (F)
LR/HR or HR	90% (9)	38.9% (7)	42.9% (3)	**43.2% (16)**	OSCC-LR/HR**, p=0.0279 (Z) BL-LR**, p=0.0048 (Z)
LR	10% (1)	61.1% (11)	**57.1% (4)**	8.1% (3)	
Hypermethylation in total patients					p=0.0001* (C)
Negative (n=73)	**95% (19)**	**75.7% (30)**	58.8% (11)	35.1% (13)	NL-Neg**, p=0.0023 (Z) BL-Neg**, p=0.0162 (Z)
Positive (n=38)	5% (1)	18.9% (7)	35.3% (6)	**64.9% (24)**	OSCC-pos**, p<0.0001 (Z)
Hypermethylation in HPV-negative patients					p=0.0015* (F)
Negative (n=37)	**50% (10)**	40.5% (15)	35.3% (6)	16.2% (6)	NL-Neg**, p=0.0105 (Z) OSCC-pos**, p=0.0007 (Z)
Positive (n=20)	0.0% (0)	10.8% (4)	23.5% (4)	**32.4% (12)**	
Hypermethylation in HPV patients					p=0.0055* (F)
Negative (n=36)	45% (9)	40.5% (15)	29.4% (5)	18.% (7)	OSCC-pos**, p=0.0006 (Z)
Positive (n=18)	5% (1)	8.1% (3)	11.8% (2)	**32.4% (12)**	

Percentages were calculated against the number of diagnosis subgroups.
*=significant test; C=chi-square test; Z=z test; **= significantly more frequent; ***= significantly less frequent; F=Fisher's exact test; A= one way ANOVA test; Sh= Scheffé post hoc test for pairwise comparisons; no lesions (NL), benign lesions (BL), potentially malignant lesions (OPMD) and cancer lesions (OSCC)

Table 3. Relationships analysis between hypermethylation in all patients and age, sex, HPV infection, symptoms, number of genotypes, and genotype risk.

Parameters	Hypermethylation		
	Negative N=73	**Positive N=38**	**p-value (test)**
Hypermethylation tot/*Age*	50.9 ± 16.0	65.0 ± 12.2	<0.0001* (T)
Hypermethylation tot/*Sex*	42.5% (31 M) 57.5% (42 F)	65.8% (25 M) 34.2% (13 F)	0.40 (C)
Hypermethylation tot/Diagnoses	26.0% (19 NL) 41.1% (30 BL) 15.1% (11 OPMD) 17.8% (13 OSCC)	2.6% (1 NL) 18.4% (7 BL) 15.8% (6 OPMD) 63.2% (24 OSCC)	p=0.0001* (C) Negative-NL**, p=0.0023 (Z) Negative-BL**, p=0.0162 (Z) Positive-OSCC**, p<0.0001(Z)
Hypermethylation tot/*HPV Infection*	50.7% (37 HPV-) 49.3% (36 HPV+)	52.6% (20 HPV-) 47.4% (18 HPV+)	0.85 (C)
Hypermethylation tot/Number of genotypes	Mean rank: 56.0	Mean rank: 55.9	0.98 (MW)
Hypermethylation tot/Genotype risk	n=36 36.1% (13 lr) 63.9% (23 hr or hr/lr)	n=18 33.3% (6 lr) 66.7% (12 hr or hr/lr)	0.84 (C)

Percentages were calculated against the number of the two groups of samples positive and negative for hypermethylation.
*=significant test (p<0.05), C=chi-square test, T=unpaired t-test.

Table 4. Multiple linear regression analysis between diagnoses and significant variables described in Table 2.

Multiple linear regression	Coefficient	Standard Error	t	R-partial	*p*-value
Multiple correlation coefficient					0.56
Diagnoses/Age	0.03	0.01	2.8	0.37	**0.0077***
Diagnoses/Genotype risk	0.30	0.28	1.1	0.15	0.29
Diagnoses/Hypermethylation tot	0.68	0.32	2.1	0.29	**0.0381***
Constant	–0.42				

* = significant test;

Table 5. Genotype, diagnosis and hypermethylation in HPV-positive patients.

Genotypes	Percentages (n) HPV positive patients = 54	Diagnosis	Hypermethylation in HPV-positive patients
6	14.8% (8)	NL= 12.5% (1/8)	0.0% (0/1)
		BL= 75.0% (6/8)	16.7% (1/6)
		OPMD= 12.5% (1/8)	0.0% (0/1)
7	1.9% (1)	BL= 100% (1/1)	0.0% (0)
11	1.9% (1)	NL= 100% (1/1)	0.0% (0)
16	25.9% (14)	NL= 28.6% (4/14)	0.0% (0/4)
		BL= 14.3% (2/14)	50.0%(1/2)
		OPMD= 7.1% (1/14)	0.0% (0/1)
		OSCC= 50.0% (7/14)	85.7% (6/7)
31	7.4% (4)	NL= 25% (1/4)	0.0% (0/1)
		BL= 25% (1/4)	0.0% (0/1)
		OSCC= 50.0% (2/4)	50.0% (1/2)
33	1.9% (1)	BL= 100% (1/1)	0.0% (0/1)
38	1.9% (1)	OPMD= 100% (1/1)	0.0% (0/1)
42	3.7% (2)	BL= 50% (1/2)	100% (1/1)
		OSCC= 50% (1/2)	0.0% (0/1)
43	1.9% (1)	BL= 100% (1/1)	0.0% (0/1)
44	5.6% (3)	NL= 33.3% (1/3)	0.0% (0/1)
		BL=33.3% (1/3)	0.0% (0/1)
		OSCC=33.3% (1/3)	100% (1/1)
51	7.4% (4)	NL= 75% (3/4)	0.0% (0/3)

		OSCC= 25% (1/4)	0.0% (0/1)
53	3.7% (2)	NL= 50% (1/2)	100% (1/1)
		OSCC= 50% (1/2)	0.0% (0/1)
54	3.7% (2)	BL= 50% (1/2)	0.0% (0/1)
		OPMD= 50% (1/2)	100% (1/1)
56	7.4% (4)	NL= 50% (2/4)	0.0% (0/2)
		BL= 25% (1/4)	0.0% (0/1)
		OSCC= 25% (1/4)	100% (1/1)
58	5.6% (3)	BL= 33.3% (1/3)	0.0% (0/1)
		OPMD= 33.3% (1/3)	0.0% (0/1)
		OSCC=33.3% (1/3)	100% (1/1)
59	3.7% (2)	NL= 50% (1/2)	0.0% (0/1)
		BL=50% (1/2)	0.0% (0/1)
61	3.7% (2)	NL= 50% (1/2)	100% (1/1)
		BL= 50% (1/2)	100% (1/1)
62	5.6% (3)	NL= 33.3% (1/3)	100% (1/1)
		OPMD= 33.3% (1/3)	100% (1/1)
		OSCC= 33.3% (1/3)	100% (1/1)
66	13.0% (7)	NL= 28.6% (2/7)	0.0% (0/2)
		OPMD= 14.3% (1/7)	0.0% (0/1)
		OSCC= 57.1% (4/7)	75% (3/4)
67	1.9% (1)	OSCC= 100% (1/1)	100% (1/1)
68	3.7% (2)	OSCC= 100% (2/2)	100% (2/2)
73	3.7% (2)	NL= 50% (1/2)	0.0% (0/1)
		OSCC= 50% (1/2)	0.0% (0/1)
81	3.7% (2)	NL= 50% (1/2)	100% (1/1)
		OSCC= 50% (1/2)	100% (1/1)
82	3.7% (2)	NL= 50% (1/2)	100% (1/1)
		BL= 50% (1/2)	0.0% (0/1)
83	1.9% (1)	OSCC= 100% (1/1)	100% (1/1)
90	1.9% (1)	BL=100% (1/1)	0.0% (0/1)
120	5.6% (3)	BL= 33.3% (1/3)	0.0% (0/1)
		OSCC= 66.7% (2/3)	100% (2/2)

no lesions (NL), benign lesions (BL), potentially malignant lesions (OPMD) and cancer lesions (OSCC)

Table 6. Mean and median scores of ΔΔCt of FAM19A4 and mir124-2 stratified for diagnosis type.

	NL	BL	OPMD	OSCC	Analysis among groups p-value (test)
					p<0.0001* (KW)
ΔΔCt ratio FAM19A4	n=20	n=37	n=17	n=37	OSCC>NL*, p<0.05 (Co)
mean±SD	0.163±0.666	0.102±0.232	0.339±0.573	1.122±1.921	OSCC>BL*, p<0.05 (Co)
median (IQR)	0.0 (0.0, 0.005)	0.0 (0.0, 0.08)	0.03 (0.0, 0.515)	0.28 (0.0, 1.088)	OSCC>OPMD*, p<0.05 (Co)
					OPMD>NL*, p<0.05 (Co)
ΔΔCt ratio hsa mir124-2	n=20	n=37	n=17	n=37	p=0.0031* (KW)
mean±SD	0.236±0.286	0.257±0.583	0.392±0.501	0.92±1.7	OSCC >NL*, p<0.05 (Co)
median (IQR)	0.14 (0.005, 0.34)	0.09 (0.018, 0.253)	0.12 (0.073, 0.72)	0.48 (0.148, 0.155)	OSCC >BL*, p<0.05 (Co)

*=significant test; KW=Kruskal-Wallis test; Co= Conover post hoc test for pairwise comparisons; no lesions (NL), benign lesions (BL), potentially malignant lesions (OPMD) and cancer lesions (OSCC)

Table 7. Mean and median scores of of ΔΔCt ratio of FAM19A4 and mir124-2 stratified for diagnosis considering the HPV risk.

Positive HPV patients	**NL**	**BL**	**OPMD**	**OSCC**	**Analysis among groups** **p-value (test)**
ΔΔCt ratio FAM19A4					
HR/HR-LR mean±SD median (IQR)	n=9 0.358±0.988 0.0 (0.0, 0.11)	n=7 0.063±0.137 0.0 (0.0, 0.043)	n=3 0.0±0.0 0.0 (0.0,0.0)	n=16 1.382±2.503 0.235 (0.0, 1.1)	p=0.065 (KW)
LR mean±SD median(IQR)	n=1 0.0 0.0	n=11 0.083±0.140 0.0 (0.0, 0.118)	n=4 0.295±0.330 0.25 (0.02, 0.57)	n=3 0.743±0.646 1.06 (0.265, 1.143)	p=0.027* (A) OSCC >BL*, p<0.05 (Co)
ΔΔCt ratio hsa mir124-2					
HR/HR-LR mean±SD median(IQR)	n=9 0.356±0.364 0.14 (0.008, 0.637)	n=7 0.584±1.254 0.13 0.038, 0.238)	n=3 0.107±0.015 0.11 (0.085, 0.118)	n=16 1.229±1.542 0.62 (0.215, 1.535)	p=0.073 (KW)
LR mean±SD median(IQR)	n=1 0.26 0.26	n=11 0.20±0.32 0.07 (0.015, 0.248)	n=4 0.458±0.433 0.38 (0.105, 0.81)	n=3 0.677±0.597 0.9 (0.225, 1.07)	p=0.30 (A)

*=significant test; KW=Kruskal-Wallis test; Co= Conover post hoc test for pairwise comparisons; A= One-way ANOVA test; Scheffé's test post hoc test for pairwise comparisons; no lesions (NL), benign lesions (BL), potentially malignant lesions (OPMD) and cancer lesions (OSCC)

Figure 5. Bar chart describing the percentage of positive (red) and negative (green) samples for hypermethylation based on protocol's cut-offs in each of the four categories, regardless of HPV positivity (OSCC: oral squamous cell carcinomas, OPMD: oral potentially malignant disorders, BL: benign lesions, NL: no lesions).

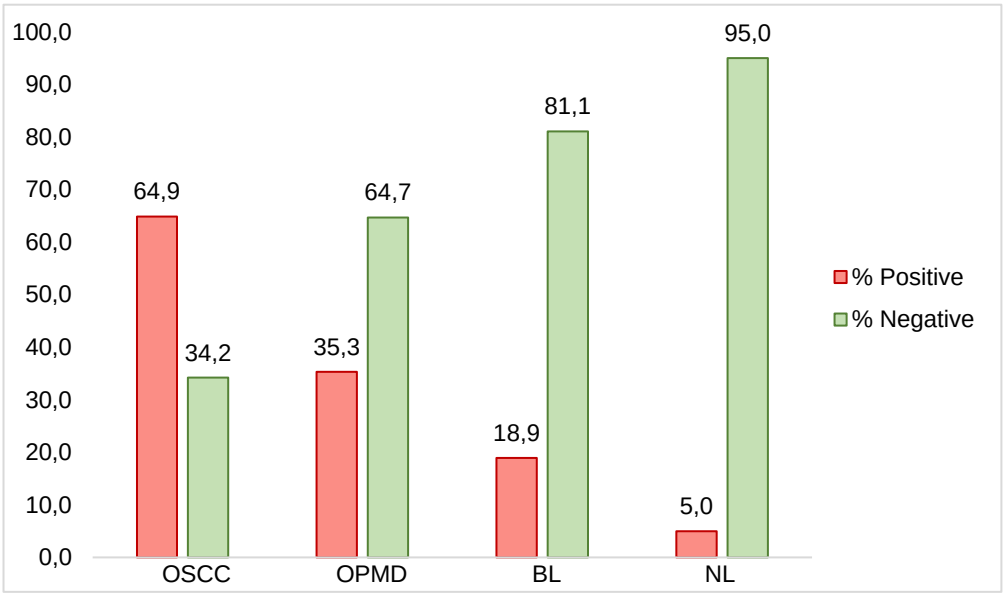


Figure 6. Box plot depicting the ΔΔCt Ratio values of FAM19A4 in the four samples category (OSCC: oral squamous cell carcinomas, OPMD: oral potentially malignant disorders, BL: benign lesions, NL: no lesions).

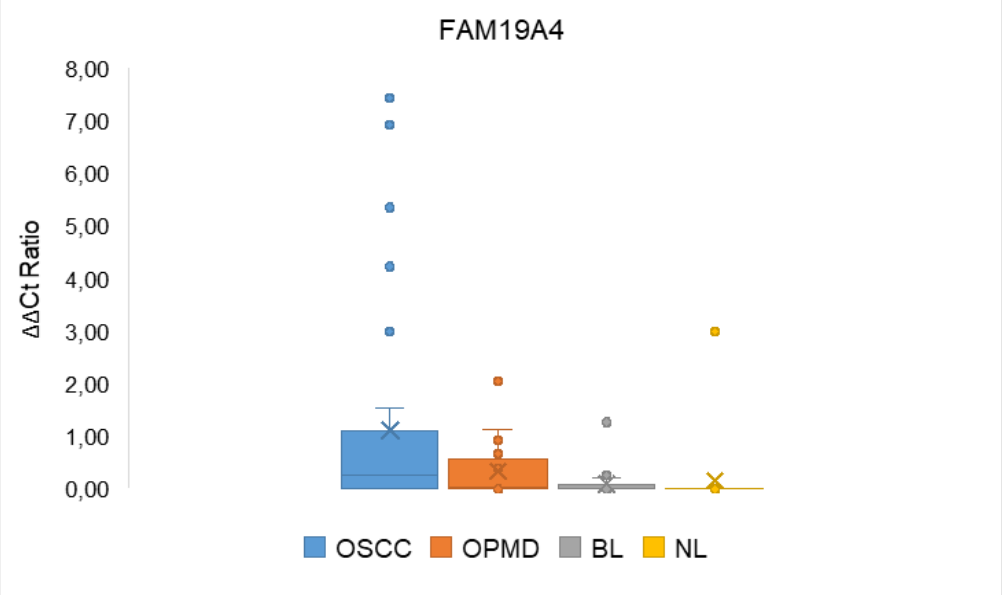


Figure 7. Box plot depicting the ΔΔCt ratio values of mir124-2 in the four samples category (OSCC: oral squamous cell carcinomas, OPMD: oral potentially malignant disorders, BL: benign lesions, NL: no lesions).

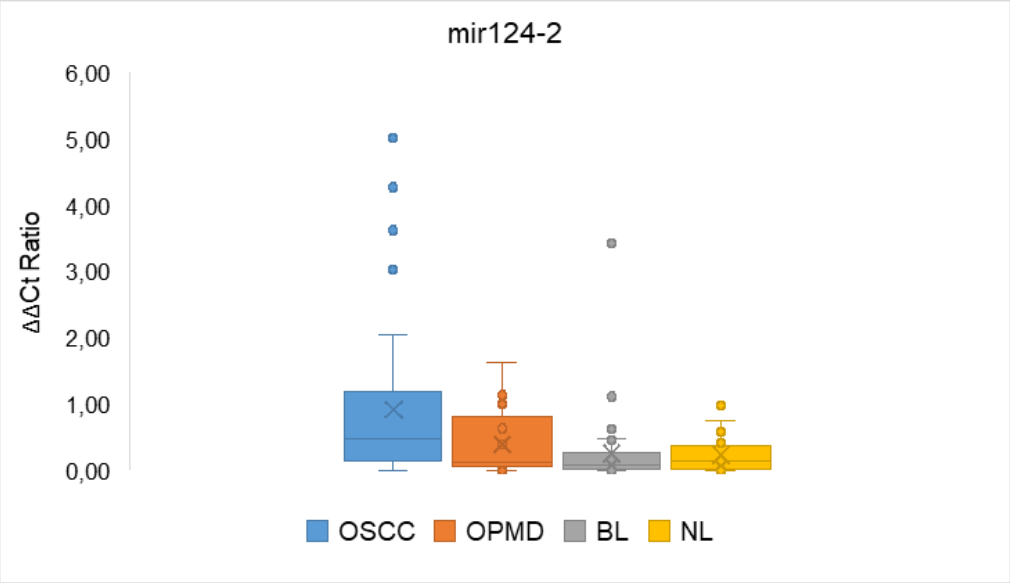


Figure 8. Box plot showing the ΔΔCt ratio values of FAM19A4 in OSCC samples positive for HPV16 compared to samples positive for all the other genotypes.

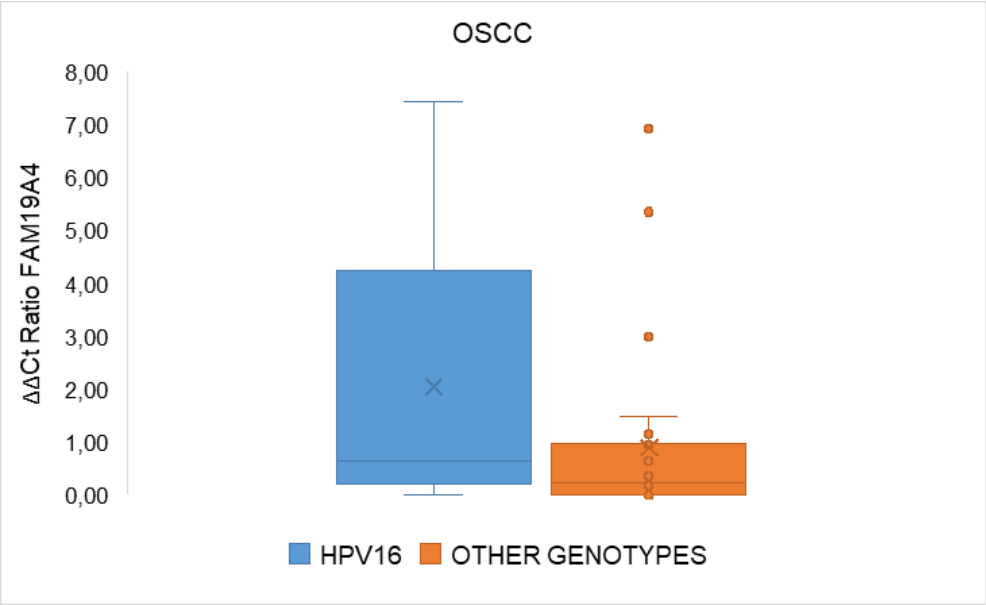
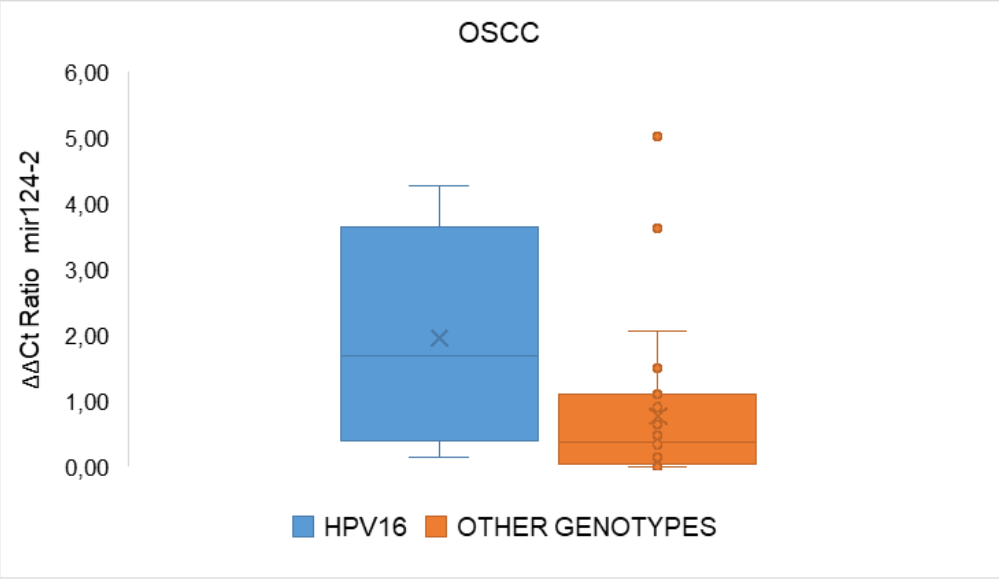


Figure 9. Box plot showing the ΔΔCt ratio values of mir124-2 in OSCC samples positive for HPV16 compared to samples positive for all the other genotypes.



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Scientific Products

Articles in Journals “in extenso” with Journal Citation Reports (JCR) Rank and Impact Factor (IF), consistent with the topic of the PhD project

1. **(Under submission) Identification of Epigenetic Biomarkers In Hpv-Related Carcinomas.**
   Buttà M., Serra N., Sucato A., Cabibi D, Campisi G., Panzarella V., Pistoia D., and Capra G.

2. **Human Papilloma Virus (HPV) Detection in Oral Rinse vs. Oral Sponge: A Preliminary Accuracy Report in Oral Cancer Patients.** *Cancers*. 2024; 16(19):3256.
   Panzarella V, Buttà M, Buttacavoli F, Capra G, Firenze A, Serra N, Giuliana G, Pizzo G, Campisi G, Mauceri R.
   IF: 4.5
   JCR Rank: Q1
   doi.org/10.3390/cancers16193256

3. **Evaluation of the Prevalence and Potential Impact of HPV Vaccines in Patients with and Without Oral Diseases: A Ten-Year Retrospective Study.** *Arch Med Res.* Published online September 5, 2024.
   Buttà M., Serra N., Mannino E., Panzarella V, Cabibi D, Campisi G, Pistoia D, and Capra G.
   IF: 4.7
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4. **Orogenital Human Papillomavirus Infection and Vaccines: A Survey of High- and Low-Risk Genotypes Not Included in Vaccines.** *Vaccines (Basel)*. 2023;11(9):1466. Published 2023 Sep 7
   Buttà M., Serra N., Panzarella V., Fasciana T. M. A., Campisi G., and Capra G.
   IF: 5.2
   JCR Rank: Q1
   doi:10.3390/vaccines11091466

Other articles in Journals "in extenso" with Journal Citation Reports (JCR) Rank and Impact Factor (IF) executed within the project time frame

1. **Human Papilloma Virus and Male Infertility: An Analysis Following World Health Organization 2021 Guidelines**. *Sci Rep* 14, 30236 (2024).
   Notari T., Buttà M., Serra N., Sucato A., Rizzo G., Capra G., and Bosco L.
   IF: 3.8
   JCR: Q1
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2. **Human Papillomavirus and Male Infertility: What Do We Know?** *Int J Mol Sci*. 2023;24(24):17562. 2023 Dec 16.
   Sucato A, Buttà M., Bosco L, Di Gregorio L, Perino A, Capra G.
   IF: 4.9
   JCR: Q1
   doi:10.3390/ijms242417562

3. **Human Papillomavirus (HPV) Infection and Its Impact on Male Infertility.** *Life*, 2022; 12(11), 1919
   Capra G., Notari T., Buttà M., Serra N., Rizzo G., and Bosco L.
   IF: 3.2
   JCR: Q2
   doi.org/10.3390/life12111919

Publications in proceedings, congress acts and awards at national and international conferences consistent with the topic of the PhD project

1. **Current options for the diagnosis of HPV-driven carcinogenesis in head and neck**
   Speaker at PHARYNGEAL CONGRESS- HPV and oropharyngeal cancer in the Mediterranean basin – Sassari, 27-28 September 2024

2. **Concurrence and concordance of Human Papillomavirus in orogenital infection**
   Buttà M., Mannino E., Cabibi D., Pistoia D., Campisi G., Panzarella V., and Capra G.
   50° NATIONAL CONGRESS AMCLI- Rimini, 24 March 2023 – 27 March 2023
   Abstract selected for oral presentation.
   Published in *Microbiologia Medica* 2023 April 11.
   doi:10.4081/mm.2023.11395

3. **Human papillomavirus oral infection and persistence in pediatric population (HOPE project)**
   Scafidi M., Giuliana G., Capra G., Buttà M., Sucato A., Minacapilli G., D'Arpa V., Panzarella V. 31° CONGRESSO NAZIONALE COLLEGIO DEI DOCENTI UNIVERSITARI DI DISCIPLINE ODONTOSTOMATOLOGICHE – Trieste 20-22 June 2024
   Honorary mention

4. **Oral potentially malignant disorders (OPMDs): HPV prevalence and vaccines**
   Buttà M., Serra N., Sucato A., Panzarella V., Mannino E., Cabibi D., Pistoia D., and Capra G 51° NATIONAL CONGRESS SIM - Cagliari 24-27 September 2023

5. **Cervical lesions and HPV oral infection: is there a relation?**
   Sucato A., Buttà M., Serra N., Immordino R., and Capra G. “” 51° NATIONAL CONGRESS SIM – Cagliari, 24-27 September 2023

6. **Distribution and prevalence of Human papillomavirus (HPV) in oral samples collected from 2001 to 2020 in Sicily**
   Buttà M., Mannino E., Campisi G., Panzarella V., Pistoia D., and Capra G. 50° NATIONAL CONGRESS SIM – Napoli, 18-21 September 2022

Other publications in proceedings, congress acts and awards at national and international conferences executed within the project time frame

1. **Male Human papillomavirus genital infection: a focus on non-vaccine genotypes**
   Buttà M., A. Sucato, L. Bosco, L. Di Gregorio, N. Serra, D. Pistoia, G. Capra. ESCMID Global, Barcelona, Spain 27–30 April 2024

2. **Correlation between sperm DNA fragmentation index, semen parameters and Human Papillomavirus: an analysis conducted under World Health Organization 2021 guidelines**.
   Notari T., Buttà M., Serra N., Rizzo G., Montano L., Capra G., and Bosco L., 39° Annual Meeting of ESHRE (European Society of Human Reproduction and Embryology), Copenhagen, Denmark 25-28 June 2023

3. **WHO 2021: DFI e nuovi parametri seminali in relazione all'infezione da Human papillomavirus (HPV).**
   Buttà M., Notari T., Serra N., Rizzo G., Montano L., Capra G., and Bosco L., 6° Congresso Nazionale SIRU (Società Italiana della Riproduzione Umana), Roma, 12-14 April 2023

4. **Prevalence of mucosal and cutaneous HPV in keratinizing subtypes of anogenital squamous-cell carcinomas.**
   Buttà M., Cabibi D., Viviano E., Pistoia D., Micciulla M.C., Formisano E., and Capra G., 49° NATIONAL CONGRESS AMCLI, (Associazione Microbiologi Clinici Italiani), 26 February - 1 March 2022